The growing story of (ARABIDOPSIS) CRINKLY 4

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Short statement: Here, we comprehensively review available literature on evolutionary, biochemical, molecular and genetic aspects of (A)CR4.

ABSTRACT

Receptor kinases play important roles in plant growth and development, but only few of them have been functionally characterised in depth. Over the past decade CRINKLY 4 (CR4)-related research peaked through a newly discovered role of ARABIDOPSIS CR4 (ACR4) in the root. Here, we comprehensively review the available (A)CR4 literature and describe its role in embryo, seed, shoot and root development, but also flag an unexpected role in plant defence. In addition, we discuss ACR4 domains and protein structure, describe known ACR4-interacting proteins and substrates, and elaborate on the transcriptional regulation of *ACR4*. Finally, we address the missing knowledge in our understanding of ACR4 signalling.

Key words: root, epidermis, flower, seed, embryo, receptor kinase, ACR4, *Arabidopsis*, maize, rice

INTRODUCTION

Cell-cell communication plays a crucial role in plant growth and development and relies to a large extent on peptide ligand−receptor kinase signalling mechanisms (Czyzewicz, et al., 2013; De Smet, et al., 2009; Murphy & De Smet, 2014; Murphy, et al., 2012; Tavormina, et al., 2015). Over 600 receptor-like kinase genes have been identified in *Arabidopsis thaliana* and similar or even higher numbers were found in other plant species (Lehti-Shiu, et al., 2009; Shiu, et al., 2004; Shiu & Bleecker, 2001; Liu, et al., 2002). Mutations in receptor kinases often lead to obvious and crucial defects as they are involved in various developmental and environmental responses, such as maintenance of root and shoot apical meristems (De Smet, et al., 2009; Stahl & Simon, 2012; Wierzba & Tax, 2013; Soyars, et al., 2016).

One such receptor kinase is ARABIDOPSIS CRINKLY4 (ACR4, AT3G59420), which belongs to the CRINKLY4 (CR4) family of receptor-like kinases. CR4 was first identified in maize (*Zea mays*), where the *cr4* mutation affects leaf epidermis differentiation (Becraft, et al., 1996). So far, CR4 family members have been identified and characterized in several vascular plants, supporting the importance of this family of receptor kinases (Nikonorova, et al., 2015; Pu, et al., 2012; Jin, et al., 2000). While it seems that this family, with all its domains (see section below), only emerged in land plants (**Figure 1**), it cannot – at the moment – be excluded that multicellular algae also contain CR4 family members, due to a lack of full genome information.

Since its initial description two decades ago, ACR4 has been shown to be involved in a wide range of biological processes. Here, we comprehensively review the CR4 family-related literature and summarize insight on the genetic, biochemical and signalling level. For this, we mainly focus on (A)CR4, the founding member of this receptor kinase family, but we also flag a few observations relating to other CR4 family members.

KEY PROTEIN DOMAINS

The 895 amino acid *Arabidopsis* ACR4 protein has a signal peptide, an extracellular domain, a single transmembrane helix, and an intracellular kinase domain; similar in all architectural features to mammalian receptor tyrosine (Tyr) kinases (RTKs) and plant receptor-like kinases (RLKs) that are predominantly serine (Ser)/threonine (Thr) kinases (Becraft, et al., 1996; Shiu & Bleecker, 2001) (**Figure 2A-B**). Orthologs in rice (OsCR4), maize (ZmCR4), and several other plant species share the same features (Tanaka, et al., 2002; Cao, et al., 2005; Nikonorova, et al., 2015).

The extracellular domain of ACR4, a putative ligand binding domain, contains seven repeat regions approximately 39 amino acids in length, followed by three cysteine (Cys)-rich regions similar to the TUMOR NECROSIS FACTOR RECEPTOR (TNFR) ligand binding domain, and is distantly related to REGULATOR OF CHROMATIN CONDENSATION 1 (Gifford, et al., 2005; McCarty & Chory, 2000). Three dimensional modelling of the ACR4 extracellular domain based on the high-resolved crystal structure of the *Arabidopsis* photoreceptor UVR8, but also structural homologues in other species, indicates a distinctive seven-bladed β-propeller, similar to structure models of CR4 family members in other green plants (McCarty & Chory, 2000; Gifford, et al., 2005; Nikonorova, et al., 2015) (**Figure 3A**). The model shows conserved, regularly spaced Cys residues in the repeats that likely contribute to structural stability in the oxidizing environment of the extracellular space by formation of disulphide bonds (**Figure 3A**). Proteins in the β-propeller domain family display huge functional diversity and play particularly important roles in protein-protein and protein-ligand interactions (Chen, et al., 2011).

A single alpha helix spanning the membrane connects the ACR4 extracellular domain to the intracellular, active serine/threonine kinase domain (**Figure 3B**). The kinase catalytic domain comprises approximately 250-300 amino acids and, based on numerous structural and biochemical studies, can be separated into 11 distinct subdomains that house roughly 10 highly conserved residues and/or motifs that contribute to the activity and stability of the molecule (Hanks & Hunter, 1995) (**Figure 3C**). The kinase domain folds into a highly conserved structure with an N-terminal lobe that is comprised predominantly of β-sheets and a C-terminal lobe that is mostly α-helical (**Figure 3B**). The tertiary structure produced at the interface of the N and C-terminal domains creates a pocket that serves as the active site of the enzyme. Conserved features include the glycine (Gly)-rich motif (P-loop) between β-strands 1 and 2 of the N-lobe (subdomain I) which function to help bind and stabilize the α and β phosphates of the bound ATP molecule (**Figure 3C**). The essential lysine (Lys) residue at position 540 in β-strand 3 of the ACR4 N lobe (subdomain II) also helps to stabilize these α and β phosphates and forms a salt bridge with the invariant glutamic acid (Glu) residue in helix-C (subdomain III) (**Figure 3B-C**). Not surprisingly, in general a mutation of this invariant Lys residue in subdomain II abolishes kinase activity (Hanks & Hunter, 1995). Interestingly, however, a mutation of the analogous site in the kinase domain of CR4 in rice does not appear to entirely abolish kinase activity (Pu, et al., 2012). The C-lobe of the kinases harbours a conserved HRD motif, termed the catalytic loop (subdomain VI B), in which the aspartic acid serves as the catalytic base during the phosphotransfer reaction and chelates a Mg^{2+} ion that bridges the α and γ phosphates (**Figure 3C**). In general, loss of this amino acid results in complete inactivity of the kinase. The activation loop of the CR4 kinases is slightly downstream of the catalytic loop and located between two conserved DFG and DPE motifs (APE in most kinases) (subdomains VII to VIII) that serve as hinge points for activation loop movement (**Figure 3B-C**). The aspartic acid (Asp) in the DFG motif functions to chelate a

Mg^{2+} ion that stabilizes the β and γ phosphates of ATP. The APE motif serves a structural role in stabilizing the C-lobe (Zheng, et al., 1993; Hanks & Hunter, 1995; Nolen, et al., 2004). These conserved features work to coordinate the efficient transfer of phosphate from ATP to the appropriate target.

Transmembrane (TM) domains in membrane-bound proteins are typically ~20-30 residues in length and consist predominantly of hydrophobic residues that adopt an α-helical conformation. They span the lipid bilayer and demarcate the extracellular and cytoplasmic domains of the membrane-anchored protein. Whereas much is known about the biochemical and structural properties of the extracellular and cytoplasmic domains of several receptors, the exact mechanism of signal transduction from the outside to the inside is not fully understood. Clearly, interactions within the membrane milieu are a particularly important aspect of the overall mechanism. In RTKs, evidence indicates that TM domains function in a dynamic fashion and have the intrinsic capacity to drive receptor dimerization (Tanner & Kyte, 1999; You, et al., 2005). Interestingly, naturally occurring mutations have been identified in the TM regions of at least two RTKs that lead to constitutive activation in the absence of ligand (Gadella & Jovin, 1995; Mischel, et al., 2002). In the rat growth factor receptor Neu, a valine (Val) to Glu mutation leads to an active dimer that is stabilized by hydrogen-bonds which renders the more stable mutant protein oncogenic (Sternberg & Gullick, 1989). The TM domain in the CR4 family comprises ~24 amino acids (**Figure 3D**). While TM domains of ACR4 and CR4 share ~50% identity there is little sequence similarity in this region with the other CRR proteins. The propensity of the TM helices of the CR4, ACR4, and ACR4 homologs (AtCRRs) to dimerize has been studied by a modified TOXCAT assay (Russ & Engelman, 1999). The TOXCAT assay is an elegant method that facilitates an *in vivo* analysis of the strength of the interaction between TM domains. In brief, a chimeric protein consisting of the TM domain of interest fused to a dimerization-dependent protein (transcription factor

ToxR) and a monomeric protein (maltose-binding protein, MBP) is expressed in the inner membrane of *E. coli* cells. Any interaction between the α-helices of the TM domain induces the dimerization of the ToxR protein which then activates the reporter gene, chloramphenicol acetyltransferase (CAT). A quantitative assessment of CAT activity in an *in vitro* assay is then correlated to the strength of the TM dimer i.e. greater the activity, higher the propensity for TM dimerization. The TM domains of the receptors vary in their ability to homodimerize with *Arabidopsis* CRR1 possessing the highest propensity to dimerize and the ACR4 TM showing the lowest potential (Stokes & Rao, 2008). Further mutagenesis studies also demonstrated the important role of specific amino acids within the TM helix of CR4 and ACR4 that profoundly affected dimer formation (Stokes & Rao, 2010) (**Figure 3D**).

In RTKs, flanking regions of the molecule, such as the juxtamembrane (JM) and carboxy-terminal domains, can regulate kinase activity (Hubbard, 2004; Thiel & Carpenter, 2007; Wybenga-Groot, et al., 2001; He, et al., 1996; Sengupta, et al., 2009; Endres, et al., 2013). *In vivo*, the kinase domain is anchored to the phospholipid bilayer and structure-function studies with RTKs such as EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) suggest that intramolecular interactions with the membrane bilayer can influence the allosteric regulation of kinase activity by the juxtamembrane domain (Sengupta, et al., 2009; Jura, et al., 2009). Importantly, the intrinsic dimerization capacity of the TM domain in ACR4, and its dynamic role in receptor dimerization, adds a layer of complexity that is not considered in structure-function studies of recombinant kinase domains. This is exemplified by observations that the TM domain of ACR4 is important for heteromerization with CLV1 (Stahl, et al., 2013).

PROTEIN ACTIVITY AND STABILITY

ACR4 has been shown to undergo rapid turnover and internalization via a BFA-sensitive pathway (Gifford, et al., 2005). Furthermore, ACR4 appears to respond similarly to CLAVATA/EMBRYO-SURROUNDING REGION 40 (CLE40) signalling as CLAVATA1 (CLV1), namely internalization and removal to the lytic vacuole (Stahl, et al., 2013). With respect to its trafficking, it seems that SUPERNUMERARY ALEURONE LAYERS 1 (SAL1) could be involved in the internalisation and degradation of CR4, since CR4 is internalised by SAL1-positive endosomes (Tian, et al., 2007).

Furthermore, function, stability and localization of ACR4 *in planta* appear to be mediated by several regions within the protein. Gifford et al (2005) have examined the role of the extracellular domain, the intracellular domain and their constituent sub-domains by expressing various deletion constructs of C-terminally fused GFP proteins and complementation assays in *acr4* mutant plants. Remarkably, a truncation construct created by deletion of 4.5 of the 7 crinkly repeats was unable to compliment *acr4* indicating that the extracellular crinkly repeat domain is essential for ACR4 function. Furthermore, the same study identified that the ACR4^{C180Y} mutation at the sole Cys residue in the fourth repeat of the propeller domain (**Figure 2B**) similarly caused a loss of function, as the *pACR4::ACR4^{C180Y}:GFP* construct was also unable to compliment the *acr4* mutant phenotype. Interestingly, given the importance of the Cys residue in disulfide bridging and protein stability, its mutation did however not appear to compromise the stability of the ACR4^{C180Y}:GFP protein, which expressed and localized to the plasma membrane in the root epidermal cells and other tissues. Similarly, deletion of the TNFR sub-domain abolished function even though the protein localized to the same membrane as the wild-type protein. Analogous deletion experiments performed within the cytoplasmic domain indicated that removal of the kinase domain and the C-terminal extension resulted in an unstable protein product with little or no detectable protein. In contrast, constructs containing either the C-

terminal deletion or the inactive kinase domain were fully functional in a complementation assay with relatively uncompromised protein expression and localization. The Gifford et al. (2005) study points to the critical role of the extracellular (putative ligand-binding) domain in the function of ACR4 while simultaneously suggesting that the kinase domain may be dispensable.

While ACR4 has all the typical domains of a receptor kinase, some data in *Arabidopsis* suggest that neither the C-terminal domain nor its kinase activity is required for ACR4 signalling (Gifford, et al., 2005). A study by Pu and Sun (2012) identifies three distinct conserved CR4 motifs in rice, maize and *Arabidopsis*. The AXK motif is thought to be essential for kinase activity, HRDLKXXN is predicted to contain a catalytic base facilitating transfer of phosphate to target proteins, and the DFG motif is thought to be involved in chelation of positive ions, enabling the correct orientation of the γ-phosphate of ATP for kinase activity (Pu & Sun, 2012; Hanks & Hunter, 1995). Substitutions of $CR4^{D652A}$ in maize, and $ACR4^{K540M}$ in *Arabidopsis*, are mutations of the DFG and AXK motifs, respectively, and both mutations abolish kinase activity (Jin, et al., 2000; Gifford, et al., 2003), although the corresponding AXK motif mutation in rice ($OsCR4^{K532E}$) did not affect autophosphorylation to the same extent. However, there are two AXK motifs present in OsCR4 (and in ZmCR4), likely explaining why the $OsCR4^{K532E}$ variant did not completely abolish kinase activity (Pu & Sun, 2012). Interestingly, Jin et al (2000) report that another maize point mutation (*cr4-651*) immediately N-terminal of the DFG motif results in a similarly strong *cr4* phenotype, indicating that the mutant phenotype may be due to steric hindrance of the DFG motif. However, the role of the kinase domain, vis-à-vis its activity or lack of activity, needs further verification in the context of the observations of Pu and Sun (2012) who showed that in rice CR4 the conserved Lys residue (K532) is not essential for *in vitro* kinase activity, but that the OsCR4 Lys mutant still shows a reduced level of kinase activity. This suggests that the point

mutant produced by Gifford et. al. (2005) may potentially possess residual kinase activity that could exert some influence in their complementation assays or that in rice alternative CR4 AXK domains may be important to conformation and activity of the kinase (Pu & Sun, 2012). In contrast, it seems that the extracellular domain of ACR4 is necessary for its function (Gifford, et al., 2005).

PROTEIN-PROTEIN INTERACTIONS AND TARGETS

There is increasing evidence that the classical paradigm of RTK function involving a single receptor−single ligand interaction may be an over-simplified model and heteromeric interactions play a significant role in expanding the signalling potential (Wieduwilt & Moasser, 2008; Schulze, et al., 2005; Pfeiffer, et al., 2002; Morgillo, et al., 2006; Saito, et al., 2001). Additionally, atypical RTKs with kinase-inactive domains can signal through heterodimerization with active RTKs (Wieduwilt & Moasser, 2008; Hynes & MacDonald, 2009; Kroiher, et al., 2001). In plants, RLKs such as FLAGELLIN SENSITIVE 2 (FLS2) and BRASSINOSTEROID INSENSITIVE 1 (BRI1) ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Chinchilla, et al., 2007; Kemmerling, et al., 2011), CLAVATA2 (CLV2) (Guo & Clark, 2010), BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Clouse, 2011; Ye, et al., 2011), the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family (Albrecht, et al., 2008) and the 5-member ethylene receptor family participate in signal transduction pathways via heteromeric interactions (Gao & Schaller, 2009). In *Arabidopsis*, the two RLKs CLV1 and ACR4 co-express in the distal root meristem and form a heteromeric complex in the plasma membrane that profoundly impacts root stemness (Stahl, et al., 2013). ACR4 and CLV1 form homomeric and heteromeric complexes depending on local concentration differences and local environments/subcellular localizations. It seems that CLV1 moderates

ACR4-dependent signalling by binding to ACR4. However, another pathway is likely active in the absence of CLV1 (Stahl, et al., 2013).

Genetic and cell biology analyses suggest that the *Arabidopsis* CR4 family of receptors, including ACR4, CRINKLY 4 RELATED 1 (CCR1 or CRR1, AT3G09780), CCR2 (or CRR2, AT2G39180), CCR3 (or CRR3, AT3G55950), and CCR4/CRINKLY 4-RELATED KINASE 1 (CRK1, AT5G47850) may act in the same genetic pathway through functional redundancy based upon gene duplication and/or through activation of signalling cascades via receptor heterodimerization (Gifford, et al., 2005; Cao, et al., 2005; De Smet, et al., 2008). Further support for potential *in vivo* interactions among members of the ACR4 family comes from *in vitro* studies demonstrating interactions between the intracellular domains of ACR4 and the CRRs using various techniques, perhaps mediated through a conserved KDSAF motif among these proteins (Meyer, et al., 2015) (**Figure 2B**). It is entirely possible that heteromeric interactions can promote preferred binding of specific ligands to the extracellular domain(s) and initiate a diverse array of signalling pathways with differential outputs. The same study also showed *in vitro* evidence for the interaction of ACR4 kinase with WUSCHEL-RELATED HOMEOBOX 5 (WOX5), a transcription factor implicated in the regulation of ACR4 signalling (Meyer, et al., 2015) (**Figure 4A**). Although the physiological relevance of this interaction is yet to be established, such focused *in vitro* studies nevertheless can drive new hypothesis-driven biological investigations.

Recently, the ACR4 interactome pinpointed a number of likely putative interactors (Yue, et al., 2016). A very strong and biologically relevant candidate protein to emerge from this study, particularly in the context of reversible protein phosphorylation which is a key element in the fidelity of signal transduction processes, is the catalytic subunit of the PROTEIN PHOSPHATASE 2A family (PP2A-3 and PP2A-4). In a study encompassing cell biology, genetics and *in vitro* biochemical approaches, compelling evidence is provided for

the interaction between ACR4 with the PP2A-3 catalytic subunit (**Figure 4A**). *In vitro* assays clearly demonstrate that PP2A-3 is in fact a substrate for ACR4 kinase and is phosphorylated on at least 9 sites (as determined by mass spectrometry) of which 5 are at serine, 3 are at threonine and 1 is at tyrosine (Yue, et al., 2016).

(AUTO)PHOSPHORYLATION

Like other RLKs, such as BRI1 (Oh, et al., 2011) and Xa21 (Liu, et al., 2002), the ACR4 kinase domain undergoes autophosphorylation via an intramolecular mechanism with at least 16 phosphorylation sites distributed across the juxtamembrane, kinase and carboxy-terminal domains (**Figure 2B**). Of particular note is the phosphorylation of two serine residues within the activation loop sequence of the kinase domain (Meyer, et al., 2011). It is well known that phosphorylation of residues within the activation segment triggers a regulatory step necessary for kinase activity in many kinases belonging to the Ser/Thr family and the Tyr kinase family (Johnson, et al., 1996; Johnson & Lewis, 2001; Burza, et al., 2006; Shah, et al., 2001; Wang, et al., 2005). This is a characteristic feature of kinases belonging to the RD family [refers to arginine (Arg, R) and aspartic acid (Asp, D) residues], which contain an Arg residue preceding the invariant catalytic Asp and are critical elements of a mechanism that stabilizes a conformation for optimal kinase activity following phosphorylation in the activation segment.

Presumably, multiple phosphorylations induce conformational changes that regulate binding sites for ATP, substrates and interaction partners involved in downstream signal transduction. Thus, in RTKs, it has been demonstrated that phosphorylated residues create docking sites for the modification-dependent recruitment of interacting proteins that initiate multifarious downstream signalling events (Huse & Kuriyan, 2002; Schulze, et al., 2005; Holland, et al., 1997). Insofar as very few RLKs have been characterized in detail at the

molecular level, the biological functions of several RLKs (Shiu & Bleecker, 2001; Haffani, et al., 2004; Afzal, et al., 2008; Tang, et al., 2008; Kim, et al., 2009) suggest that activation of the kinase domain also elicits an amplification of the extracellular signal via recruitment of cytoplasmic downstream protein targets and their subsequent phosphorylation. The phosphorylated effector proteins can then act to modulate transcriptional activity in the nucleus. In an effort to dissect the functional role of phosphorylated residues in ACR4 as potential sites of interactions with other proteins, a combinatorial phage-peptide library screening approach to identify peptide sequences/motifs interacting with the phosphorylated Ser residue in the JM domain of the kinase was used (Meyer, et al., 2013). This study identified the motif LxxLL as a recognition motif that is present within the kinase domain of ACR4 and presumably participates in a regulatory intramolecular interaction between the JM and the N-terminal lobe of the KD. Intriguingly, the LxxLL motif has been demonstrated to be central to protein-protein interactions among many proteins in both plants and animals, particularly in a number of the LRRIII subfamily of the leucine-rich repeat (LRR) RLKs and mammalian receptor signalling proteins (Cubas, et al., 1999; Dong, et al., 2004; Savkur & Burris, 2004; Plevin, et al., 2005; Kim, et al., 2009). More importantly, inasmuch as the LxxLL motif occurs in at least one of the ACR4 homologs and is also present in many other RLKs, phosphatases and transcription factors involved in plant growth and development, a compelling case could be put forth for ACR4-mediated signal transduction driven by cross-talk and heteromeric protein-protein interactions. It has been speculated that protein-protein interactions between transmembrane (TM) regions of ACR4 and CRR2 may facilitate the observed phosphorylation of the inactive kinase domain of CRR2 (Cao, et al., 2005; Stokes & Rao, 2008; Stokes & Rao, 2010). Further, it has recently been demonstrated that the LLSLL motif present in the ACR4 kinase domain is able to bind a KDSAF motif in CRR3 using peptide interactions and HD exchange (Meyer, et al., 2015). Since interactions between

Arabidopsis TM domains are relatively weak compared to interactions of maize CR4 TM domains, it is possible that the LxxLL motif present in the JM domain (Meyer, et al., 2013) serves to further stabilise protein-protein interactions between ACR4 and interacting proteins in order for phosphorylation to occur.

Interestingly, *in vitro* phosphorylation analyses indicate that intracellular domains of ABNORMAL LEAF SHAPE 2 (ALE2) and ACR4 are able to mutually trans-phosphorylate; potentially indicating dimerization of the two domains and formation of a receptor complex involved in defining epidermal identity during ovule development (Lemmon & Schlessinger, 2010; Tanaka, et al., 2007).

Previously, through the lack of interaction with KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP), it was suggested that dephosphorylation is not a major means of down regulating ACR4 or CR4 (Braun, et al., 1997; Gifford, et al., 2005). However, recently it was shown that PP2A (or at least the human variant) is able to dephosphorylate ACR4. Furthermore, the ACR4 phosphostatus seems to affect its membrane localization (Yue, et al., 2016). Overall, the Yue et al. (2016) study has defined a hypothesis involving a balance between the phosphorylation and dephosphorylation processes in regulating the membrane localization of ACR4 (**Figure 4A**).

LOCALIZATION AT PLASMODESMATA

Interestingly, next to its membrane localization, ACR4 has been shown to localize to plasmodesmata, potentially indicating that ACR4−CLV1 heteromeric complexes may act to restrict mobility of signalling molecules responsible for maintenance of stem cell fate from the quiescent centre to the surrounding columella stem cells via the plasmodesmata (Stahl, et al., 2013; Stahl & Faulkner, 2015; Williams & De Smet, 2013) (**Figure 4B**). Similarly, in *in*

vitro grown maize endosperm tissue, expression of the *γ-Zein::CR4:HA:FLAG:AcGFP* construct is detected in the membrane and endosomes of endosperm cells, specifically concentrating at plasmodesmata on the anticlinal intersection between aleurone cells (Tian, et al., 2007).

EXPRESSION AND TRANSCRIPTIONAL REGULATION

ACR4 is expressed in different cells and organs in the plant, suggesting it functions in multiple developmental processes. In this section, we summarize this and include aspects of *ACR4* transcriptional regulation. In the *Arabidopsis* root, *ACR4* has 3 important expression domains: in the stem cell niche, in the epidermis and during lateral root initiation. *ACR4* is specifically expressed in the small daughter cells after the first asymmetric pericycle cell division (De Smet, et al., 2008). This expression pattern is associated with formative cell divisions, as no *ACR4* expression is observed when pericycle cells are proliferating (De Smet, et al., 2008; De Smet, et al., 2010). In the root apical meristem, *ACR4* is expressed in the columella and other initials (De Smet, et al., 2008; Stahl, et al., 2009; Stahl & Simon, 2012). Finally, *ACR4* is also expressed in the root epidermis, but no clear role for ACR4 in the root epidermis has been described so far (Gifford, et al., 2005). Immunostaining of wild-type maize root sections with antibodies raised against the extracellular domain of CR4 indicate that *CR4* is also expressed in maize roots, where it localises to the plasma membrane and endosomes (Tian, et al., 2007).

In *Arabidopsis*, *ACR4* expression is observed in the L1 layer of the developing embryo, inflorescence and floral meristems, sepal margins, and additionally in the inner and outer integuments, funiculus, and endothelium of mature ovules (Gifford, et al., 2003). In maize, RT-PCR indicates expression in the leaf, ear, tassel and stalk tissues (Jin, et al., 2000;

Kang, et al., 2002). Additionally, *in situ* hybridisation of the *CR4* transcript indicates expression in the shoot apical meristem and young leaf primordia, and in the epidermis, vasculature and leaf margins of mature leaves (Becraft, et al., 2001).

Very little is known about the transcriptional regulation of *ACR4*, but it seems that a network structure where *ACR4* expression is regulated by ATML1 and PDF2 in a negative feedback model is important for epidermal identity (San-Bento, et al., 2014). In this context, it seems that the L1 box is required for normal epidermal expression of *ACR4* during embryogenesis (San-Bento, et al., 2014), and this is in line with earlier work where it was suggested that ATML1 and PDF2 positively regulate *ACR4* expression via the L1 box (Abe, et al., 2003; Tanaka, et al., 2002) (**Figure 4C**). However, other results also suggest a negative regulation of *ACR4* expression by ATML1 and PDF2 (San-Bento, et al., 2014). Interestingly, *in situ* hybridisation indicates decreased expression of *ATML1* in *ale2* embryos (Tanaka, et al., 2007). Given that the ACR4 and ALE2 intracellular domains interact *in vitro*, ACR4−ALE2-mediated regulation of *ATML1* may potentially reflect a feedback loop capable of regulating *ACR4* expression (Tanaka, et al., 2007) (**Figure 4C**).

In addition to this, *ACR4* expression is up-regulated by CLE40 (Stahl, et al., 2009), but at the moment it is not clear what the involved transcription factors are. Interestingly, *CRK1* expression is negatively regulated by the plant hormones auxin, ABA and cytokinin (Schäfer & Schmülling, 2002). Especially with respect to cytokinin, there is an important role for okadaic acid sensitive phosphatases (such as PP2A) (Schäfer & Schmülling, 2002). However, Chang *et al* (2015) show that while cytokinin-deficient plants display reduced *ACR4* expression, *ACR4* does not appear to be a primary cytokinin response gene (Chang, et al., 2015). Furthermore, an RT-PCR based analysis of leaf tissues indicates that expression of *CR4* in maize aerial tissues is only observed after 24 hours of light induction in 7 day dark-germinated seedlings, indicating that *CR4* expression may be light-induced in these tissues

(Kang, et al., 2002). Additionally, no expression of *ACR4* is detected in the *dek1* mutant embryos (Johnson, et al., 2005) suggesting that DEK1 is required for *ACR4* expression and subsequent determination of epidermal cell fate, which is further evidenced by reduced *ACR4* transcript presence and apparent loss of epidermal identity in *DEK1* RNAi lines (Johnson, et al., 2005). Finally, a recent study in *Populus trichocarpa* showed that an *ACR4* orthologue is down regulated by treatment with the HISTONE DEACETYLASE-specific inhibitor trichostatin A (TSA), suggesting that the acetylation status of histones may provide further control over *ACR4* expression (Ma, et al., 2016).

ROLES OF (A)CR4 IN PLANT GROWTH AND DEVELOPMENT

In this section, we describe the key processes in which (A)CR4 is involved during plant growth and development (**Figure 5**).

Root development

In columella stem cells (CSCs), ACR4 is implicated in regulating asymmetric cell division, as *acr4* displays a disorganised and/or irregularly differentiating columella (De Smet, et al., 2008; Stahl, et al., 2013). While ACR4 is required for CLE40 signalling activity (Stahl, et al., 2009), it remains to be shown that CLE40 directly binds ACR4. Nevertheless, given the genetic data it seems clear that both act in the same pathway (together with CLV1) regulating columella stem cell differentiation by regulation of *WOX5* expression (**Figure 5C**). A possible mechanism is that ACR4 relays the CLE40 signal and also sequesters CLE40 to protect the quiescent centre (QC) around which the stem cells are located from too much peptide.

During early lateral root initiation, ACR4 (in a redundant way with other family members) similarly influences formative asymmetric cell division, which normally results in daughter cells of different identities that give rise to the cell lineages required for lateral root formation (De Smet, et al., 2008). This results in lateral roots being initiated close to each other and often in the normally excluded opposite positions, and stretches of two-layered pericycle or fused primordia (De Smet, et al., 2008). Thus, ACR4 appears to be involved in preventing surrounding cells from dividing, and also in the initiation itself.

Shoot epidermis development

(A)CR4 also functions to define epidermal identity during the vegetative phase of plant growth (**Figure 5A-B**). In maize, abnormal expansion and overabundant division of cells in regions of the shoot apical meristem epidermis in the *cr4* lineage leads to a disorganised structure on the cellular scale, which becomes visible at the macro scale as the irregularities in the cellular structure give rise to the characteristic short "crinkly" leaves (Becraft, et al., 2001; Kang, et al., 2002; Jin, et al., 2000). This is, however, not the only effect observed on the aerial tissues of maize, since wart-like growths are also observed in the leaves of maize *cr4* mutants, which cross sectional analysis reveals as masses of large, undifferentiated and disorganised cell structures, similar to tumour growths (Jin, et al., 2000). In addition to the effect observed in leaf epidermal tissues, *cr4* mutants display abnormalities in trichome, bulliform and mesophyll cells, suggesting that CR4 signalling in the epidermis is able to affect neighbouring tissues (Becraft, et al., 2001; Jin, et al., 2000; Cao, et al., 2005). Interestingly, cross-sectional analysis reveals that the cell wall structure of the maize *cr4* leaf epidermis is irregular, varying in thickness several fold over a few microns (Jin, et al., 2000), demonstrating a similar epidermal tissue phenotype to that observed in the aleurone layer of

seeds (see below) (Cao, et al., 2005; Becraft & Asuncion-Crabb, 2000), suggesting that *CR4* regulates formative divisions of the epidermis and is responsible for maintaining an organised structure.

In *Arabidopsis*, there is no visible alteration to *acr4* aerial tissues on the macro scale, however, microscopic analysis indicates extrusion of epidermal cells from the leaf tips, and toluidine blue staining indicates decreased deposition of cuticle on the leaf surface (Watanabe, et al., 2004). This suggests that - similar to its role of signalling in the seed coat - ACR4 is responsible for determination of epidermal cell fate in *Arabidopsis* leaves.

Embryo and seed development

In addition to the role of regulation of division of meristematic tissues during the vegetative phase of growth, (A)CR4 is also involved in embryonic development (Cao, et al., 2005; Watanabe, et al., 2004; Tian, et al., 2007; Johnson, et al., 2005; Becraft & Asuncion-Crabb, 2000; Gifford, et al., 2003). In the *acr4* background, there are several development defects during embryonic development, which lead to increased rates (40-85%) of seed abortion (Gifford, et al., 2003; Watanabe, et al., 2004). This observation was further supported by Tanaka et al (2007), who additionally observe a compounded effect on seed sterility in the *acr4 ale2* double mutant. The *ale2* siliques contained no seeds and were unable to be rescued by the presence of wild-type ACR4. However, the decreased seed production observed in the *ale2/+* heterozygous mutant was further decreased in the *acr4 ale2/+* background (Tanaka, et al., 2007). Scanning electron microscopy images of the seed coat reveal an irregular, rough surface structure of *acr4* seeds compared with a smooth surface in the wild-type, potentially due to the disrupted organisation of the integument cell lineage in the *acr4* background, which is occasionally severe enough to leave the nucellus exposed (Gifford, et al., 2003; Watanabe,

et al., 2004; Cao, et al., 2005). Additionally, cross sections of *acr4* ovules reveal that the internal structure is similarly disorganised, with irregular cell layers and fused ovules being the causative factors leading to seed abortion (Gifford, et al., 2003; Watanabe, et al., 2004). Disorganised ovule development is also observed in *ale2* mutants, and this disruption is further increased in the *acr4 ale2* background, suggesting that both proteins act on the same or overlapping pathways responsible for determining epidermal identity during embryogenesis (Tanaka, et al., 2007). Application of the hydrophilic toluidine blue stain indicated that the surface of *acr4* seeds is water permeable, suggesting disrupted production of cuticle (Watanabe, et al., 2004). Extrapolating from this it is possible that ACR4 is required to retain integument epidermal identity (Watanabe, et al., 2004).

Similar disorganisation in seed coat development is observed in the maize *cr4* background. In these plants, differentiation of the endosperm into aleurone and starchy endosperm is partially disrupted, forming mosaic patches of starchy endosperm instead of aleurone at the seed surface (Cao, et al., 2005; Becraft & Asuncion-Crabb, 2000). Interestingly, the *defective kernel 1 (dek1)* mutant lineage in maize forms no aleurone, and does not express *CR4* in the embryo, suggesting that DEK1 is responsible for governing CR4 activity (Johnson, et al., 2005; Becraft & Asuncion-Crabb, 2000).

In maize *cr4* mutants, silk development is also disrupted, since mutants exhibit short, deformed papillar hairs, and in more severe mutations, multiple silks fuse together, suggesting a loss of epidermal identity (Jin, et al., 2000).

In rice *CR4* RNAi (*OsCR4i*) lines, seed development is disrupted by two mechanisms. In *OsCR4i* lines, not only is the aleurone layer disrupted similar to that of maize, but the development of the palea and lemma structures encasing the developing seed is also disrupted (Pu, et al., 2012). In *OsCR4i* lines, the interlock does not form correctly between the palea and lemma, resulting in an open hull spikelet exposing the fragile inner floral organ before

pollination, and later the developing seed (Pu, et al., 2012). This leads to a large number of unpollinated gynoecia, and also increases risk of damage during post fertilisation development, which causes increased numbers of pollinated seed to fill incorrectly, resulting in a very low viable seed yield (Pu, et al., 2012). Structural analysis of the palea and lemma structures reveals wart-like protrusions of large undifferentiated cells on the inner surface, suggestive of disrupted control of division and loss of epidermal cell identity in these cells (Pu, et al., 2012).

Other roles

In addition to being an important developmental regulator, ACR4 also appears to play a role – directly or indirectly – in plant defence, since *acr4* displays increased resistance to *Botrytis* infection. This is thought to be due to increased expression of *LIPOXYGENASE2*, an essential enzyme for jasmonic acid (JA) synthesis (Zereen & Ingram, 2012). Since application of JA is able to affect root architecture, producing a short-root phenotype (Wasternack & Hause, 2013), it is possible that downstream effectors of *ACR4* signalling may be involved in localised regulation of JA synthesis.

OUTSTANDING QUESTIONS

While (A)CR4 has an extracellular domain that is a putative ligand binding domain, a ligand has yet to be identified. Notwithstanding CLE40 has been put forward in this context (Stahl, et al., 2009), there is no conclusive biochemical data to support this. Future analyses will have to reveal if ACR4 on its own is ligand binding and/or if this requires an interacting partner, such as CLV1. And if this is the case, what the nature of the ligand is. Additionally, while

550 *CLE40* is involved in regulation of CSC division in the RAM, there is no expression observed
551 in lateral root primordia, suggesting the possibility that multiple ligands are able to trigger
552 *ACR4* signalling in a tissue-specific manner, a situation that may be facilitated by heteromeric
553 interactions with different co-receptors. Further to this, although *WOX5* is regulated by
554 CLE40−ACR4 signalling, little is known about the immediate downstream targets of ACR4
555 or the signalling pathway responsible for regulating *WOX5* expression. In this context,
556 (A)CR4 localization at plasmodesmata, the model of ACR4 regulating a stemness factor
557 (Stahl & Simon, 2013) together with the mobility of WOX5 (Pi, et al., 2015) and the fact that
558 ACR4 can phosphorylate WOX5 (*in vitro*) (Meyer, et al., 2015) offers exciting leads to
559 further explore this.

560 Also in a more general context, the molecular and biochemical pathways ACR4
561 signalling impacts on have not been uncovered. Importantly, from a molecular standpoint, a
562 comprehensive *in vitro* biochemical and biophysical study of the entire recombinant receptor
563 can provide valuable clues towards an understanding of *in vivo* physiological functions.
564 Despite recent studies on the properties of the juxtamembrane domain, kinase domain and the
565 transmembrane domain (Meyer, et al., 2013; Meyer, et al., 2015; Stokes & Rao, 2008; Stokes
566 & Rao, 2010), important questions regarding the role of the subdomains and the role of the
567 phosphorylation sites remain to be answered. Ultimately, the three-dimensional structure of
568 the ACR4 kinase domain, in the phosphorylated and unphosphorylated state, will provide a
569 better understanding of the finer aspects of kinase activity and its regulation. More
570 importantly, elucidation of the crystal structure of the extracellular domain of ACR4 can
571 catalyse the identification of ligand(s), the molecular basis for ligand binding, activation of
572 the receptor and concomitant activation of the intracellular kinase domain. In addition, the
573 identification of components of the downstream signalling pathway is essential. The recent
574 identification of the catalytic subunit of the protein phosphatase PP2A-3 as an interacting

protein and a regulator of *in vivo* ACR4 function (Yue, et al., 2016) opens up the opportunity to delve further into the biochemical properties of the PP2A holoenzyme, phosphatase activity and mechanistic aspects of regulation of kinase activity. Thus far, little is known about the role of the C-terminal domain (CTD) in kinase autoregulation and activation. It is common among RTKs to have their CTDs phosphorylated at tyrosine residues in response to receptor activation. These sites can then recruit Src Homology 2 (SH2) or Phosphotyrosine Binding (PTB) domain containing target proteins. As exemplified in the four-member EGF receptor family, multiple sites within their respective CTDs are phosphorylated and act to attract downstream targets (Schulze, et al., 2005; Jones, et al., 2006). Therefore, we can invoke the likelihood that some of the autophosphorylation sites in the CTD could serve to recruit downstream signalling targets. Furthermore, the intrinsic dimerization capacity of the TM domain and molecular details of its dynamic role in affecting receptor function is yet to be ascertained. As mentioned earlier, Stokes & Rao (2009, 2010) have demonstrated the differential dimerization propensities of the TM domains of the ACR4 family and Stahl et al., (2013) have shown that *in vivo*, the TM domain in ACR4 is important for heteromerization with CLV1. The development of the Nanodisc technology (Bayburt & Sligar, 2010; Bayburt & Sligar, 2003) now facilitates the reconstitution of receptor kinases in a membrane environment and provides a tool to examine the mechanism of receptor activation *in vitro*. Particularly exciting will be the ability to delineate the role of TM domains in the formation of homomeric and heteromeric complexes in a membrane-bound environment, and to further understand kinase activity and protein-protein interaction in the context of the TM domain.

Finally, in the future, it will be interesting to explore the role(s) of CR4 family members in, for example, *Physcomitrella patens* and assess to which extent its function is conserved.

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FIGURES AND FIGURE LEGENDS

Figure 1. Schematic phylogenetic tree to indicate absence or presence of CR4 family members in indicated clades. Collapsed tree based on detailed results in Nikonorova, et al., 2015).

Figure 2. Details on ACR4 protein. **(A)** Cartoon of the subdomains of the ACR4 protein: in the extracellular region, the 'Crinkly' repeats (light blue) and TUMOR NECROSIS FACTOR RECEPTOR (TNFR)-like domain (orange); in the intracellular region, the juxtamembrane domain (purple), kinase domain (green), and C-terminal domain (red), and a transmembrane helix (yellow) connects the extracellular and intracellular portions of the protein**. (B)** The 895-amino acid sequence of ACR4. The sequence is color coded as in A. The signal sequence for membrane targeting is highlighted (white). The CRINKLY domain cysteine (Cys) residue (at position 180) critical for ACR4 function is underscored in red. Key residues that can be phosphorylated *in vitro* are underscored in white. The KDSAF motif is underscored in yellow.

Figure 3. (A) 3D-structure model of ACR4, based on the 1.7 Å crystal structure of the *A. thaliana* UVR8 photoreceptor (PDB: 4D9S). Visualization was performed using Chimera 1.10.2 (Pettersen, et al., 2004). Conserved cysteine (Cys) residues are indicated as grey sticks. **(B)** General structure of a kinase domain. The model for the ACR4 kinase domain (residues 512-786 of the RLK sequence) was built by homology modeling using Discovery Studio (Biovia, San Diego, USA) and visualized on the same platform, as described in Meyer et al., (2013). The kinase domain forms a bilobal structure with an N-lobe (blue) and a C-lobe (red). The N-lobe is primarily comprised of β-sheets, whereas the C-lobe is predominantly α-helical. Secondary structures surrounding the active site are labeled. **(C)** The subdomain architecture

of the ACR4 kinase domain. The 11 subdomains of the ACR4 kinase domain are highlighted in varying colors. Residues essential to catalytic activity are depicted (black, bold). Regions involved in kinase activation and catalysis are underlined in black. Underlined regions in orange indicate *Arabidopsis* AXK, HRDIKSSN and DFG conserved motifs important to kinase activity as flagged by Pu and Sun (2012). **(D)** Transmembrane **(**TM) domains of maize CR4 (ZmCR4), *Arabidopsis* CR4 (ACR4) and *Arabidopsis* homologs (CRR). Residues in ACR4 that impact the stability are underlined.

Figure 4. Aspects of ACR4 regulation and targets. **(A)** Schematic representation of ACR4 interactions with the PP2A holoenzyme complex and WOX5. ACR4 is able to interact with and phosphorylate WOX5 (*in vitro*) and PP2A-3 (*in vivo/in vitro*), which forms part of the PP2A holoenzyme complex, which in turn dephosphorylates ACR4. These interactions impact on localization and/or activity. **(B)** Simplified representation of ACR4 and CLV1 interacting (and possibly acting) at plasmodesmata. PM, plasma membrane; ER, endoplasmic reticulum; and PD, plasmodesma. **(C)** Regulation of *ACR4* expression in *Arabidopsis* and/or maize, and relevant inputs. The dash dotted arrows indicate that (likely) one or more intermediate steps are required. The dotted arrow indicates the steps from expression to protein. The L1 box is indicated.

Figure 5. Roles of ACR4 in plants. **(A-B)** Schematic overview of roles of (A)CR4 in dicot (A) and monocot plants (B). **(C)** Root apical meristem with the quiescent centre (yellow/green) and columella stem cells (blue). The (possible) signalling cascade regulating *Arabidopsis* columella stem cell fate is shown.