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Bruce D. Kohorn
Bowdoin College

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REVIEW PAPER

Cell wall-associated kinases and pectin perception

Bruce D. Kohorn

Department of Biology, Bowdoin College, Brunswick, ME 04011, USA

To whom correspondence should be addressed. E-mail: bkohorn@bowdoin.edu

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Abstract

The pectin matrix of the angiosperm cell wall is regulated in both synthesis and modification and greatly influences the direction and extent of cell growth. Pathogens, herbivory and mechanical stresses all influence this pectin matrix and consequently plant form and function. The cell wall-associated kinases (WAKs) bind to pectin and regulate cell expansion or stress responses depending upon the state of the pectin. This review explores the WAKs in the context of cell wall biology and signal transduction pathways.

Key words: *Arabidopsis thaliana*, cell wall, pectin, receptors, signal transduction, WAKs.

Introduction

The extra-cellular matrix or cell wall of angiosperms contain cellulose that is synthesized by complexes on the plasma membrane, and hemicellulose, pectin, and a number of other complex carbohydrates that are synthesized in the Golgi and subsequently secreted (Kohorn, 2000; Somerville *et al.*, 2004; Caffall and Mohnen, 2009; Harholt *et al.*, 2010; Keegstra, 2010; Wolf *et al.*, 2012*a*; Wolf and Hofte, 2014). These polymers are then assembled into cell walls by processes that remain to be well characterized. The pectins are secreted as methyl-esterified polymers, but are then partially de-esterified in a temporally and spatially regulated fashion to allow cross-linking with calcium. Together with a host of pectin-degrading enzymes that are targeted at specific times and locations, the cell can regulate the flexibility of the wall, and thereby affect cell enlargement (Kohorn, 2000; Somerville *et al.*, 2004; Caffall and Mohnen, 2009; Harholt *et al.*, 2010). Pectin is a primary target of invading pathogens and can be disrupted by mechanical forces, and these induce a plant stress response (Espino *et al.*, 2010; Wolf *et al.*, 2012*a*; Ferrari *et al.*, 2013; Bethke *et al.*, 2014; Malinovsky *et al.*, 2014; Wolf and Hofte, 2014). The cell wall-associated kinases (WAKs) not only bind to cross-linked pectin in *Arabidopsis thaliana* (Arabidopsis) cell walls, but also to pathogen- and damage-induced pectin fragments, or oligo-galacturonides (OGs) (Kohorn and Kohorn, 2012; Ferrari *et al.*, 2013). The binding of WAKs to these two types of pectin triggers two different types of responses: (i) native pectin interactions regulate cell expansion during development, and (ii) OGs activate a stress response pathway. This review will explore the role WAKs play in sensing the pectin matrix of the cell wall, and how WAKs can distinguish and respond to different types of pectin during development.

Monitoring the cell wall

It is clear now that plants have multiple mechanisms by which they respond to changes in the cell wall, and some of these events have been modeled using examples of yeast that have a number of integrated cell wall integrity sensing systems (Seifert and Blaukopf, 2010; Hamann and Denness, 2011; Engelsdorf and Hamann, 2014; Hamann, 2015). Over the past few years, a growing number of proteins have been

identified that regulate the ability of a plant to compensate for cell wall deficiencies. Some receptors have also been isolated in screens for developmental effects and have revealed a cell wall relationship. There are numerous recent excellent reviews of these receptors (Hamann and Denness, 2011; Wolf *et al.*, 2012*a*; Engelsdorf and Hamann, 2014; Wolf and Hofte, 2014; Hamann, 2015); examples include the receptor kinases THE1, FER, HERK, ANX, and RLP44, which have been termed cell wall integrity sensors (Guo et al., 2009*a*, *b*; Miyazaki *et al.*, 2009; Hematy *et al.*, 2007; Hematy and Hofte, 2008; Wolf *et al.*, 2012*b*, 2014; Haruta *et al.*, 2014; Shih *et al.*, 2014; Wolf and Hofte, 2014). Unlike the WAKs, these integrity sensors are often members of the leucine-rich receptor kinase family, and some have a malectin carbohydrate binding type domain. RLP44 interacts with components of the brassinosteroid signaling pathway (Wolf and Hofte, 2014). FER is known to bind a peptide ligand, and is active in numerous developmental events including fertilization (Haruta *et al.*, 2014). These potential plant cell wall sensors are also integral parts of signaling pathways that control developmental mechanisms and responses to environmental influences, but how these individual pathways intersect is still to be determined. As yet, no physical or genetic relationship is known between these cell wall integrity receptors and the WAKs, the latter of which are distinguished from the other receptors by the presence of their unique epidermal growth factor (EGF) repeats (Sampoli Benitez and Komives, 2000) in the extracellular domain, and by their ability to both bind and respond to pectin (Kohorn and Kohorn, 2012).

Pectins

The enzymes that polymerize pectin, a methyl esterified α-(1–4) D-galacturonic acid polymer, are located in the Golgi. Pectin is then secreted to somehow associate with cellulose, hemicellulose and proteins, to form the plant cell wall (Ferrari *et al.*, 2013; Harholt *et al.*, 2010; Mohnen, 2008; Willats *et al.*, 2001). Pectin methyl-esterases (PMEs) are selectively and temporally secreted into the cell wall to remove methyl groups to create de-esterified polymers with a negative charge, which are subsequently cross-linked by the binding of calcium. The formation of these networks can change the structural properties of the pectin matrix and affect cell growth in root hairs, pollen tubes (Bosch *et al.*, 2005; Winship *et al.*, 2010; Wolf and Hofte, 2014) and leaf cells (Peaucelle *et al.*, 2011, 2012).

The pectin network can also be digested by polygalacturonases secreted at specific locations and times thereby loosening the cell wall and allowing turgor-driven cell expansion. The HAE/HSL2 receptors and their IDA ligand induce the secretion of pectin degrading enzymes in abscission zones at the root cap, petiole and sepal base (Kumpf *et al.*, 2013), and while subsequent events are not known they may well involve the newly generated pectin fragments. The HAE/HSL2 receptors also mediate the emergence of lateral roots and perhaps other developmental events yet to be identified. The inductive activity of pectin fragments in developmental processes has been suggested for many years (Yamazaki *et al.*, 1983; Willats *et al.*, 2001; Mohnen, 2008; Harholt *et al.*, 2010; Ferrari *et al.*, 2013), but only recently has direct mechanistic evidence arisen supporting the idea that plant-regulated generation of these polymers play an integral part of developmental processes. It remains to be determined how many other events in development directly involve or are triggered by pectin fragments, and how these fragments are able to influence subsequent events.

The role of pectin fragments generated by invading pathogens that secrete plant-targeted polygalacturonases has received greater attention in recent years (Ferrari *et al.*, 2013). Pathogen-induced pectin fragments, or oligogalacturonides (OGs) with a degree of polymerization (dp) of 9–5, are recognized by the plant and induce a defense response (Denoux *et al.*, 2008; Ferrari *et al.*, 2013; Benedetti *et al.*, 2015). Physical disruption through wounding or herbivory can also trigger the accumulation of OGs, and these events as well as pathogens can generate a variety of different sized OGs. However it is not known if these are all sensed by the same mechanism, or stimulate similar responses, and this has been extensively reviewed elsewhere (Davidsson *et al.*, 2013; Ferrari *et al.*, 2013). Nevertheless, the importance of pectin fragments in relation to both developmental mechanisms and to environmental disturbances needs to be explored further.

WAKs bind to pectin

WAKs were first defined by their high affinity for the cross-linked pectin fraction of the cell wall (He *et al.*, 1996). A 30 kb Arabidopsis locus encodes the five WAKs that share 85% identity in the kinase domains, and 65% identity in the extracellular domain, including the conserved spacing of cysteines that form EGF repeats (He *et al.*, 1999; Sampoli Benitez and Komives, 2000; Kohorn, 2001; Kohorn and Kohorn, 2012). While WAK1 and 2 are the most abundantly expressed isoforms, *WAK1* is expressed most in the vasculature while *WAK2* is also expressed in organ junctions, abscission zones and meristems (Anderson *et al.*, 2001; Wagner and Kohorn, 2001; Kohorn and Kohorn, 2012). Subsequent experiments using purified WAK1 and WAK2 showed that the extracellular domains bound OGs of dp 9–15 with far higher affinity than longer pectin polymers (Decreux and Messiaen, 2005; Decreux *et al.*, 2006; Kohorn *et al.*, 2006, 2009). This *in vitro* binding was dependent upon a series of lysines and hence presumably the interaction of this positive charge with the negatively charged pectin. This is supported also by the finding that WAKs bind de-esterified pectins with higher affinity than those that are esterified. However, it remains to be determined how WAKs bind pectins, as no structural similarity has been found with other carbohydrate-binding protein domains. It is not known if the WAK pectin binding domains are modified by sugar addition, although they do contain predicted N- and O-linked glycosylation signals (He *et al.*, 1999).

WAKs and the response to pectin

It has been known for some time that WAKs are required for both cell expansion and response to pathogens. But it

is only recently that these biological roles have been linked and understood in the context of their binding to both cross-linked pectin in the cell wall and to OGs generated by pathogens. Several types of *WAK* mutants indicate WAKs are required for cell expansion. Antisense RNA directed to all five *WAKs* reduced WAK protein levels to 50% of wild type and led to a loss of leaf cell expansion (Lally *et al.*, 2001; Wagner and Kohorn, 2001). Mutants of *wak2* alone cause shorter roots, and a reduction in vacuolar invertase, raising the possibility that WAKs indirectly control turgor-driven expansion (Kohorn *et al.*, 2006, 2009; Kohorn and Kohorn, 2012). On the other hand, a dominant allele of *WAK2*, $WAK2^{cTAP}$, has a hyperactive receptor that induced a stress response. Mutations in the pectin-binding domain or kinase catalytic site of the dominant allele affected receptor activity, indicating that both pectin binding and kinase activity are required. Moreover, the $WAK2^{cTAP}$ phenotype was suppressed by a null allele of *pme3*, suggesting that de-esterified pectin was activating the receptor. Additional genetic analysis of the Arabidopsis WAKs has been hampered by their close linkage, which makes it difficult to combine alleles. That individual loss-of-function alleles have weak or no phenotype may be due to redundancy within the WAK family, but may also be due to other genes and signaling systems that compensate for the loss of WAK. The use of CRISPR cas9 technology should help to resolve this issue (Feng *et al.*, 2014).

Since WAKs bind pectin both *in vivo* and *in vitro*, it is reasonable to suggest that WAKs might be pectin receptors, and this receptor activity might explain the mutant phenotypes. The idea is corroborated by several reports. The first was the observation that pectin induced the transcription of numerous genes in a *WAK2*-dependent fashion in protoplasts. Secondly, when the WAK1 extracellular domain was fused to the EFR cytoplasmic kinase domain and transiently expressed in tobacco leaves (Brutus *et al.*, 2010), OGs induced events indicative of the EFR kinase activation. This report suggested that the WAK extracellular domain was responding to OGs.

An explanation of the two apparent roles of WAK – regulating cell expansion and a response to pathogens – may lie in the two types of pectin that WAKs bind. We suggest that the developmental role (cell expansion) involves binding of WAKs to native pectin in cell walls. It is not clear if this pectin is at all digested by plant modifying enzymes, nor is it known how this signaling pathway is activated, except that it requires MPK3 and invertase induction (Kohorn *et al.*, 2006, 2012). When OGs are generated by pathogens that target de-esterified pectins, we proposed that WAKs, with a demonstrated higher *in vitro* affinity for OGs over longer pectin polymers, bind these newly released fragments and activate a stress response. The model predicts that plants compromised in their ability to de-esterify pectin (*pme3* mutants) would be more responsive and bind to OGs as the WAKs would be released more readily from native pectins since they had lower affinity for the esterified polymers. Indeed this is what was observed when the transcription of *FADlox* was used as a reporter to measure the transcriptional effects of OG treatments of Arabidopsis wild type and *pme3* mutant seedlings (Kohorn *et al.*, 2014; Kohorn, 2015).

Signal pathway components

The puzzle is how one receptor type can distinguish and respond in different ways to different types of pectin, during development and the response to environmental disturbance. The goal is to understand if this is achieved through different WAKs each binding different pectins, and in combination with different co-receptors and ligands to activate alternate pathways.

To identify proteins involved in WAK-mediated signal transduction the genetic interaction between *WAK* alleles and over 30 candidate genes were explored (Kohorn *et al.*, 2014). We found that null alleles of *mpk6, eds1, pad4* suppressed the $WAK2^{cTAP}$ hyperactive dominant allele that induces dwarfism, curled leaves and a constitutive stress response. Surprisingly *mpk6* but not *mpk*3 suppressed $WAK2^{cTAP}$, indicating that these kinases, thought previously to be redundant, have distinguishable functions (Kohorn *et al.*, 2012). The transcription factors EDS1 and PAD4 regulate the response to pathogens, showing that the WAK and other pathogen-sensing pathways might converge at this step (Heidrich *et al.*, 2011). A model for how these proteins are involved in WAK signaling is shown in Fig. 1.

To further identify how WAKs might activate events in the cytoplasm that lead to a response to OGs, a phospho-proteomic analysis identified 50 proteins that were induced to be

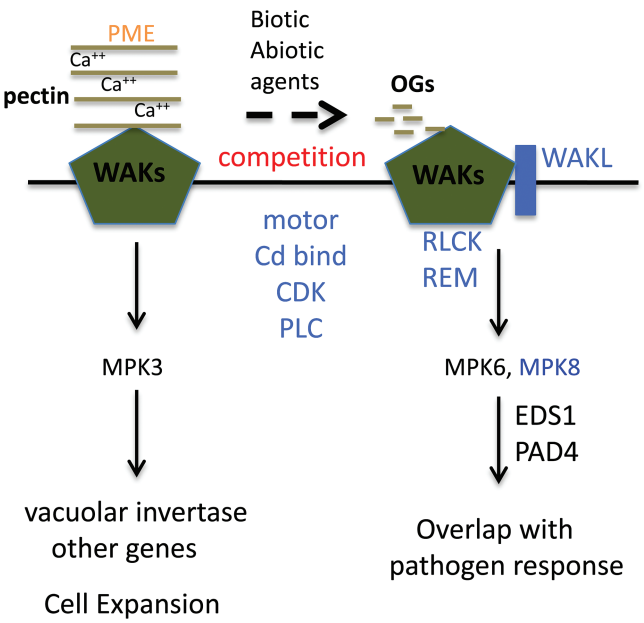

Fig. 1. A model for WAK perception of pectin. Pectin polymers (brown) can be cross-linked in the cell wall with Ca^{++}, and WAKs (green) bind these pectins and signal via the activation of vacuolar invertase and numerous other induced proteins to aid in cell expansion. The methyl esterification state of the pectin is modulated by pectin methylesterases (PMEs) and WAKs bind de-methylated pectin with higher affinity. Pectin is fragmented by biotic and abiotic events and the oligo-galacturonides (OGs), have a higher affinity for the WAKs and induce a stress response. Speculative components downstream of WAK are shown in blue (see text for details).

phosphorylated by OG exposure, relative to untreated plants (Kohorn, unpublished). Some but not most of these are also phosphorylated when plants are exposed to the bacterial elicitor Flg22 indicating that OGs activate events that are in common to other elicitors and therefore the signal transduction pathways share common mechanisms. However, most of the new phosphorylation events were unique to OG treatment indicating that this signal transduction pathway is in part distinct from others. Genetic analysis of these OG-induced phosphorylation events confirmed that two cytoplasmic kinases, two membrane associated scaffold proteins of the Remorin family, a phospholipase C, a CDPK, an unknown cadmium response protein, and a motor protein were required for an efficient OG-induced response. Further studies will help to place these proteins into an OG-induced signal transduction scheme, and this is modeled in Fig. 1.

Protein ligands?

While WAKs do bind to pectin and this activates a response, it remains to be determined whether WAKs also bind additional ligands. One possible candidate is Glycine-Rich Protein 3 (GRP3). The Arabidopsis WAK1 extracellular domain binds to GRP3 in a yeast two-hybrid assay (Park *et al.*, 2001), but the remaining four WAKs do not (Anderson *et al.*, 2001). GRP3 and WAK1 are found in a 450 kDa complex (Park *et al.*, 2001) although it is not clear how the insoluble WAKs were released from the wall so as to be isolated in such a complex. GRPs are characteristically high in glycine and represented by over 50 genes in Arabidopsis. Whereas many of these GRPs are nuclear, at least half have signal sequences that direct them to be secreted (Anderson *et al.*, 2001; Mousavi and Hotta, 2005; Mangeon *et al.*, 2010). Of these secreted GRPs, only six genes have any amino acid identity to GRP3 outside of the glycine-rich domain, and these are clustered on one 90 kb locus on chromosome 2. Of these GRP3-like genes, one (GRP3S) has 83% identity while the five others have ~65% identity with GRP3. GRP3S is shorter than GRP3 due to a deletion of 29 amino acids, and binds to WAK1 but not WAK2 *in vitro*. Seven of the GRPs that do not contain similarity to GRP3 beyond the glycine-rich domain do not bind to WAK1 *in vitro*, indicating that glycine richness is not sufficient for binding (Anderson and Kohorn, unpublished). *GRP3* and *GRP3S* expression, as assayed by *in situ* hybridization and RTPCR, overlaps that of *WAK1* (Anderson *et al.*, 2001). Arabidopsis lines homozygous for T-DNA insertions in either the *GRP3* or *GRP3S* gene show no obvious phenotype. It is tempting to speculate that the tandem array of GRPs that include *GRP3* and *GRP3S* might act as corresponding binding partners to the tandem array of WAKs and the relationship between GRP, pectin and WAKs warrants further exploration.

WAK-like genes

The five Arabidopsis WAKs are characterized by their conserved EGF containing the extracellular domain, a trans-membrane region, and a conserved kinase domain. Importantly, they are cell wall-associated, and evidence suggests that this is due to a region of the amino terminus that has conserved lysine residues promoting interaction with pectin (Decreux *et al.*, 2006; Kohorn *et al.*, 2012). The Arabidopsis genome also contains 21 other receptor-like proteins whose extracellular domains show little sequence similarity to the WAKs except for the presence of both conserved and degenerate EGF repeats, and their kinases are similar (Verica and He, 2002). Since the conservation appears to be in the EGF and kinase domains they may instead comprise an EGF superfamily, with the WAKs forming a subset of this superfamily that specifically binds to the cell wall. Indeed no reports have demonstrated if the WAKLs are wall associated but further characterization may well find some that do (Verica and He, 2002; Verica *et al.*, 2003).

WAK protein is detected in numerous angiosperms, but not in algae (He *et al.*, 1996), and indeed WAK-like genes have also been identified in numerous flowering plants including maize, rice, and tomato (Zhang *et al.*, 2005; Rosli *et al.*, 2013; Hurni *et al.*, 2015; Zuo *et al.*, 2015). The criterion used to identify the WAKs in other species includes the identification of a predicted coding region for a WAK-like receptor kinase, and EGF repeats in the encoded extracellular region, but no reports yet indicate these encoded proteins are cell wall-associated. Until these WAK-like genes are fully characterized it may be more useful to classify them as an EGF-containing receptor kinase superfamily since subfamilies may encode receptors of different functions. The maize and rice *WAK-like (WAKL)* genes are far more numerous (over 100) than those in Arabidopsis and are present in multiple tandem arrays. Significantly, a number of reports demonstrate a direct role of the *WAKLs* in disease resistance (Diener and Ausubel, 2005; Zhang *et al.*, 2005; Li *et al.*, 2009; Hurni *et al.*, 2015; Zuo *et al.*, 2015). The disease-related maize and rice WAKLs are distinguished from the WAKs in Arabidopsis not only in the diverged extracellular domain but also by their non-RD kinases domains, implying the kinases act via different signaling pathways. In the one case tested, the maize WAKLs are not cell wall-associated as they easily fractionate with the plasma membrane, suggesting that while these receptors are part of the EGF superfamily, they are not WAKs (Zuo *et al.*, 2015). Indeed, while there is very low similarity between the pectin-binding domain (termed GUB for galacturonic acid binding) of Arabidopsis WAK2 and the maize WAK2-like, the critical lysines residues (Decreux *et al.*, 2006) are missing in the maize isoform. However, it is very intriguing that like WAK2 in Arabidopsis, the maize WAK that confers pathogen resistance may also control turgor (Zuo *et al.*, 2015). The significance of the similarities and differences may well be understood upon further characterization of the maize and Arabidopsis mechanisms of WAK/WAKL signal transduction. Thus it seems that in the very least the plant EGF receptor superfamily serves to perceive pathogen invasion, and that the WAKs may be a specific subset of this EGF family that bind pectins. Like some of the WAKLs, WAKs are involved in the response to pathogens; further studies revealing the role of WAKLs in pathogenesis may provide insight into the Arabidopsis WAK cluster of five isoforms. Of most interest

would be the identification of a similar, functional pectin-binding domain in a WAKL cluster of maize or rice.

Summary

The WAKs appear to be regulating both cell expansion and response to pathogens, and these two disparate events are related by the affinity of WAKs for pectin. An association of undefined nature with cross-linked cell wall pectin is required for a WAK-dependent cell expansion involving MPK3 and vacuolar invertase. But the generation of OGs leads to the binding of WAKs to these pectin fragments and the development of a stress response.

A model suggests that newly generated pectin fragments compete with longer pectins to alter the WAK-dependent responses so as to regulate cell expansion or switch to a stress response. The question now remains as to how the two different types of pectins can trigger one receptor type to activate different paths. It is possible that part of the mechanism lies in the heterogeneity of the WAK family, as there are five WAKs tightly clustered in a 30 kb Arabidopsis locus. But it is also possible that WAKs associate with different co-receptors to distinguish the pectin, and these receptor complexes have different downstream partners. Perhaps the WAKLs serve as some of these co-receptors, and this idea can be tested. In addition, the relationship between WAK-activated pathways and the growing number of cell wall integrity receptors needs to be explored as there is as yet no apparent overlap. One could also predict that in addition to sites of pathogen invasion WAKs would be influenced at locations of developmentally controlled pectin fragmentation, such as abscission zones and lateral root emergence, both locations where WAK2 is abundant. This raises the possibility that pectin fragment activation of WAKs does not always lead to a stress response, and represents an intermediate in a continuum of pectin perception, from polymers to small fragments, thereby acting as a sensor of the pectin component of the cell wall.

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