

1 Crystal structures of Arabidopsis and Physcomitrella CR4 reveal the molecular architecture
2 of CRINKLY4 receptor kinases.

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19 Abstract

20 Plant-unique receptor kinases harbor conserved cytoplasmic kinase domains and sequence-
 21 diverse ectodomains. Here we report crystal structures of CRINKLY4-type ectodomains from
 22 *Arabidopsis* ACR4 and *Physcomitrella patens* PpCR4 at 1.95 Å and 2.70 Å resolution,
 23 respectively. Monomeric CRINKLY4 ectodomains harbor a N-terminal WD40 domain and a
 24 cysteine-rich domain (CRD) connected by a short linker. The WD40 domain forms a seven-
 25 bladed β -propeller with the N-terminal strand buried in its center. Each propeller blade is
 26 stabilized by a disulfide bond and contributes to the formation of a putative ligand binding
 27 groove. The CRD forms a β -sandwich structure stabilized by six disulfide bonds and shares
 28 low structural homology with tumor necrosis factor receptor domains. Quantitative binding
 29 assays reveal that ACR4 is not a direct receptor for the peptide hormone CLE40. An ACR4
 30 variant lacking the entire CRD can rescue the known *acr4-2* mutant phenotype, as can
 31 expression of PpCR4. Together, an evolutionary conserved signaling function for CRINKLY4
 32 receptor kinases is encoded in its WD40 domain.

Introduction

Plants have evolved a unique set of membrane receptor kinases (RKs) that regulate diverse aspects of growth and development, form the first layer of the plant immune system and mediate symbiotic interactions. RKs contain a single membrane-spanning helix, a conserved dual-specificity cytoplasmic kinase domain and sequence-diverse extracellular domains (ectodomains) involved in signal perception and receptor activation¹. The three-dimensional structures and functions of plant RKs with leucine-rich repeat (LRR) ectodomains have been characterized in detail, yielding a molecular understanding of their ligand binding and receptor activation mechanisms².

Crystal structures of non-LRR RKs have been reported for lysine-motif domain containing immune and symbiosis receptors involved in the perception of N-acetyl-D-glucosamin-containing ligands³⁻⁵. S-locus receptor kinases involved in self recognition during flower pollination have been structurally characterized to contain β -barrel lectin domains and growth factor-like domains, all contributing to the specific recognition of a cysteine-rich signaling peptide⁶. Two other classes of RKs with lectin domain-containing extracellular domains have subsequently been characterized⁷: The CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASES (CRKs) contain a tandem arrangement of DOMAIN OF UNKNOWN FUNCTION 26 (DUF26) lectin domains, which may be involved in the recognition of a carbohydrate ligand⁸. The *Catharanthus roseus* receptor kinase 1-like (CrRLK1L) family contains a tandem arrangement of malectin domains⁹ involved in the sensing of cysteine-rich RAPID ALKALINIZATION FACTOR peptides¹⁰, which can be distinctly bound to either LORELEI-like GLYCOLPHOSPHATIDYLINOSITOL (GPI)-ANCHORED PROTEINS¹⁰ or to the LRR domains of extensins¹¹.

Plant-unique CRINKLY4 (CR4) -type RKs show an unusual ectodomain structure radically different from the known LRR, LysM and lectin receptor kinases described above. The founding member of this family was identified by mapping the *crinkly4* mutation affecting leaf epidermis differentiation in maize¹². The putative receptor kinase CR4 was initially shown to contain an active cytoplasmic protein kinase module as well as an ectodomain with distant sequence homology to tumor necrosis factor receptor (TNFR) domains^{12,13}. TNFR type I and II receptors contain a cysteine-rich ectodomain that folds into several ~40 amino-acid segments. Each segment contains 6 conserved cysteines engaged in disulfide bonds¹⁴ and can act as binding sites for growth factors¹⁵. The sequence similarities between the CRINKLY4 and TNFR ectodomains suggested a role for maize CR4 in growth factor-triggered cell differentiation responses^{13,16}. Anti-sense knock-down or insertion mutation-based knock-out of *ACR4*, the Arabidopsis ortholog of maize CR4, again resulted in epidermis differentiation defects, leading to, for example, abnormal embryo and seed development¹⁷⁻¹⁹. *ACR4* localizes to the plasma membrane and to endosomes¹⁷⁻²⁰ and is a

catalytically active protein kinase^{18,21–23}. Sequence analysis of four ACR4 homologs in Arabidopsis indicated the presence of a conserved N-terminal β -propeller structure in CRINKLY4 ectodomains²¹. Subsequent structure-function studies revealed that kinase null mutations as well as deletion of the putative TNFR domain complemented the *acr4-2* null mutant phenotype^{18,20,24,25}. In contrast, partial deletion of the putative β -propeller domain or mutation of the conserved Cys180 in the β -propeller to tyrosine could not rescue the *acr4-2* phenotype^{20,26}, suggesting an important functional role for the N-terminal segment of the ACR4 ectodomain.

β -propeller domains are often involved in protein – ligand or protein – protein interactions²⁷ and thus different interaction partners for ACR4 ectodomain have been proposed, following the identification of root specific functions for ACR4^{28,29}. Specifically, the plant peptide hormone CLAVATA3/ESR-RELATED 40 (CLE40) controls expression of the transcription factor WUSCHEL RELATED HOMEBOX 5 (WOX5) to regulate root stem cell proliferation²⁹. CLE40's signaling capacity depends on the presence of ACR4 and ACR4 has been proposed to act as a direct receptor for CLE40 in the root^{29–31}. Moreover, ACR4 has been reported to physically interact with the CLAVATA3 (CLV3) / CLE peptide receptor CLAVATA1 (CLV1), forming heteromeric complexes at the plasma-membrane³². In the same study, ACR4 homo-oligomers were observed³². PROTEIN PHOSPHATASE 2A-3 (PP2A-3) and WOX5 have been identified as direct interaction partners for the ACR4 cytoplasmic domain^{33,34}. Here, we uncover the architecture of plant-unique CRINKLY4 RKs by solving crystal structures of ACR4 and *Physcomitrella patens*³⁵ PpCR4.

Results

For protein X-ray crystallographic analysis, we produced the ectodomains of ACR4 (ACR4^{WD40-CRD}, residues 1 – 423) and PpCR4 (PpCR4^{WD40-CRD}, residues 1 – 405), the isolated β -propeller domain of ACR4 (ACR4^{WD40}, residues 1 – 334) and the kinase domain of ACR4 (ACR4^{kinase}, residues 497 – 792) by secreted and cytoplasmic expression in insect cells, respectively. (see Methods) (Fig. 1a). All proteins were purified to homogeneity and the autophosphorylation activity of ACR4^{kinase} could be confirmed (Fig. 1b,c). No crystals were obtained for ACR4^{kinase} and initial crystals of ACR4^{WD40-CRD} and ACR4^{WD40} diffracted poorly. Enzymatic deglycosylation of ACR4^{WD40} yielded a new crystal form diffracting to 1.95 Å resolution. The structure was determined using the multiple anomalous dispersion method on a single crystal derivatized with a platinum compound (see Methods, Supplementary Table 1). Next, enzymatic deglycosylation of PpCR4^{WD40-CRD} yielded crystals diffracting to 2.7 Å resolution, enabling us to trace the entire CRINKLY4 ectodomain (Supplementary Table 1).

99 The N-terminal β -propeller domain of ACR4 and PpCR4 folded into a seven-bladed WD40
100 domain²⁷ (Fig. 1d), as previously speculated²⁰. Each blade is stabilized by a highly conserved
101 disulfide bridge and connected by small loop regions, possibly an evolutionary adaptation to the
102 extracellular environment (Fig. 1d, Supplementary Fig. 1). Cys180, which is found mutated to
103 tyrosine in the *acr4-7* mutant²⁰, forms a disulfide bond in the 4th blade (Fig. 1d). The N- and C-
104 terminal blades are not connected by disulfide bonds (Fig. 1d). The most N-terminal β -strand is
105 buried in the center of the propeller and is highly conserved among all known CRINKLY4
106 receptors³⁶ (Fig. 1d, Supplementary Fig. 1). Several small loops connecting the different blades of
107 the WD40 domain appear partially disordered in our ACR4 and PpCR4 structures (Fig. 1d,e).

108 The C-terminal CRD comprises PpCR4 residues 313-401 and folds into a well defined β -
109 sandwich structure stabilized by six invariant disulfide bridges (Fig. 1e, Supplementary Fig. 1, see
110 below). The WD40 and CRD domains are connected by a short linker region (Fig. 1e). Analysis of
111 crystal lattice arrangements with the program PISA³⁷ and analytical size-exclusion chromatography
112 experiments (Supplementary Fig. 2) together indicate that the ACR4 and PpCR4 ectodomains
113 behave as monomers in solution. All surface exposed cysteines in ACR4 and PpCR4 contribute to
114 disulfide bond formation (Fig. 1d,e; Supplementary Fig. 1). The N-glycosylation pattern differs
115 between ACR4 and PpCR4 (Fig. 1e, Supplementary Fig. 1). Taken together, a compact WD40 and a
116 cysteine-rich domain represent structural fingerprints of monomeric CRINKLY4 ectodomains.

117 Structural homology searches against ACR4^{WD40} using the program DALI³⁸ returned the
118 extracellular WD40 domain of the secreted β -lactamase inhibitor protein II BLIP-II from the soil
119 bacterium *Streptomyces exfoliatus* as top hit (DALI Z-score 23.2, root mean square deviation
120 [r.m.s.d.] is ~ 2.2 Å comparing 192 corresponding C α atoms) (Supplementary Fig. 3)³⁹. A previously
121 reported homology model of ACR4^{WD40} had been based on the BLIP-II structure²⁰. The UV-B
122 photoreceptor UV-B – RESISTANCE 8 (UVR8) represents the closest structural homolog in plants
123 (Dali Z-score 22.1, r.m.s.d. is ~ 2.4 Å comparing 218 corresponding C α atoms) (Supplementary
124 Fig.3)⁴⁰. ACR4^{WD40} however lacks the UVR8 tryptophan cage involved in UV-B light sensing^{40,41}
125 and both BLIP-II and UVR8 are devoid of the buried N-terminal strand and the conserved disulfide
126 bridge pattern present in ACR4^{WD40}. Thus, the pore-filling N-terminus and the invariant blade
127 disulfide bonds are unique structural features of extracellular CRINKLY4 WD40 domains.

128 We next studied the interaction of ACR4^{WD40-CRD} with its proposed ligand CLE40²⁹⁻³². As
129 ACR4 has been previously reported to form hetero-oligomers with the LRR-RK CLV1, we sought
130 to include the CLV1 ectodomain in these experiments, but we could not produce well-behaving
131 protein samples of the AtCLV1 ectodomain by secreted expression in insect cells (Supplementary
132 Fig. 4), and consequently could not use the CLV1 ectodomain for biochemical or crystallographic

133 experiments. We thus replaced CLV1 with the LRR ectodomain of the sequence-related CLE
134 peptide receptor BARELY ANY MERISTEM (BAM1) in our *in vitro* binding experiments (Fig. 2a).
135 We found that CLE40 binds the AtBAM1 ectodomain with a dissociation constant (K_d) of $\sim 1 \mu\text{M}$
136 (Fig. 2b) but shows no detectable binding to the ACR4 ectodomain in quantitative grating-coupled
137 interferometry (Fig. 2b) and isothermal titration calorimetry (Fig. 2c) assays. Thus, CLE40 does not
138 represent a direct ligand for the ACR4 ectodomain.

139 Using the previously documented seed retardation phenotype of the *acr4-2* mutant^{18,20} we
140 next carried out genetic complementation analyses using different constructs expressed from the
141 *ACR4* promoter. In agreement with an earlier report²⁰, a construct in which the entire cytoplasmic
142 domain of ACR4 had been deleted could not rescue the seed development phenotypes of *acr4-2*
143 plants (Fig. 3a). Full-length ACR4 lacking kinase activity partially restored seed development in
144 *acr4-2* plants (Fig. 3a). Strikingly, expression of full-length PpCR4, the ectodomain of which shares
145 only 40% sequence identity at the amino-acid level with ACR4^{WD40-CRD}, from the *ACR4* promoter
146 could partially complement *acr4-2* phenotypes as well. Together, these experiments reinforce an
147 evolutionary conserved function for CRINKLY4 RKs, which are however not strictly dependent on
148 the protein kinase activity of the receptor.

149 The 2.7 Å crystal structure of the entire ectodomain from PpCR4 enabled us to further
150 characterize the ~ 90 amino-acid CRINKLY4 CRD (Fig. 3b). A structural homology search with
151 DALI³⁸ indeed identified several TNFR domains as top hits, but with very low DALI Z-scores (4.1-
152 2.9). Structural superposition of PpCR4^{CRD} with the previously reported structure of a type I TNF
153 receptor extracellular domain revealed that only a small portion of the CRINKLY4 aligns with
154 canonical TNFR domains (r.m.s.d. is ~ 1 Å comparing 20 corresponding C α atoms, Fig. 3b). The
155 segment includes a small β -hairpin and two conserved disulfide bridges located at the center of the
156 CRINKLY4 CRD (Fig. 3c). Structural superposition of the eight molecules in the asymmetric unit
157 of our PpCR4^{WD40-CRD} crystal structure (Supplementary Table 1) revealed only subtle movements of
158 the CRD versus the WD40 domain (r.m.s.d. is ~ 0.3 - 0.5 Å comparing 360 corresponding C α atoms,
159 Supplementary Fig. 5). In line with this, we located a small WD40 – CRD domain interface using
160 PISA³⁷ (total buried surface area is ~ 900 Å²). The interface is formed by the C-terminus of the CRD
161 (PpCR4 residues 385-401) that makes mainly hydrophobic interactions with a small groove located
162 between the N- and C-terminal blade of the WD40 domain (Supplementary Fig. 6). Additional
163 contacts originate from a small α -helix in the CRD and several loop regions in PpCR4^{WD40}
164 (Supplementary Fig. 6).

165 Using the now experimentally determined domain boundaries of the ACR4 CRD
166 (Supplementary Figs. 7 and 1), we re-performed complementation assays of the *acr4-2* mutant with

167 a construct in which the entire CRD was omitted (ACR4 Δ CRD). As previously reported^{20,24}, we
 168 found that ACR4 Δ CRD can rescue the seed development phenotype of *acr4-2* plants (Fig. 3a).
 169 Recently, mutation of the cysteine residues in ACR4^{WD40} and ACR4^{CRD} involved in the formation of
 170 disulfide bonds in our structures (Fig.1, 3b,c) to alanine resulted in a functional receptor for seed
 171 development²⁴. We monitored migration of the purified ACR4^{WD40-CRD} ectodomain under oxidizing
 172 and strongly reducing conditions in analytical size exclusion chromatography experiments and
 173 found that reduction of ACR4^{WD40-CRD} did not induce aggregation of the receptor (Fig. 3d,e).
 174 Together, the CRINKLY4 CRD only shares weak structural homology with animal TNFR domains,
 175 has a conserved domain interface with the WD40 domain and is dispensable for seed development.
 176 The conserved disulfide bonds appear to be involved in structural stabilization. The domain
 177 interface between the WD40 domain and the CRD is conserved among CRINKLY4 receptors from
 178 different species (Supplementary Figs. 1, 6).

179 While the CRD domain appears to be dispensable for at least some of ACR4's physiological
 180 functions, our and previous findings^{20,24} argue for an important role of the structurally unique WD40
 181 domain in CRINKLY4 receptors. We located evolutionary conserved, surface exposed residues at
 182 the 'back side' of the ACR4 WD40 domain (Fig. 4a, Supplementary Fig. 1), which in our
 183 PpCR4^{WD40-CRD} structure is in contact with the CRD (Fig. 1e). We replaced individual residues by
 184 alanine or glutamine, respectively and assessed the ability of the resulting mutant proteins to
 185 complement the *acr4-2* seed development phenotype (Fig. 4a-c). We analyzed three independent
 186 homozygous T3 lines per mutant receptor and found that most mutations behaved similar to wild
 187 type (Fig. 4b) and that none of mutants tested displayed the strong loss-of-function phenotype of
 188 *acr4-2* plants (Fig. 4b,c). Plants in which either Tyr157 or Asn158/Asn196 were mutated had seed
 189 numbers per silique that were significantly reduced compared to wild type (Fig. 4b). While there
 190 was no electron density for a N-glycan at position Asn158 in the ACR4^{WD40} structure (see Methods),
 191 the corresponding Asn150 in PpCR4 was found glycosylated (Fig. 1e). ACR4 Asn196 is predicted
 192 to be N-glycosylated as well⁴², suggesting that the weak loss-of-function phenotypes observed in
 193 our Tyr157/Asn158 and Asn196 point mutants may be caused by an altered N-glycosylation pattern
 194 of the receptor (Fig. 4b,c).

195 We next analyzed the molecular surface of the 'front side' of ACR4^{WD40}, which represents
 196 another canonical binding surface for peptide and protein ligands in many cytoplasmic or nuclear
 197 localized WD40 proteins²⁷. We located a large binding groove in ACR4^{WD40} formed by the WD40
 198 domain core and by small surrounding loop regions, which appear similar in our ACR4^{WD40} and
 199 PpCR4^{WD40-CRD} WD40 domain structures (r.m.s.d. is ~1.4 Å comparing 246 corresponding C α atoms,
 200 Supplementary Fig. 7). The very low degree of sequence surface conservation in the putative

binding groove in apo ACR4^{WD40} renders mutational analysis of the full-length receptor *in planta* difficult (Supplementary Figs. 1, 8). The binding groove is however larger and deeper compared to the VP-peptide binding site in the structurally related WD40 domain of the light-signaling E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Fig. 4d)⁴³. It may thus provide an interaction platform for high molecular weight ligand.

Discussion

Our crystal structures (Fig. 1d,e) and reverse genetic analyses (Fig. 3a) together reveal an evolutionary conserved domain architecture for plant-unique CRINKLY4 receptor kinases³⁶. The CRINKLY4 WD40 domain differs from known cytoplasmic and extracellular WD40 domains^{27,39,40,43}, with its seven blades being stabilized by disulfide bridges and the hydrophobic core of the domain being reinforced by insertion of the protein's N-terminus (Fig. 1d,e). We speculate that these unique structural features represent an adaptation to CRINKLY4 ectodomains being exposed to the plant cell wall environment. Previous²⁰ and our genetic data argue for an important function for the ACR4 WD40 domain in seed development (Fig. 3a). A large groove located on the 'front side' of ACR4 may be involved in the binding of a ligand (Fig. 4d). This ligand could be a small molecule, a protein or a peptide, and may be larger than the octameric peptide motifs recognized by COP1 (Fig. 4d). The low degree of sequence conservation of residues contributing to the formation of the binding groove in the WD40 domain (Fig. 4d, Supplementary Fig. 1) and the fact that PpCR4 can functionally replace ACR4's function in Arabidopsis seed development (Fig. 3a) together indicate that CRINKLY4 receptors may sense a family of structurally conserved ligands.

Our quantitative binding assays reveal that the previously proposed peptide ligand CLE40 cannot directly interact with the ACR4 ectodomain (Fig. 2), but we cannot rule out that CLE40 binds the CLV1 ectodomain in a signaling complex also containing ACR4^{29,30,32}. The architecture and cellular functions of CLV1 – ACR4 signaling complexes remain to be elucidated, with recombinant expression and purification of the CLV1 ectodomain representing a significant challenge (Supplementary Fig. 4). BAM1 cannot fully replace CLV1 in quantitative biochemical assays, as it binds CLE40 only with moderate affinity (Fig. 2). In contrast, the CLE family member CLE9 binds BAM1 with nanomolar affinity^{44,45}. In solution and in the absence of ligand, CRINKLY4 ectodomains behave as monomers (Fig. 3e, Supplementary Fig. 2). The previously observed ACR4 homo-oligomers³² may thus be generated by ligand-induced oligomerisation of several CRINKLY4 ectodomains and/or be stabilized by interaction of the CRINKLY4 trans-membrane helices, as previously suggested^{46,47}.

Analysis of the CRINKLY4 cysteine-rich domain revealed only weak structural homology with animal TNFR domains (Fig. 3b)¹⁴. In line with this, we could not locate proteins with homology to tumor necrosis factors in the *Arabidopsis* or *Physcomitrella patens* genomes^{48,49}. The CRINKLY4 CRD contains six conserved disulfide bridges (Fig. 1e, Supplementary Fig. 1), which in our PpCR4^{WD40-CRD} structure appear to be involved in structural stabilization (Fig. 3b,c). However, CRINKLY4 ectodomains can withstand reducing conditions (Fig. 3e), and thus the putative function of the CRD could indeed be regulated by changes in the cell wall redox environment²⁴.

Enzymatic assays of the CRINKLY4 cytoplasmic domains obtained from prokaryotic^{18,21–23} or eukaryotic expression hosts (Fig. 1b,c) clearly identify CRINKLY4s as active protein kinases. Our and previous²⁰ reverse genetic experiments suggest that the ACR4 cytoplasmic domain has to be present for normal seed development in *Arabidopsis*, yet its catalytic activity seems to be dispensable (Fig. 1e). Similar observations have been made for CrRLK1L-family receptor kinases^{50–52}. The mechanistic implications are poorly understood, but the involvement of protein phosphatases in both CR4 and CrRLK1L-mediated signal transduction^{33,53,54} suggests that the cytoplasmic kinases domains of these receptors may act as scaffolding proteins that can become phosphorylated despite not requiring auto- and trans-phosphorylation activity themselves.

Genetic interactions between ACR4 and other receptor kinases such as ABNORMAL LEAF SHAPE 2 (ALE2)⁵⁵ the LRR-RKs CLV1^{29,30,32} and GSO1/GSO2^{45,56} have so far not yielded a mechanistic understanding of CRINKLY4's signaling functions. Also, no ligand candidate for ACR4 or for its homologs in *Arabidopsis* has emerged from forward genetic screens²¹. Our identification of a putative ligand binding pocket in ACR4^{WD40} now reinforces the notion that *bona fide* ligands for CR4s may exist and that their identification may be achieved using a combination of genetic and biochemical approaches.

Material and Methods

Protein expression and purification

ACR4 coding sequences for the WD40 domain (residues 1 – 334) and its entire ectodomain (residues 1 – 423) were amplified from *A. thaliana* cDNA. PpCR4^{WD40-CRD} (residues 1 – 405), AtCLV1 (residues 25 – 621) and BAM1 (residues 20 – 637) were synthesized by Geneart (Germany) with codon usage optimized for expression in *Trichoplusia ni*. The constructs of ACR4 and PpCR4 were cloned in a modified pFastBac vector (Geneva Biotech), containing a TEV (tobacco etch virus protease) cleavable C-terminal StrepII – 9x His tag. ACR4^{WD40-CRD}, CLV1 and BAM1 were also cloned into the vector holding a native signal peptide or the *Drosophila melanogaster* BiP secretion signal peptide, respectively, a C-terminal TEV cleavable StrepII – 10x

269 His tag and a non-cleavable Avi-tag^{57,58}. *Trichoplusia ni* (strain Tnao38) cells⁵⁹ were infected with a
 270 multiplicity of infection (MOI) of 1 at a density of 2×10^6 cells ml⁻¹ and incubated 26h at 28 °C and
 271 48h at 22 °C. The secreted protein was purified from the supernatant by Ni²⁺ affinity
 272 chromatography on a HisTrap Excel column (GE healthcare), equilibrated in 50 mM KP_i pH 7.6,
 273 250 mM NaCl, 1 mM 2-Mercaptoethanol, followed by StrepII affinity chromatography on a Strep-
 274 Tactin XT Superflow high affinity column (IBA), equilibrated in 20 mM Tris pH 8.0, 250 mM
 275 NaCl, 1 mM EDTA. The tag was cleaved with His-tagged TEV protease at 4 °C overnight and
 276 removed by a second Ni²⁺ affinity chromatography step. Proteins were then further purified by size-
 277 exclusion chromatography on either a Superdex 200 increase 10/300 GL, Hi Load 16/600 Superdex
 278 200 pg, or a HiLoad 26/600 pg column (GE Healthcare), equilibrated in 20 mM HEPES pH 7.5,
 279 150 mM NaCl. For crystallization, ACR4^{WD40} and PpCR4^{WD40-CRD} were dialyzed in 20 mM sodium
 280 citrate pH 5.0, 150 mM NaCl and treated with Endoglycosidase H, F1, and F3 to cleave sugar
 281 chains. Proteins were then purified by ion exchange chromatography on a HiTrapSP HP column
 282 (GE Healthcare), equilibrated in 20 mM Citrate pH 5.0, 25 mM NaCl for ACR4^{WD40} or 20 mM
 283 Citrate pH 3.5, 25 mM NaCl for PpCR4^{WD40-CRD}, respectively. Fractions were pooled, concentrated
 284 and further purified by size-exclusion chromatography.

285

286 ***In vitro* kinase phosphorylation assay**

287 Coding sequence of ACR4 kinase domain (residues 497 – 792) was amplified from *A.*
 288 *thaliana* cDNA and cloned in a modified pFastBac vector harboring a TEV cleavable N-terminal
 289 maltose binding protein (MBP) – StrepII – 10x His tag. Point mutation was introduced into the
 290 ACR4 (Asp659 → Asn; hereafter ACR4^{D659N}, Supplementary Table 2) coding sequence using the
 291 primer extension method for site-directed mutagenesis, rendering the kinase inactive⁶⁰. Insect cells
 292 were infected with a MOI of 1 at a density of 2×10^6 cells ml⁻¹ and incubated 26h at 28 °C and 48h
 293 at 22 °C. Cells were pelleted by centrifugation at 4,000 x g, 4 °C for 15 min and resuspended in
 294 buffer A (20 mM HEPES pH 7.5, 500 mM NaCl, 4 mM MgCl₂ and 2 mM 2-Mercaptoethanol)
 295 supplemented with 50 µg ml⁻¹ DNase I, 10 %(v/v) glycerol and 1 tablet of protease inhibitor
 296 cocktail (cOmplete, Roche), followed by sonication. The cell lysate was centrifuged at 35,000 x g, 4
 297 °C for 60 min and the protein was purified from the supernatant by Ni²⁺ affinity chromatography
 298 with buffer A, followed by StrepII affinity chromatography. For ACR4^{D659N}, the 10x His – StrepII –
 299 MBP tag was cleaved with His-tagged TEV protease at 4 °C overnight and removed by Ni²⁺ affinity
 300 chromatography. Proteins were then further purified by size-exclusion chromatography on a
 301 Superdex 200 increase 10/300 GL column equilibrated in 20 mM Tris-HCl pH 8, 250 mM NaCl, 4
 302 mM MgCl₂ and 0.5 mM TCEP. Monomeric peak fractions were collected and concentrated for

analyses. For *in vitro* kinase assays, 2 µg of MBP-ACR4 and 1 µg of ACR4^{D659N} were used in a reaction volume of 20 µl. The reactions were started by addition of 5 µCi [γ-³²P]-ATP (Perkin-Elmer, Waltham, MA), incubated at room temperature for 45 min and terminated by the addition of 6x SDS loading dye, immediately followed by heating the samples at 95 °C. Proteins were separated by SDS-PAGE in 4 – 15 % gradient gels (TGX, Biorad) and ³²P-derived signals were visualized by exposing the gel to an X-ray film (SuperRX, Fujifilm).

Crystallization and data collection

Crystals of the deglycosylated ACR4^{WD40} and PpCR4^{WD40-CRD} developed at room temperature in hanging drops composed of 1 µl protein solution (ACR4^{WD40}, 20 mg/ml; PpCR4^{WD40-CRD}, 16 mg/ml) 1 µl of crystallization buffer (16 % PEG 6,000, 0.01 M tri-sodium citrate pH 5.0 for ACR4^{WD40}; 15 % PEG 4,000, 0.2 M imidazole malate pH 7.0 in the case of PpCR4^{WD40-CRD}) suspended above 1.0 ml of the latter as reservoir solution and using microseeding protocols. Crystals were cryo-protected by serial transfer into crystallization buffer supplemented with 20 % (v/v) ethylene glycol and snap-frozen in liquid nitrogen. For heavy-atom derivatization, crystals of ACR4^{WD40} were transferred in the crystallization buffer containing 2 mM K₂[Pt(CNS)₆] and incubated for 2.5h. Crystals were cryo-protected by serial transfer into crystallization buffer supplemented with 20 % (v/v) glycerol and cryo-cooled in liquid nitrogen. Platinum multi-wavelength anomalous diffraction (MAD) data were collected to 3.2 Å resolution was collected at beam-line PXIII at the Swiss Light Source (SLS), Villigen, CH. A native data for ACR4^{WD40} and PpCR4^{WD40-CRD} were recorded at a resolution of 1.95 Å and 2.70 Å, respectively (Supplementary Table 1). Data processing and scaling were done with XDS and XSCALE⁶¹.

Structure solution and refinement

Nine consistent Pt sites were located in three wavelength MAD data using the program SHELXD⁶² followed by site refinement and phasing in SHARP⁶³. The resulting heavy atom sites and starting phases (FOM was 0.35 to 3.2 Å resolution) were input into phenix.autobuild⁶⁴ for non-crystallographic symmetry (NCS) averaging, phase extension, density modification (FOM was 0.75 to 1.95 Å resolution) and iterative model building. The refined (Refmac5⁶⁵) model comprises four ACR4^{WD40} molecules in the asymmetric unit with an associated solvent content of 0.42. The space group *P* 2₁ with a β angle of 90.1° was validated using the programs POINTLESS⁶⁶ and ZANUDA⁶⁷. The structure of PpCR4^{WD40-CRD} was solved using the molecular replacement method using an ACR4^{WD40} monomer as search model in calculations with the program PHASER⁶⁸. The solution comprises eight PpCR4^{WD40-CRD} molecules in the asymmetric unit. The structure was

completed in alternating cycles of manual model building in COOT⁶⁹ and restrained NCS refinement in phenix.refine⁷⁰. Ile156 represents a Ramachandran plot outlier in each chain, but is well defined by electron density. Structural diagrams were prepared in Pymol (<https://sourceforge.net/projects/pymol/>) and ChimeraX⁷¹.

Biotinylation of proteins

The respective proteins (20 – 100 μ M) were biotinylated with biotin ligase BirA⁵⁸ (2 μ M) for 1h at 25 °C, in a volume of 200 μ l; 25 mM Tris pH 8, 150 mM NaCl, 5 mM MgCl₂, 2 mM 2-Mercaptoethanol, 0.15 mM Biotin, 2 mM ATP, followed by size-exclusion chromatography to purify the biotinylated proteins.

Grating – coupled interferometry

GCI experiments were performed with the Creoptix WAVE system (Creoptix AG, Switzerland), using 4PCP WAVE chips (thin quasiplanar polycarboxylate surface; Creoptix, Switzerland). Chips were conditioned with borate buffer (100 mM sodium borate pH 9.0, 1 M NaCl; Xantec, Germany) and activated with 1:1 mix of 400 mM *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride and 100 mM *N*-hydroxysuccinimide (Xantec, Germany) for 7 min. Streptavidin (50 μ g ml⁻¹; Sigma, Germany) in 10 mM sodium acetate pH 5.0 (Sigma, Germany) was immobilized on the chip surfaces and passivated with 0.5 % BSA (Roche, Switzerland) in 10 mM sodium acetate pH 5.0, followed by final quenching with 1M ethanolamine pH 8.0 (Xantec, Germany) for 7 min. Biotinylated ligands (20 – 50 μ g ml⁻¹) was captured by streptavidin immobilized on the chip surface. All kinetic analyses were performed at 25°C with a 1:2 dilution series from 10 μ M of CLE40 peptides in 20 mM citrate pH 5.0, 250 mM NaCl, 0.01 % Tween 20. Blank injections were used for double referencing and a dimethylsulfoxide (DMSO) calibration curve for bulk correction. Analysis and correction of the obtained data was performed using the Creoptix WAVE control software (correction applied: X and Y offset; DMSO calibration; double referencing). Mass transport binding models with bulk correction were used. Experiments were performed in triplicates.

Isothermal titration calorimetry

All ITC experiments were performed on a MicroCal PEAQ-ITC (Malvern Panalytical) with a 200 μ l sample cell and a 40 μ l injection syringe at 25 °C. Proteins were dialyzed into ITC buffer (20 mM sodium citrate pH 5.0, 250 mM NaCl) prior to all experiments. The CLE40 peptide (RQV[Hyp]TGSDPLHH) was synthesized (Peptide Specialty Labs GmbH) and dissolved directly

in buffer. The dissolved peptide concentration was measured by right-angle light scattering (OMNISEC RESOLVE / REVEAL combined system, Malvern Panalytical). The protein concentrations were calculated based on their absorbance at 280 nm and their corresponding molar extinction coefficient. A typical experiment consisted of injecting 19 injections of 2 µl of 1000 µM CLE40 into the cell containing 100 µM ACR4. Experiments were performed in triplicates.

Plant materials and generation of transgenic lines

Arabidopsis thaliana ecotype Columbia (Col-0) and SAIL_240_B04 (*acr4-2*¹⁸) were used for all experiments. *ACR4* gene (residues 1 – 895) and *ACR4* promoter region (*pACR4*, 1847 bp upstream from ATG) were amplified from *A. thaliana* genomic DNA. *PpCR4* (residues 1 – 893) gene with *Physcomitrella patens* CDS was synthesized (Geneart, Germany). The coding sequences were cloned in a pDONR 221 Gateway vector (Invitrogen) and *pACR4* sequence was cloned in a pDONR P4-P1R Gateway vector (Invitrogen). *ACR4* variants carrying deletion or point mutations were generated using the primer extension method. pDONR P2R-P3 Gateway vector harboring mCitrine or 6x HA tag were used to attach C-terminal tag. Expression constructs were generated with LR Gateway Cloning (Invitrogen) in pH7m34GW⁷²; *pACR4::ACR4* (residues 1 – 895)-mCitrine, *pACR4::ACR4_ΔCyto* (residues 1 – 492)-mCitrine, *pACR4::PpCR4* (residues 1 – 893)-mCitrine, *pACR4::ACR4_ΔCRD* (residues 1 – 895 with deletion 335 – 423)-mCitrine, *pACR4::ACR4_K540R-HA*, *pACR4::ACR4_D84A-mCitrine*, *pACR4::ACR4_F105A-mCitrine*, *pACR4::ACR4_D127A-mCitrine*, *pACR4::ACR4_Y157A-mCitrine*, *pACR4::ACR4_N158A-mCitrine*, *pACR4::ACR4_Y157A*, *N158A-mCitrine*, *pACR4::ACR4_Y218A-mCitrine*, *pACR4::ACR4_N158Q*, *N196Q-HA*. They were transformed in *acr4-2* background by floral dipping method⁷³ with *Agrobacterium tumefaciens* strain GV3101 (Supplementary Table 3).

Seed counting and statistical analysis

Plants were germinated on 0.5 MS (Murashige and Skoog) agar plates after 3 days in dark at 4°C. Seedling were transferred to soil and grown at 22°C, under long days (16 h light / 8 h dark) for 6 weeks. A top opened flower was defined as position 1 and a silique at position 12 was collected for analyses in a blind manner. 10 siliques were sampled for independent lines as biological replicates and seeds were counted under a stereo microscope. The simultaneous comparisons of the different transgenic lines vs wild type were performed using the Dunnett procedure⁷⁴ for the primary endpoint number seeds per silique using the count transformation model⁷⁵. The Comprehensive R Archive Network packages multcomp⁷⁶ and cotram⁷⁵ were used in R, version 3.6.3.

405 **Data availability**

406 Data supporting the findings of this manuscript are available from the corresponding authors
407 upon reasonable request. A reporting summary for this article is available as a Supplementary
408 Information file. Coordinates and structure factors have been deposited in the Protein Data Bank
409 (PDB) with accession codes 7A0J (ACR4^{WD40}) and 7A0K (PpCR4^{WD40-CRD}).

410

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417

418 **Author contributions**

419 MH and SO designed the study, SO performed all biochemical and genetic experiments, SO
420 and MH phased and refined the structures, LAH performed the statistical analysis and SO and MH
421 wrote the manuscript.

422

423 **Conflict of interest**

424 The authors declare no conflict of interest.

425

426 **Figure legends**

427 **Figure 1. CRINKLY 4 receptor kinases harbor structurally unique β -propeller and cysteine-**
428 **rich domains.**

429 **a**, ACR4 domain scheme: SP, signal peptide; WD40, WD40 domain; CRD, cysteine-rich domain;
430 TM, transmembrane helix; JM, juxtamembrane region; CT, C-terminal tail. **b**, SDS-PAGE analysis
431 of purified CRINKLY4 proteins expressed in insect cells. **c**, Autoradiography *in vitro* kinase assay
432 of the wild-type ACR4 kinase domain fused to maltose-binding protein (MBP), and of the unfused
433 kinase domain carrying a point mutation (Asp659 \rightarrow Asn) in the active site. The coomassie-stained
434 gel loading control is shown in b (lanes on the right of the dotted line). **d**, Ribbon diagrams of
435 ACR4^{WD40} in two orientations and colored from N- (yellow) to C-terminus (green). Disulfide bonds
436 are shown in bonds representation and highlighted by yellow circles. **e**, Structure of PpCR4^{WD40-CRD}
437 shown in two different orientations and colored in blue (WD40 domain) and yellow (CRD),

438 respectively. The N-glycans visible in the electron density map are depicted in bonds representation
439 (in gray).c

440

441 **Figure 2. The ACR4 ectodomain does not bind the peptide hormone CLE40 *in vitro*.**

442 **a**, SDS-PAGE analysis of the biotinylated ACR4^{WD40-CRD} and AtBAM1^{LRR} ectodomains used for
443 binding experiments. **b**, Quantitative grating-coupled interferometry (GCI) binding assay of a
444 synthetic CLE40 peptide versus ACR4^{WD-CRD} and BAM1^{LRR}. Shown are sensorgrams with raw data
445 in red and their respective fits in black. Table summaries of kinetic parameters are shown alongside
446 (D_c , density of captured protein; k_t , mass transport coefficient; k_{on} , association rate constant; k_{off} ,
447 dissociation rate constant; K_d , dissociation constant; n.d., no detectable binding, n=3). **c**, Isothermal
448 titration calorimetry (ITC) experiment of ACR4^{WD-CRD} versus CLE40. No binding was detected in
449 this assay (n=3).

450

451 **Figure 3. CRINKLY4 ectodomains harbor an evolutionary conserved function.**

452 **a**, Reverse genetic rescue experiments of the seed development phenotype of *acr4-2*. Left panel:
453 Seed development phenotypes of wild type, *acr4-2* and a complemented line. Right panel: Ten
454 siliques per transgenic line from three independent homozygous T3 lines were pooled and plotted as
455 beeswarm plots with the bold line representing mean, whiskers indicating the standard deviation,
456 and circles depicting the raw data. Seed counts per silique significantly different from wild type
457 were determined by simultaneous comparisons of several mutants against wild type using the
458 Dunnett procedure (indicated by an asterisk). **b**, Ribbon diagram overview of PpCR4^{WD40-CRD} (colors
459 as in Fig. 1) and close-up view of the CRD superimposed to a type I TNF receptor ectodomain
460 (PDB-ID 1NCF⁷⁷; in gray). The six invariant disulfide bridges of CRINKLY4 CRDs are shown in
461 green, the disulfide bonds in TNFR are shown in gray (in bonds representation). **c**, Superposition of
462 the structurally homologous PpCR4^{CRD} (in yellow) and TNFR (in gray) core segments (r.m.s.d. is ~1
463 Å comparing 20 corresponding C α atoms). **d**, Analytical size-exclusion chromatography of
464 ACR4^{WD40-CRD} in the pre- or absence of Tris(2-carboxyethyl)phosphine (TCEP). Void (V_0), total (V_t),
465 and elution volumes for molecular-mass standards (Al, Aldolase, 158 kDa; Co, Conalbumin, 75
466 kDa; Ov, Ovalbumin, 44 kDa; CA, Carbonic Anhydrase, 29 kDa; R, Ribonuclease A, 13.7 kDa; Ap,
467 Aprotinin; 6.5 kDa) are indicated. **e**, SDS-PAGE analysis of fractions shown in d.

468

469 **Figure 4. The CRINKLY4 WD40 domain contains a putative ligand binding groove.**

470 **a**, Ribbon diagram of ACR4^{WD40} (in blue) with surface exposed conserved residues shown in bonds
471 representation (in orange) at the exposed surface. Blade numbers are indicated. **b**, Effect on surface

point-mutations on ACR4-mediated seed production. Ten siliques per transgenic line from three independent homozygous T3 complementation lines were pooled and plotted as beeswarm plots with the bold line representing mean, whiskers indicating the standard deviation, and circles depicting the raw data. The plots for wild type, *acr4-2* and ACR4 were generated from same data sets shown in Fig. 3a. Seed counts per silique significantly different from wild type were determined by simultaneous comparisons of several mutants against wild type using the Dunnett procedure (indicated by an asterisk). **c**, Molecular surface of the ACR4^{WD40} β -propeller domain ‘back side’ (in light blue). The positions of the mutated residues are highlighted in orange. **d**, Comparison of the ‘front sides’ of the structurally related WD40 domains of COP1 (PDB-ID 6QTO⁴³ left panel) and ACR4 (right panel, r.m.s.d is ~3.5 comparing 205 corresponding C α atoms). The COP1 VP-peptide ligand derived from the transcription factor HY5 is shown in yellow. Note the large and deep putative binding groove in the corresponding surface area in ACR4^{WD40}.

Supplementary Figure 1. Structure-based multiple sequence alignment of CRINKLY4 receptor ectodomains from different species.

Structure based T-COFFEE⁷⁸ sequence alignment and including a secondary structure assignment calculated with DSSP⁷⁹ (WD40 domain in blue, CRD in yellow). Invariant cysteine residues contributing to disulfide bonds in the WD40 domain or CRD domain are highlighted in yellow and green, respectively. Residues analyzed with point mutations in this study are shown in orange. Conserved residues in the WD40 – CRD domain interface are depicted in gray. Asterisks denote the location of experimentally confirmed N-glycosylation sites. Red arrows represent domain boundaries for the Δ TNFR/CRD deletion constructs in previous reports: (1)²⁴, (2)²⁰. ACR4 (*Arabidopsis thaliana*) UNIPROT-ID (<http://uniprot.org>) Q9LX29; PpCR4 (*Physcomitrella patens*) A9RKG8; ZmCR4 (*Zea mays*) O24585; OsCR4 (*Oryza sativa*) Q75J39; SmCR4 (*Selaginella moellendorffii*) D8T625. Note that the annotated SmCR4 sequence may be incomplete.

Supplementary Figure 2. CRINKLY4 receptor ectodomains behave as monomers in solution.

Analytical size-exclusion chromatography of the ACR4^{WD40-CRD}, ACR4^{WD40} and PpCR4^{WD40-CRD} in the presence or absence of enzymatic deglycosylation. The void volume (V₀), the total column volume (V_t), and the elution volumes for molecular-mass standards (Al, Aldolase, 158 kDa; Co, Conalbumin, 75 kDa; Ov, Ovalbumin, 44 kDa; CA, Carbonic Anhydrase, 29 kDa; R, Ribonuclease A, 13.7 kDa; Ap, Aprotinin; 6.5 kDa) are indicated.

Supplementary Figure 3. ACR4^{WD40} shares structural features with known WD40 domains.

506 Structural superposition of ACR4^{WD40} (blue ribbon diagram) with **a**, the secreted β -lactamase
 507 inhibitor protein II BLIP-II (PDB-ID 1JTD³⁹, in yellow) from the bacterium *Streptomyces exfoliatus*
 508 (r.m.s.d. is ~ 2.2 Å comparing 192 corresponding C α atoms), and **b**, with the WD40 domain of the
 509 UV-B photoreceptor UVR8 (PDB-ID 4D9S⁴⁰, r.m.s.d. is ~ 2.4 Å comparing 218 corresponding C α
 510 atoms). Note that SeBLIP-II and UVR8 shares the blade number and overall architecture with
 511 ACR4^{WD40}, but lack the buried N-terminal strand and the conserved disulfide bonds stabilizing each
 512 blade.

513

514 **Supplementary Figure 4. Expression and purification attempts of the AtCLV1 LRR**
 515 **ectodomain.**

516 Shown are immunoblot analyses monitoring the secreted expression of the AtCLV1 ectodomain
 517 (see Methods) with an anti-His antibody (left panels, Day, days post infection, MOI, multiplicity of
 518 infection; SN, supernatant; P, pellet). Right panel: Preparative size-exclusion chromatography of the
 519 purified AtCLV1 ectodomain reveals the presence of large aggregates. The void (V₀), total (V_t), and
 520 elution volumes for molecular-mass standards (Al, Aldolase, 158 kDa; Co, Conalbumin, 75 kDa;
 521 Ov, Ovalbumin, 44 kDa; CA, Carbonic Anhydrase, 29 kDa; R, Ribonuclease A, 13.7 kDa; Ap,
 522 Aprotinin; 6.5 kDa) are indicated.

523

524 **Supplementary Figure 5. Only small WD40 - CRD inter-domain movements can be observed**
 525 **in the PpCR4 crystal structure.**

526 Structural superposition of the eight molecules located in the asymmetric unit of the PpCR4^{WD40-CRD}
 527 crystal structure (r.m.s.d. is ~ 0.3 - 0.5 Å comparing 360 corresponding C α atoms). Individual
 528 molecules are shown in different colors as C α traces.

529

530 **Supplementary Figure 6. Overview of the WD40 – CRD domain interface in the PpCRD^{WD40-}**
 531 **CRD structure.**

532 Shown is a ribbon diagram of the PpCR4 ectodomain (colored according to Fig. 1e) with selected
 533 interface residues shown in bonds representation. Hydrogen bonds and salt bridges are indicated by
 534 dotted lines.

535

536 **Supplementary Figure 7. Structural visualization of the TNFR/CRD domain boundaries used**
 537 **in this and in previous studies.**

538 Ribbon diagram of PpCR4^{WD40-CRD} with the WD40 domain shown in blue and the experimentally
 539 determined CRD domain boundaries shown in yellow (left panel). The previously used TNFR

540 domain boundaries^{20,24} derived from sequence analysis (in orange) omit the most N-terminal β -
541 strand in the CRD (in blue, indicated by a black arrow).

542

543 **Supplementary Figure 8. Structurally conserved loop regions contribute to the formation of a**
544 **putative ligand binding groove in CRINKLY4 WD40 domains.**

545 **a**, Structural superposition of the isolated WD40 domain from ACR4 (blue) and PpCR4 (light gray,
546 r.m.s.d. is ~ 1.4 Å comparing 246 corresponding C $_{\alpha}$ atoms reveals the loop regions contributing to
547 the formation of a putative ligand binding groove to adopt similar orientations in both structures. **b**,
548 A temperature (B-) factor plot of PpCR4^{WD40-CRD} (molecule chain A) reveals little structural
549 flexibility for the secondary structure elements forming part of the putative binding groove, while
550 the partially disordered loops connecting the blades of the β -propeller and the loops connecting the
551 CRD appear mobile in the PpCR4^{WD40-CRD} crystal structure.

552 **Supplementary Table 1. Crystallographic data collection and refinement statistics.**

PDB-ID	ACR4^{WD40}	PpCR4^{WD40-CRD}
Data collection	7A0J	7A0K
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	75.0, 88.0, 88.6, 90, 90.1, 90	88.6, 184.0, 98.2
α, β, γ (°)	90, 90.1, 90	90, 96.1, 90
Resolution (Å)	48.05 – 1.95 (2.07 – 1.95)	45.87 – 2.70 (2.86 – 2.70)
<i>R</i> _{meas} [#]	0.125 (1.94)	0.151 (1.89)
CC(1/2) [#]	1.0 (0.4)	1.0 (0.46)
<i>I</i> /σ <i>I</i> [#]	8.75 (0.91)	12.01 (0.98)
Completeness (%) [#]	99.7 (98.3)	99.9 (99.7)
Redundancy [#]	6.8 (6.6)	7.0 (6.6)
Wilson B-factor [#]	40.2	71.5
Refinement		
Resolution (Å)	48.05 – 1.95	45.87 – 2.70
No. reflections	79,774	85,621
<i>R</i> _{work} / <i>R</i> _{free} ^{\$}	0.22 (0.24)	0.22 (0.25)
No. atoms		
protein	7,980	20,414
carbohydrate/buffer	106	524
solvent	270	134
Res. B-factors ^{\$}		
protein	53.7	90.8
carbohydrate/buffer	61.6	97.6
solvent	48.4	66.1
R.m.s deviations ^{\$}		
bond lengths (Å)	0.0135	0.0027
bond angles (°)	1.64	0.60
Ramachandran plot ^{\$} :		
most favored regions (%)	97.0	96.1
outliers (%)	0	0.2
MolProbity score ^{\$}	1.06	1.27

553 [#]as defined in XDS⁶¹

554 ^{\$}as defined Refmac5⁶⁵ or phenix.refine⁷⁰

555 ^{\$}as defined in Molprobity⁸⁰

556 Supplementary Table 2. Primers used in this study

Primer Name	Sequence	Description
ACR4prom_B4-F	GGGGACAACCTTTGTATAGAAAAGTTGACGAGATAGTCAAGAAATGGCCTTTC	cloning of ACR4 promoter region
ACR4prom_B1r-R	GGGGACTGCTTTTTGTACAAAAGTCTCTTTTCAAAGTCAACACACACG	cloning of ACR4 promoter region
ACR4cds_B1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGAATGAGAATGTTGAAACGAGAG	cloning of ACR4 coding sequence
ACR4cds_B2r-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAAATTATGATGCAAGAACAAGC	cloning of ACR4 coding sequence
ACR4delK_B2rR	GGGGACCACTTTGTACAAGAAAGCTGGGTATTGCAGCTCATCAAGATC	Δcyto construct for transgenic plant
ACR4_K540R_For	GCAGTGAGAAGAGCGATAATGTCATCAGACAAACAGAAG	site directed mutagenesis for transgenic plant
ACR4_K540R_Rev	CGCTCTTCTCACTGCAACAGTGGTTCCATCTCTCAG	site directed mutagenesis for transgenic plant
ACR4_delTNFR_Fw	CCTGCTTCTATCCCTAAGTTTGGTCACTGCAGCTAC	ΔCRD construct for transgenic plant
ACR4_delTNFR_Rv	CAGTGACCAAACTTAGGGATAGAAGCAGGGAAACC	ΔCRD construct for transgenic plant
ACR4_D84A_F	GGGTGGAGCTGGGTTTATGTGTGGGC	site directed mutagenesis for transgenic plant
ACR4_D84A_R	ATAAACCCAGCTCCACCCGTAAACCG	site directed mutagenesis for transgenic plant
ACR4_F105A_F	CAGTGCAGCTATTCAAATGGGAGTTTCCTC	site directed mutagenesis for transgenic plant
ACR4_F105A_R	ATTTGAATAGCTGCACTGTTTCCCCAAC	site directed mutagenesis for transgenic plant
ACR4_D127A_F	TGCTGGTGCTTACCATCTTTGTGGTTTGAG	site directed mutagenesis for transgenic plant
ACR4_D127A_R	AGATGGTAAGCACCAGCACTAACTTCTAAATAC	site directed mutagenesis for transgenic plant
ACR4_Y157A_F	TTGGGGTGCTAATATGACAAGAACTTTGTCTTTG	site directed mutagenesis for transgenic plant
ACR4_Y157A_R	GTCATATTAGCACCCCAACAATCAACAAG	site directed mutagenesis for transgenic plant
ACR4_N158A_F	GGGTTACGCTATGACAAGAACTTTGTCTTTG	site directed mutagenesis for transgenic plant
ACR4_N158A_R	CTTGTCATAGCGTAACCCCAACAATCAAC	site directed mutagenesis for transgenic plant
ACR4_Y157,N158A_F	TTGGGGTGCTGCTATGACAAGAACTTTGTCTTTG	site directed mutagenesis for transgenic plant
ACR4_Y157,N158A_R	CTTGTCATAGCAGCACCCCAACAATCAACAAG	site directed mutagenesis for transgenic plant
ACR4_Y218A_F	TGGTGGAGCTCATGTTTGTGGCAITCTTG	site directed mutagenesis for transgenic plant
ACR4_Y218A_R	ACAAACATGAGCTCCACCAGCTGCAATTTTC	site directed mutagenesis for transgenic plant
ACR4_N158Q_For	GGTTACCAGATGACAAGAACTTTGTCTTTGATAAGCAG	site directed mutagenesis for transgenic plant
ACR4_N158Q_Rev	TGTCATCTGGTAACCCCAACAATCAACAAG	site directed mutagenesis for transgenic plant
ACR4_N196Q_For	GATGAGCAGAGTAGTCAAGTAATCAGTTTAATCCCCAAG	site directed mutagenesis for transgenic plant
ACR4_N196Q_Rev	ACTACTCTGCTCATCTCCCCAACAGAAAACCGAC	site directed mutagenesis for transgenic plant
ACR4_pBB2_ins_f	TTATTATACCGTCCCACCATCGGGCGGGATGAGAATGTTGAAACGAGAG	protein expression in insect cell
334-pBB2_Rv	CCCTGGAAGTACAGGTTCTCGAGTTAAGGGATAGAAGCAGGGAAC	protein expression in insect cell
ACR4_pBB2_423r	CATGCAGAGCCCTGGAAGTACAGGTTCTCGAGTCCTTTTTCCTTGCCTCCAC	protein expression in insect cell
ACR4_423_Avi_Rv	AGCCTCGAAGATGTCGTTTCAGACCCTCGAGTCCTTTTTCCTTGCTCCACTGGTAGCC	protein expression in insect cell
ACR4_497-F_Nco1	CGGCCATGGCTAGAGTTTCACTTATGAGGAACCTG	protein expression in insect cell
ACR4_792-R_Not1	TTAGCGGCCGCTTATTATAGCTGTGCAAGCGCTCG	protein expression in insect cell
PpCR4_pBB2_Fw	ATACCGTCCCACCATCGGGCGGGAGCTCATGCCTGTACTCGTGCG	protein expression in insect cell
PpCR4_P405_Rv	TCGAAGATGTCGTTTCAGACCCTCGAGTGGAGCCTTTGAAGGGTTATAAC	protein expression in insect cell
CLV1co_T25_Fw	TGTTGGCCTCTCGCTCGGGGCTACCATGGGATACACCGACATGAGGTGC	protein expression in insect cell

CLV1co_P621_Rv	AGCCTCGAAGATGTCGTTTCAGACCCTCGAGAGGGCAGGACA CACGG	protein expression in insect cell
BAM1_BiP_F	CTTTGTTGGCCTCTCGCTCGGGGCTACCATGGGACGACCAATC TCCGAG	protein expression in insect cell
BAM1_Avi_R	AGCCTCGAAGATGTCGTTTCAGACCCTCGAGTGATAAAGGTCC TTTACTATGACTC	protein expression in insect cell
ACR4_D659N_For	GTAGCTAACTTTGGTCTCTCCTTACTTGGTCCTGTCG	site directed mutagenesis for kinase-dead recombinant protein
ACR4_D659N_Rev	GACCAAAGTTAGCTACTCGAGCATTGTGTCTTCATC	site directed mutagenesis for kinase-dead recombinant protein

F, For, Fw: Forward; R, Rev, Rv: Reverse

Supplementary Table 3. Transgenic lines generated in this study

Name	residues (amino acids)	Tag	Resistance	Genetic background
ACR4	1 – 895	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 Δcyto	1 – 492	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 kinase dead	1 – 895 (K540R)	6x HA	Hygromycin	<i>acr4-2</i>
ACR4 ΔCRD	1 – 895 with deletion 335 – 423	mCitrine	Hygromycin	<i>acr4-2</i>
PpCR4	1 – 893	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 D84A	1 – 895 (D84A)	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 F105A	1 – 895 (F105A)	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 D127A	1 – 895 (D127A)	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 Y157A	1 – 895 (Y157A)	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 N158A	1 – 895 (N158A)	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 Y157A, N158A	1 – 895 (Y157A/N158A)	mCitrine	Hygromycin	<i>acr4-2</i>
ACR4 N158Q, N196Q	1 – 895 (N158Q/N196Q)	6x HA	Hygromycin	<i>acr4-2</i>
ACR4 Y218A	1 – 895 (Y218A)	mCitrine	Hygromycin	<i>acr4-2</i>

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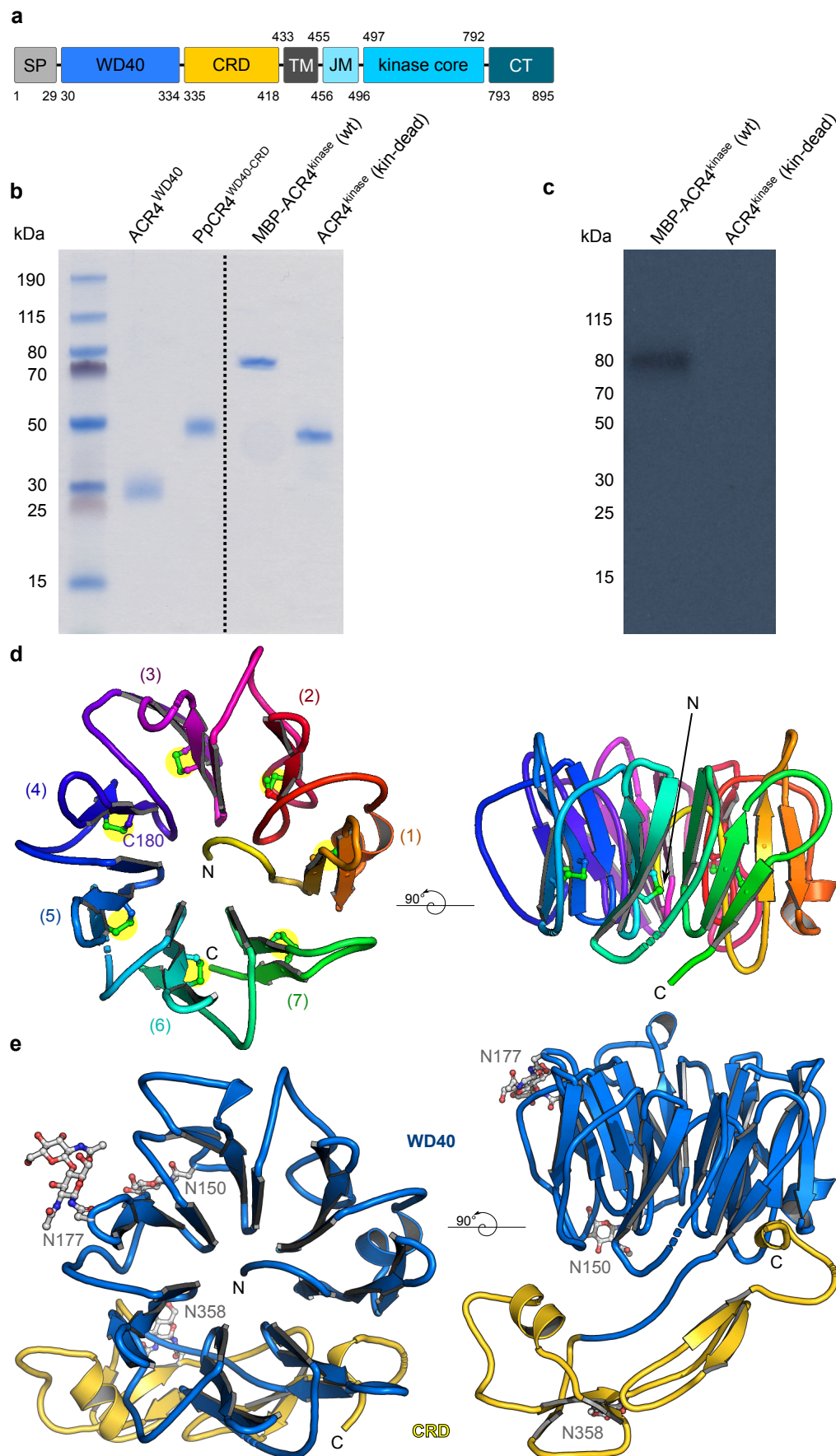


Figure 1. CRINKLY 4 receptor kinases harbor structurally unique β -propeller and cysteine-rich domains.

a, ACR4 domain scheme: SP, signal peptide; WD40, WD40 domain; CRD, cysteine-rich domain; TM, transmembrane helix; JM, juxtamembrane region; CT, C-terminal tail. **b**, SDS-PAGE analysis of purified CRINKLY4 proteins expressed in insect cells. **c**, Autoradiography *in vitro* kinase assay of the wild-type ACR4 kinase domain fused to maltose-binding protein (MBP), and of the unfused kinase domain carrying a point mutation (Asp659Asn) in the active site. The coomassie-stained gel loading control is shown in **b** (lanes on the right of the dotted line). **d**, Ribbon diagrams of ACR4^{WD40} in two orientations and colored from N- (yellow) to C-terminus (green). Disulfide bonds are shown in bonds representation and highlighted by yellow circles. **e**, Structure of PpCR4^{WD40-CRD} shown in two different orientation and colored in blue (WD40 domain) and yellow (CRD), respectively. The N-glycans visible in the electron density map are depicted in bonds representation (in gray).c++

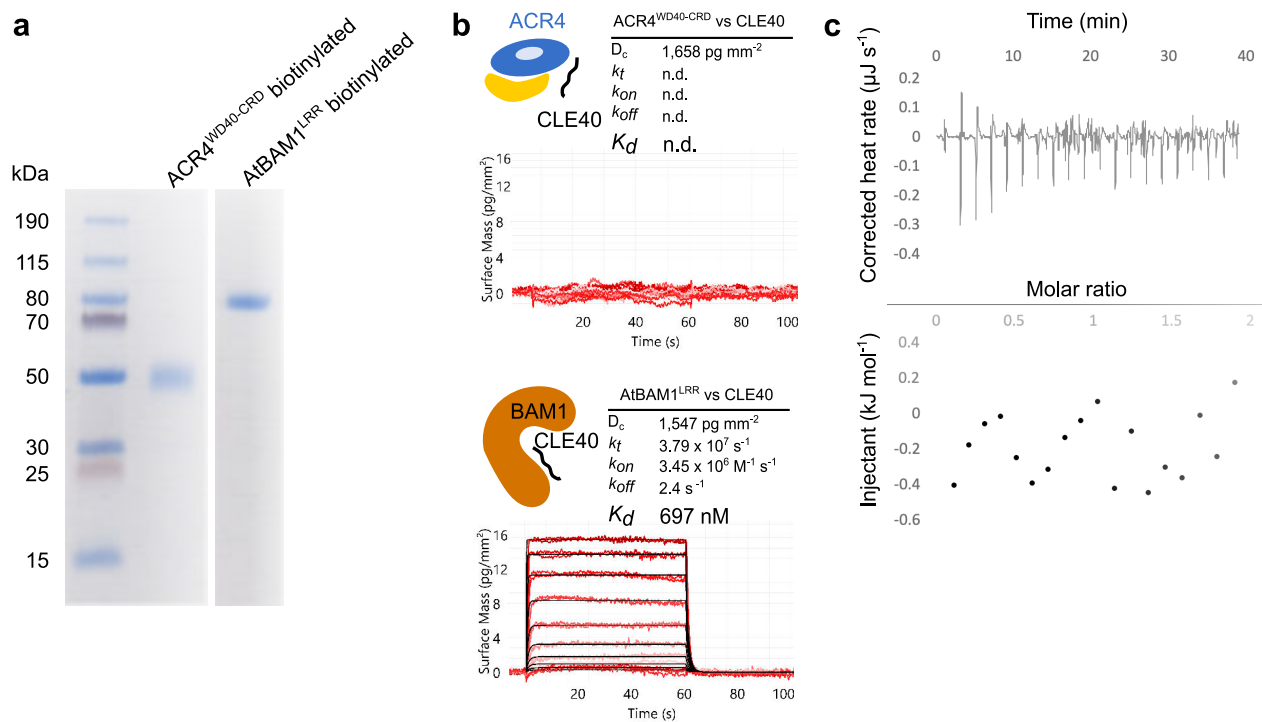


Figure 2. The ACR4 ectodomain does not bind the peptide hormone CLE40 *in vitro*.

a, SDS-PAGE analysis of the biotinylated ACR4^{WD40-CRD} and AtBAM1^{LRR} ectodomains used for binding experiments. **b**, Quantitative grating-coupled interferometry (GCI) binding assay of a synthetic CLE40 peptide versus ACR4^{WD40-CRD} and BAM1^{LRR}. Shown are sensorgrams with raw data in red and their respective fits in black. Table summaries of kinetic parameters are shown alongside (D_c , density of captured protein; k_t , mass transport coefficient; k_{on} , association rate constant; k_{off} , dissociation rate constant; K_d , dissociation constant; n.d., no detectable binding, n=3). **c**, Isothermal titration calorimetry (ITC) experiment of ACR4^{WD40-CRD} versus CLE40. No binding was detected in this assay (n=3).

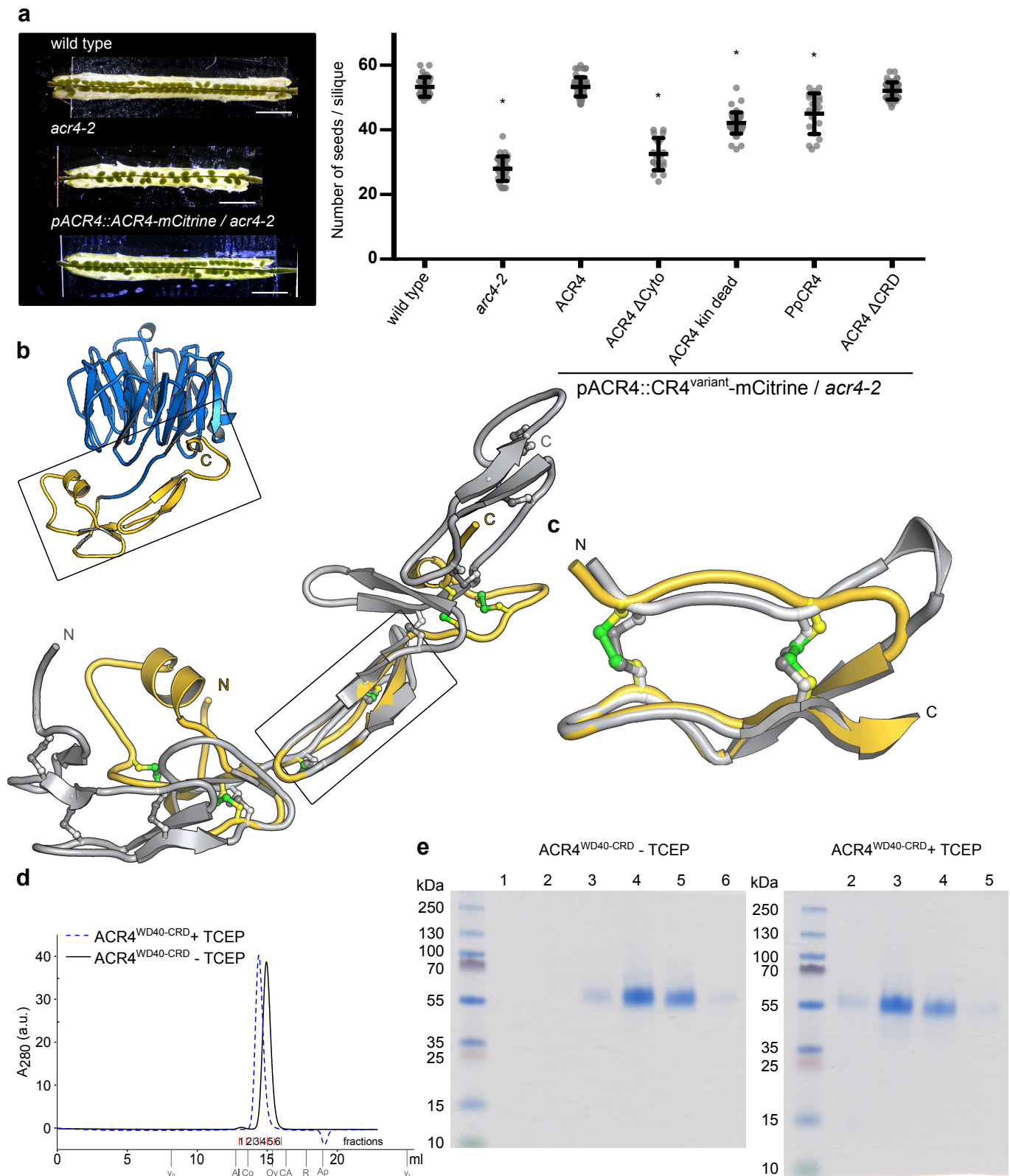


Figure 3. CRINKLY4 ectodomains harbor an evolutionary conserved function.

a, Reverse genetic rescue experiments of the seed filling phenotype of *acr4-2*. Left panel: Seed development phenotypes of wild type, *acr4-2* and a complemented line. Right panel: Ten siliques per transgenic line from three independent homozygous T3 lines were pooled and plotted as beeswarm plots with the bold line representing mean, whiskers indicating the standard deviation, and circles depicting the raw data. Seed counts per silique significantly different from wild type were determined by simultaneous comparisons of several mutants against wild type using the Dunnnett procedure (indicated by an asterisk). **b**, Ribbon diagram overview of PpCR4^{WD40-CRD} (colors as in Fig. 1) and close-up view of the CRD superimposed to a type I TNF receptor ectodomain (PDB-ID 1NCF⁷⁷; in gray). The six invariant disulfide bridges of CRINKLY4 CRDs are shown in green, the disulfide bonds in TNFR are shown in gray (in bonds representation). **c**, Superposition of the structurally homologous PpCR4^{CRD} (in yellow) and TNFR (in gray) core segments (r.m.s.d. is ~1 Å comparing 20 corresponding C α atoms). **d**, Analytical size-exclusion chromatography of ACR4^{WD40-CRD} in the pre- or absence of Tris(2-carboxyethyl)phosphine (TCEP). Void (V₀), total (V_t), and elution volumes for molecular-mass standards (Al, Aldolase, 158 kDa; Co, Conalbumin, 75 kDa; Ov, Ovalbumin, 44 kDa; CA, Carbonic Anhydrase, 29 kDa; R, Ribonuclease A, 13.7 kDa; Ap, Aprotinin; 6.5 kDa) are indicated. **e**, SDS-PAGE analysis of fractions shown in d.

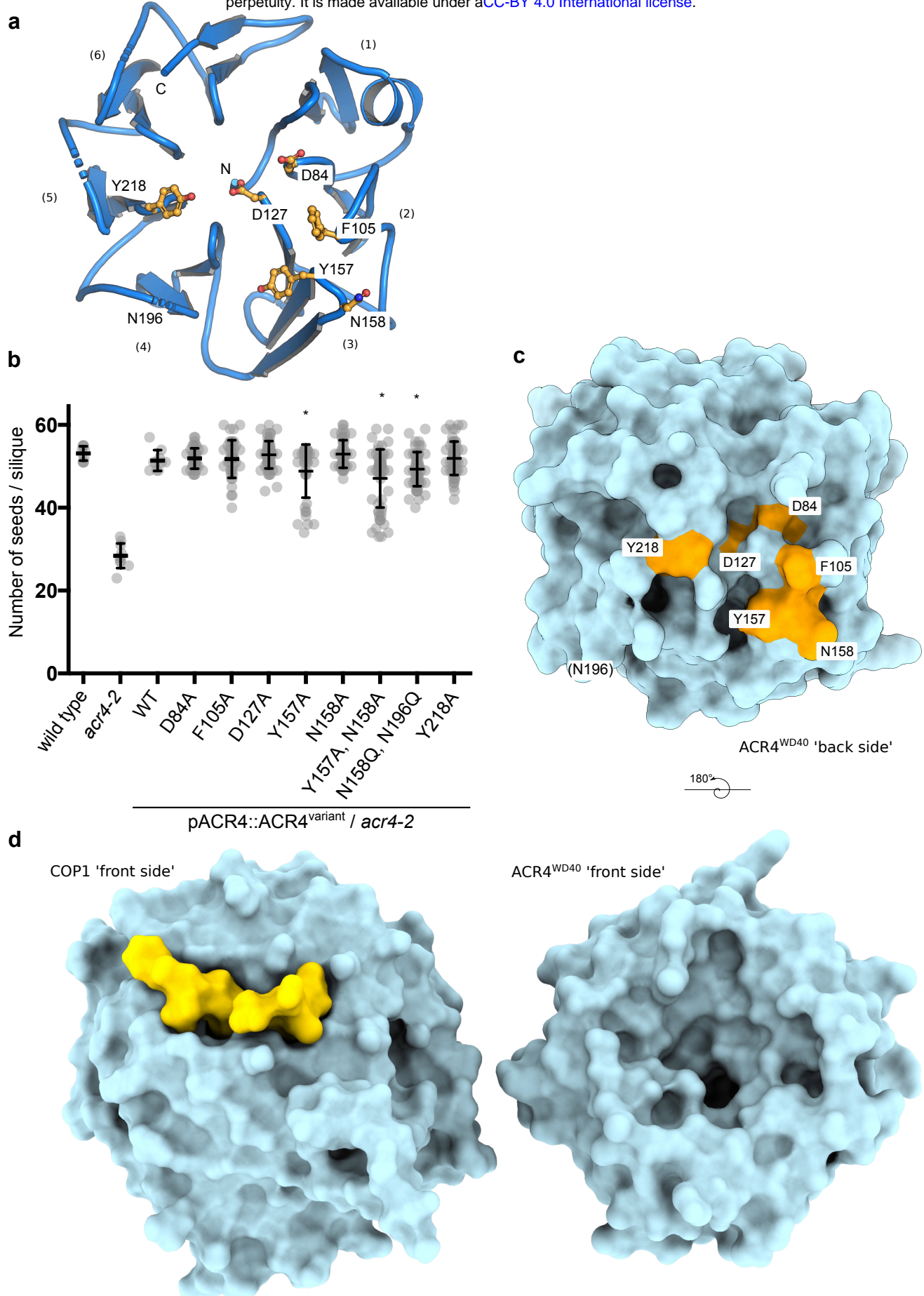
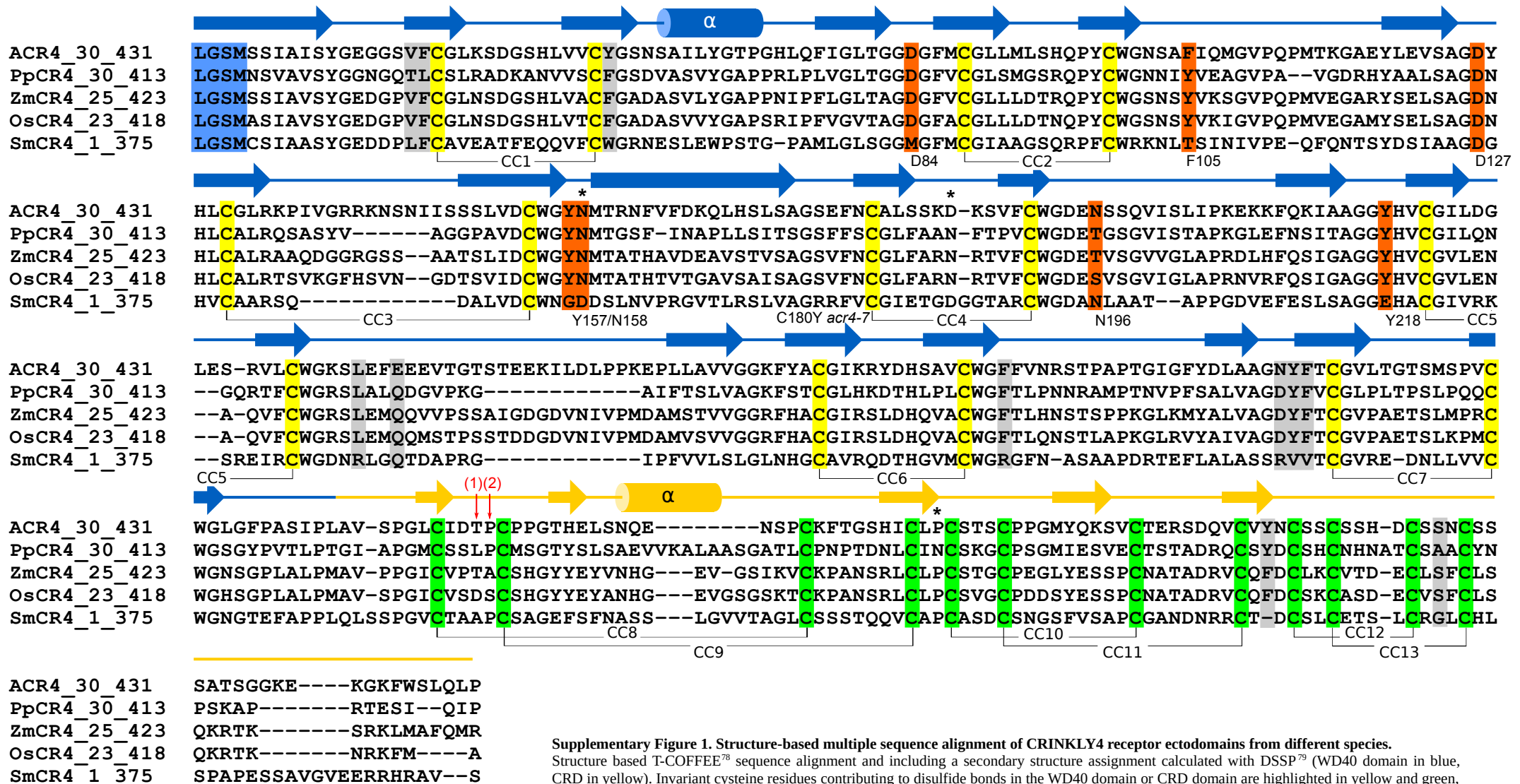
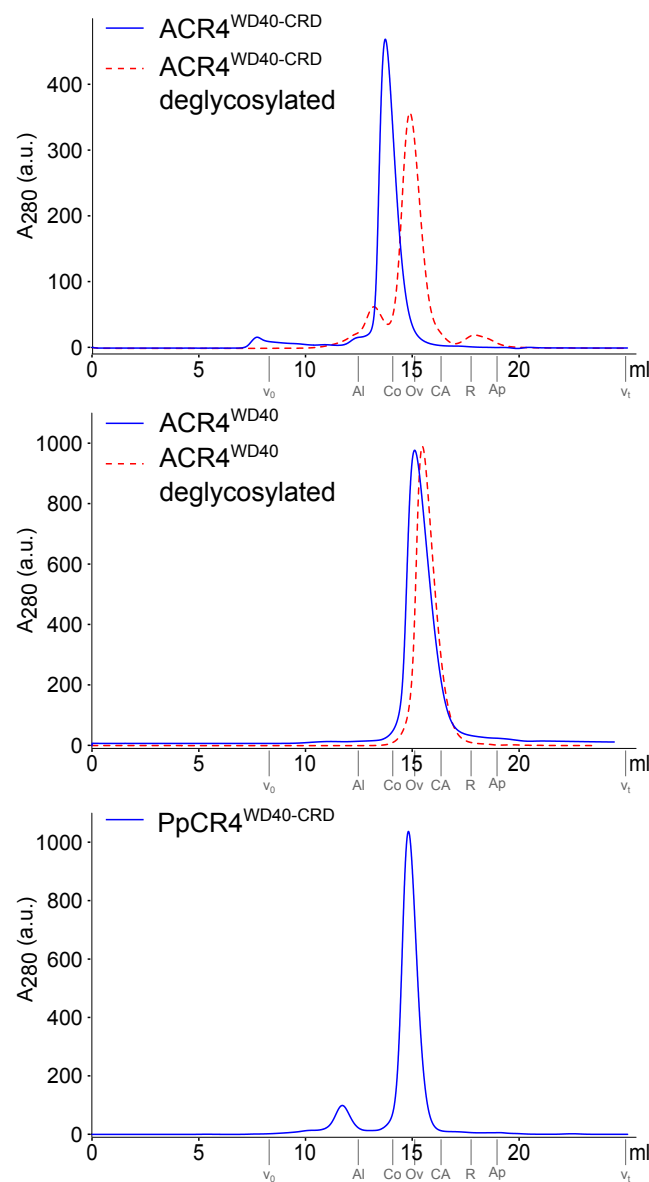


Figure 4. The CRINKLY4 WD40 domain contains a putative ligand binding groove.

a, Ribbon diagram of ACR4^{WD40} (in blue) with surface exposed conserved residues shown in bonds representation (in orange) at the exposed surface. Blade numbers are indicated. **b**, Effect on surface point-mutations on ACR4-mediated seed production. Ten siliques per transgenic line from three independent homozygous T3 complementation lines were pooled and plotted as beeswarm plots with the bold line representing mean, whiskers indicating the standard deviation, and circles depicting the raw data. The plots for wild type, *acr4-2* and ACR4 were generated from same data sets shown in Fig. 3a. Seed counts per silique significantly different from wild type were determined by simultaneous comparisons of several mutants against wild type using the Dunnett procedure (indicated by an asterisk). **c**, Molecular surface of the ACR4^{WD40} β-propeller domain 'back side' (in light blue). The positions of the mutated residues are highlighted in orange. **d**, Comparison of the 'front sides' of the structurally related WD40 domains of COP1 (PDB-ID 6QTO⁴³ left panel) and ACR4 (right panel, r.m.s.d is ~3.5 comparing 205 corresponding C_α atoms). The COP1 VP-peptide ligand derived from the transcription factor HY5 is shown in yellow. Note the large and deep putative binding groove in the corresponding surface area in ACR4^{WD40}.

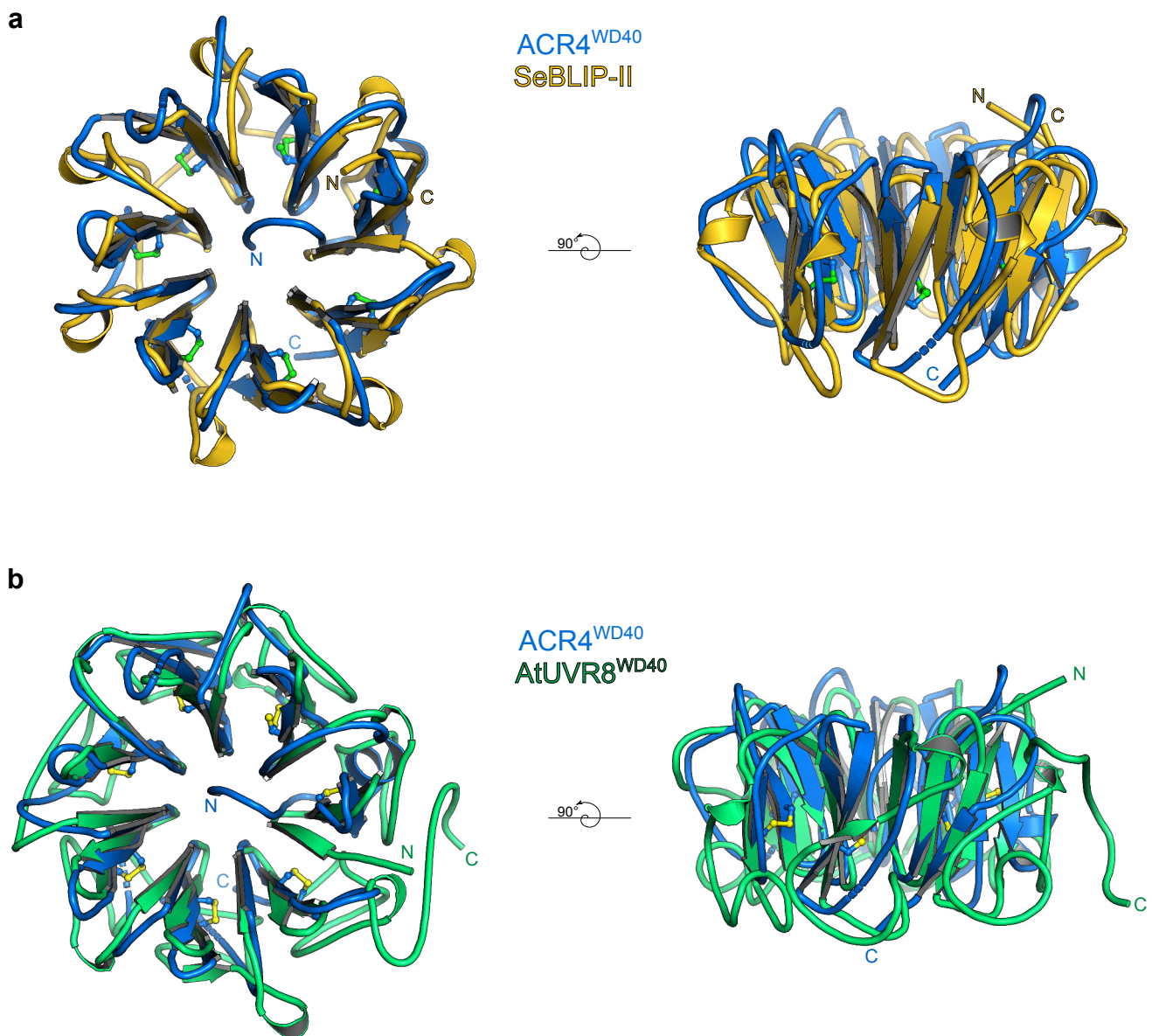


Supplementary Figure 1. Structure-based multiple sequence alignment of CRINKLY4 receptor ectodomains from different species. Structure based T-COFFEE⁷⁸ sequence alignment and including a secondary structure assignment calculated with DSSP⁷⁹ (WD40 domain in blue, CRD in yellow). Invariant cysteine residues contributing to disulfide bonds in the WD40 domain or CRD domain are highlighted in yellow and green, respectively. Residues analyzed with point mutations in this study are shown in orange. Conserved residues in the WD40 – CRD domain interface are depicted in gray. Asterisks denote the location of experimentally confirmed N-glycosylation sites. Red arrows represent domain boundaries for the TNFR/CRD deletion constructs in previous reports: (1)²⁴, (2)²⁰. ACR4 (*Arabidopsis thaliana*) UNIPROT-ID (<http://uniprot.org>) Q9LX29; PpCR4 (*Physcomitrella patens*) A9RK88; ZmCR4 (*Zea mays*) O24585; OsCR4 (*Oryza sativa*) Q75J39; SmCR4 (*Selaginella moellendorffii*) D8T625. Note that the annotated SmCR4 sequence may be incomplete.



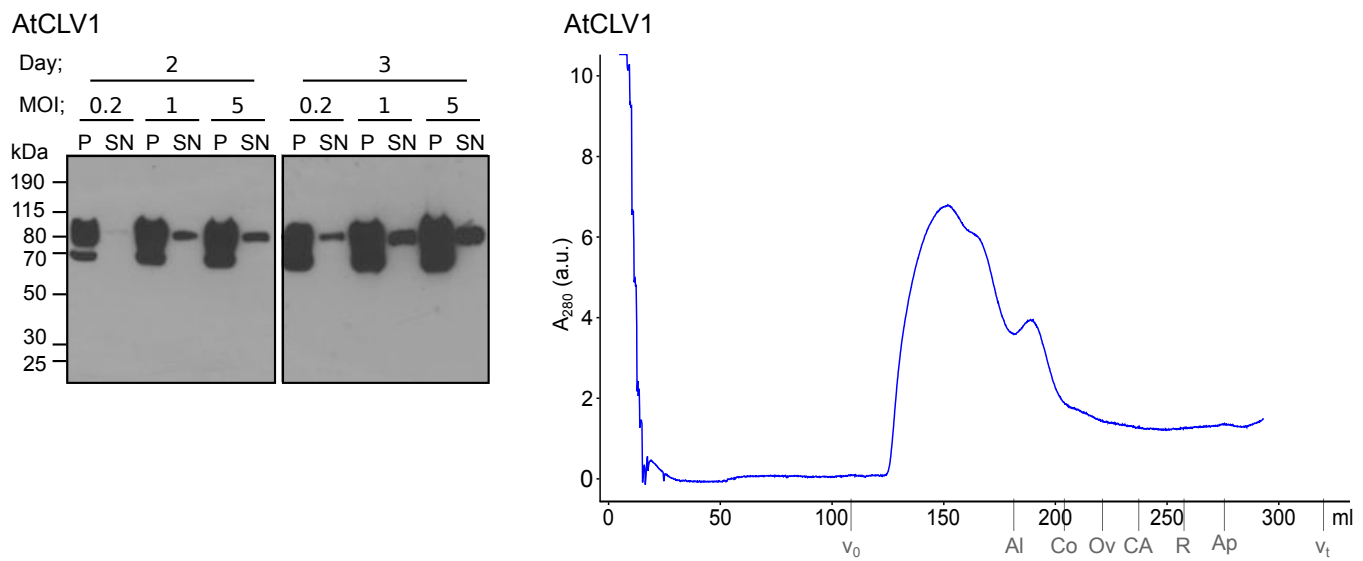
Supplementary Figure 2. CRINKLY4 receptor ectodomains behave as monomers in solution.

Analytical size-exclusion chromatography of the ACR4^{WD40-CRD}, ACR4^{WD40} and PpCR4^{WD40-CRD} in the presence or absence of enzymatic deglycosylation. The void volume (V₀), the total column volume (V_t), and the elution volumes for molecular-mass standards (Al, Aldolase, 158 kDa; Co, Conalbumin, 75 kDa; Ov, Ovalbumin, 44 kDa; CA, Carbonic Anhydrase, 29 kDa; R, Ribonuclease A, 13.7 kDa; Ap, Aprotinin; 6.5 kDa) are indicated.



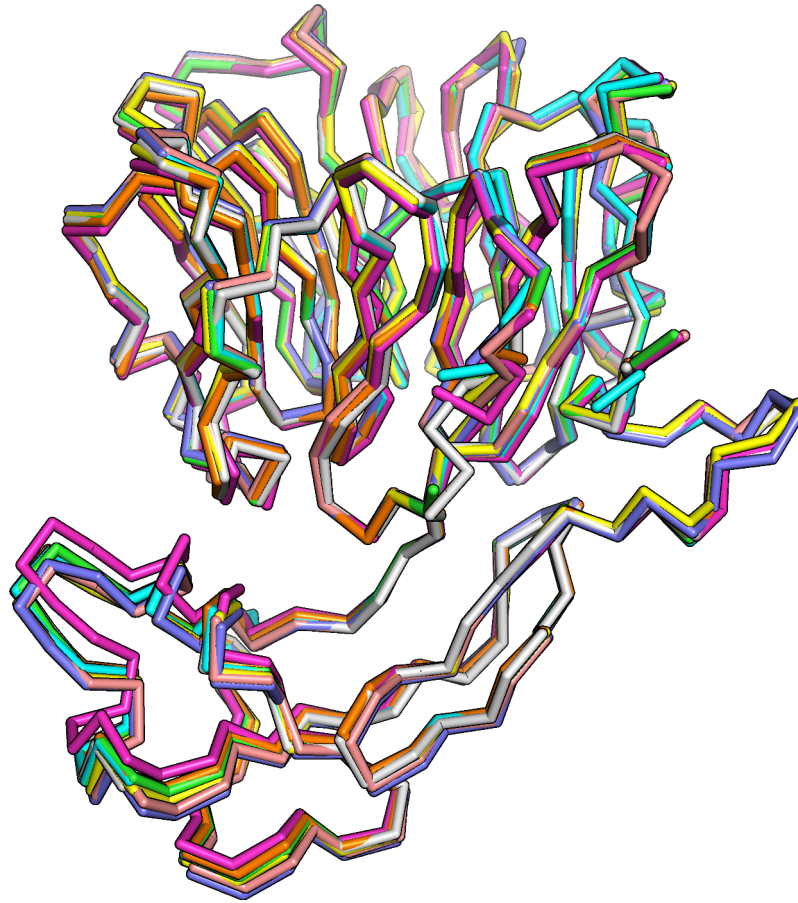
Supplementary Figure 3. ACR4^{WD40} shares structural features with known WD40 domains.

Structural superposition of ACR4^{WD40} (blue ribbon diagram) with **a**, the secreted β -lactamase inhibitor protein II BLIP-II (PDB-ID 1JTD³⁹, in yellow) from the bacterium *Streptomyces exfoliatus* (r.m.s.d. is ~ 2.2 Å comparing 192 corresponding C α atoms), and **b**, with the WD40 domain of the UV-B photoreceptor UVR8 (PDB-ID 4D9S⁴⁰, r.m.s.d. is ~ 2.4 Å comparing 218 corresponding C α atoms). Note that SeBLIP-II and UVR8 shares the blade number and overall architecture with ACR4^{WD40}, but lack the buried N-terminal strand and the conserved disulfide bonds stabilizing each blade.



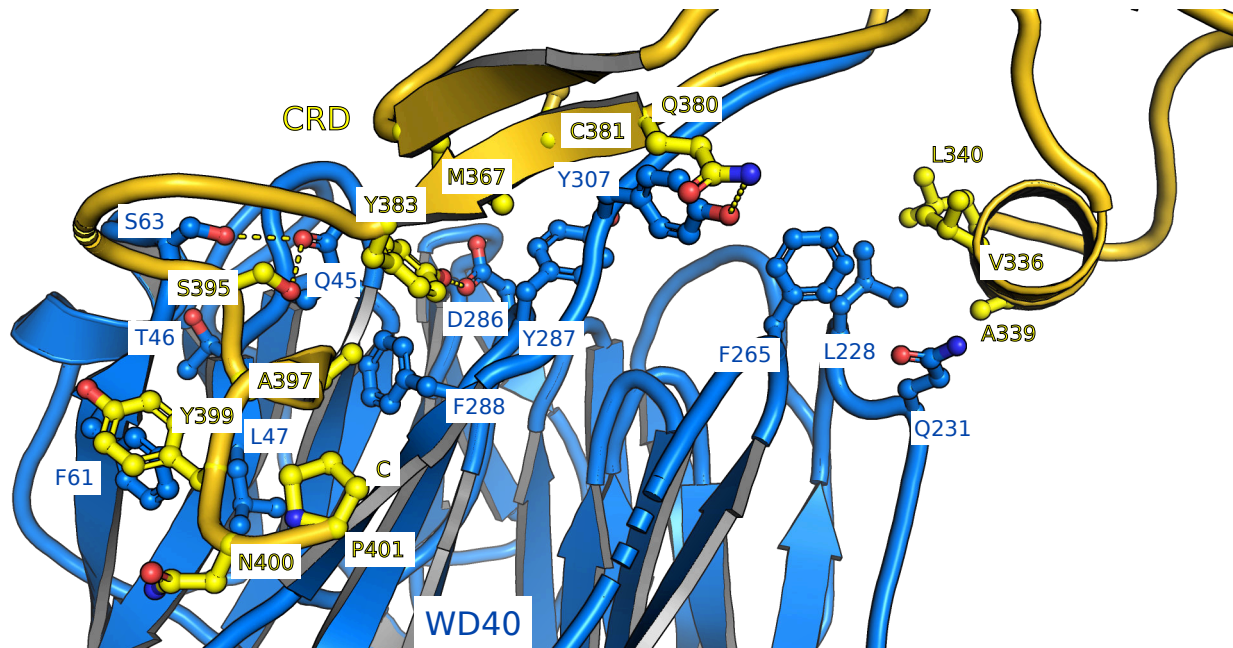
Supplementary Figure 4. Expression and purification attempts of the AtCLV1 LRR ectodomain.

Shown are immunoblot analyses monitoring the secreted expression of the AtCLV1 ectodomain (see Methods) with an anti-His antibody (left panels, Day, days post infection, MOI, multiplicity of infection; SN, supernatant; P, pellet). Right panel: Preparative size-exclusion chromatography of the purified AtCLV1 ectodomain reveals the presence of large aggregates. The void (V_0), total (V_t), and elution volumes for molecular-mass standards (Al, Aldolase, 158 kDa; Co, Conalbumin, 75 kDa; Ov, Ovalbumin, 44 kDa; CA, Carbonic Anhydrase, 29 kDa; R, Ribonuclease A, 13.7 kDa; Ap, Aprotinin; 6.5 kDa) are indicated.



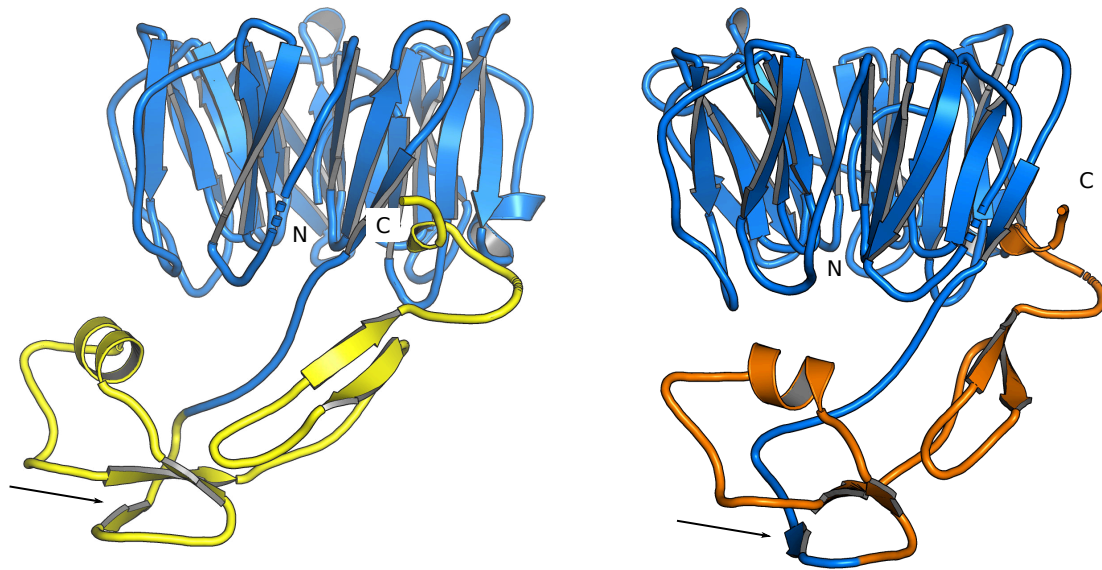
Supplementary Figure 5. Only small WD40 - CRD inter-domain movements can be observed in the PpCR4 crystal structure.

Structural superposition of the eight molecules located in the asymmetric unit of the PpCR4^{WD40-CRD} crystal structure (r.m.s.d. is ~0.3-0.5 Å comparing 360 corresponding C_α atoms). Individual molecules are shown in different colors as C_α traces.



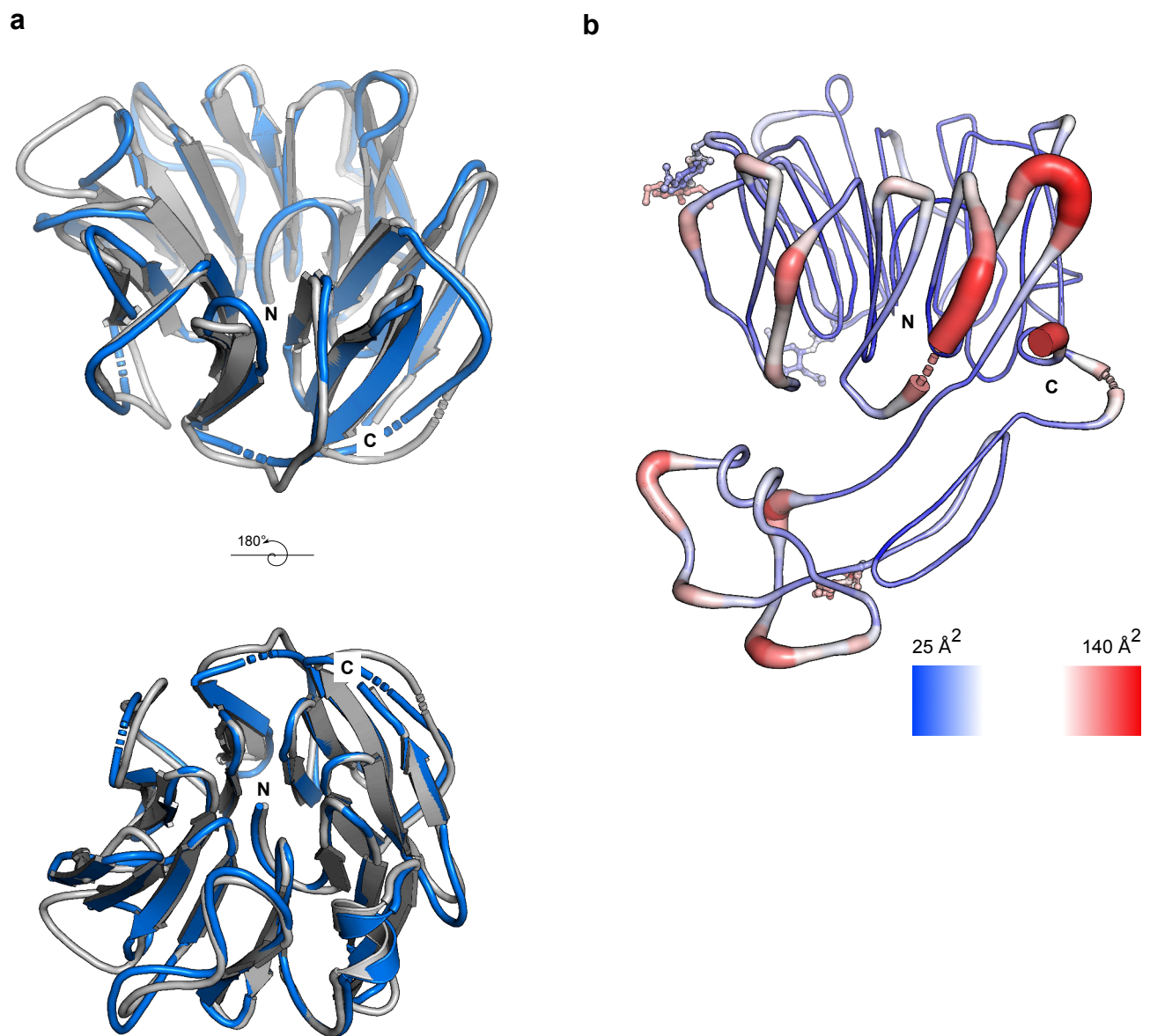
Supplementary Figure 6. Overview of the WD40 – CRD domain interface in the PpCRD^{WD40-CRD} structure.

Shown is a ribbon diagram of the PpCR4 ectodomain (colored according to Fig. 1e) with selected interface residues shown in bonds representation. Hydrogen bonds and salt bridges are indicated by dotted lines.



Supplementary Figure 7. Structural visualization of the TNFR/CRD domain boundaries used in this and in previous studies.

Ribbon diagram of PpCR4^{WD40-CRD} with the WD40 domain shown in blue and the experimentally determined CRD domain boundaries shown in yellow (left panel). The previously used TNFR domain boundaries^{20,24} derived from sequence analysis (in orange) omit the most N-terminal β -strand in the CRD (in blue, indicated by a black arrow).



Supplementary Figure 8. Structurally conserved loop regions contribute to the formation of a putative ligand binding groove in CRINKLY4 WD40 domains.

a, Structural superposition of the isolated WD40 domain from ACR4 (blue) and PpCR4 (light gray, r.m.s.d. is ~ 1.4 Å comparing 246 corresponding C $_{\alpha}$ atoms) reveals the loop regions contributing to the formation of a putative ligand binding groove to adopt similar orientations in both structures. **b**, A temperature (B-) factor plot of PpCR4^{WD40-CRD} (molecule chain A) reveals little structural flexibility for the secondary structure elements forming part of the putative binding groove, while the partially disordered loops connecting the blades of the β -propeller and the loops connecting the CRD appear mobile in the PpCR4^{WD40-CRD} crystal structure.