Plant Cell Physiol. 46(2): 268–278 (2005)
doi:10.1093/pcp/pci026, available online at www.pcp.oupjournals.org

Wall-associated Kinase WAK1 Interacts with Cell Wall Pectins in a Calcium-induced Conformation

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Wall-associated kinase 1 (WAK1) is a transmembrane protein containing a cytoplasmic Ser/Thr kinase domain and an extracellular domain in contact with the pectin fraction of the plant cell walls. In order to characterize further the interaction of WAK1 with pectin, a 564 bp DNA sequence corresponding to amino acids 67–254 of the extracellular domain of WAK1 from *Arabidopsis thaliana* was cloned and expressed as a soluble recombinant peptide in yeast. Using enzyme-linked immunosorbent assays (ELISA), we show that peptide $WAK_{67–254}$ binds to polygalacturonic acid (PGA), oligogalacturonides, pectins extracted from *A. thaliana* cell walls and to structurally related alginates. Our results suggest that both ionic and steric interactions are required to match the relatively linear pectin backbone. Binding of $WAK_{67–254}$ to PGA, oligogalacturonides and alginates occurred only in the presence of calcium and in ionic conditions promoting the formation of calcium bridges between oligo-and polymers (also known as 'egg-boxes'). The conditions inhibiting the formation of calcium bridges (EDTA treatment, calcium substitution, high NaCl concentrations, depolymerization and methylesterification of pectins) also inhibited the binding of $WAK_{67–254}$ to calcium-induced egg-boxes. The relevance of this non-covalent link between $WAK_{67–254}$ and cell wall pectins is discussed in terms of cell elongation, cell differentiation and host–pathogen interactions.

Keywords: *Arabidopsis thaliana* — Cell wall — Egg-box — Pectin — Receptor kinase — Wall-associated kinase

Abbreviations: DM, degree of methylesterification; DP, degree of polymerization; ELISA, enzyme-linked immunosorbent assay; GRP, glycine-rich protein; GulA, guluronic acid; HRGP, hydroxyproline-rich glycoprotein; INA, 2,6-dichloroisonicotinic acid; ManA, mannuronic acid; PGA, polygalacturonic acid; PMSF, phenylmethylsulfonyl fluoride; TMB, 3,3′,5,5′-tetramethylbenzidine; WAK, wall-associated kinase; WAKL, WAK-like.

Introduction

Analysis of the *Arabidopsis* genome unravelled several hundred open reading frames encoding putative receptor kinases (Morris and Walker 2003). Except for a limited number of receptor kinases involved in specific gene-for-gene (Song et al. 1995, Liu et al. 2002) or general pathogen recognition (Gomez-Gomez et al. 2001, Gomez-Gomez and Boller 2002), the perception of brassinosteroids (He et al. 2000, Clouse 2002), cytokinins (Yamada et al. 2001, Schafer and Schmulling 2002) ethylene (Gamble et al. 1998, Hua and Meyerowitz 1998, Hua et al. 1998) and systemin (Scheer and Ryan 2002), and those controlling pollen germination (Giranton et al. 2000, Kemp and Doughty 2003), somatic embryogenesis (Hecht et al. 2001), nodulation (Endre et al. 2002, Krusell et al. 2002, Stracke et al. 2002) and meristem identity (Rojo et al. 2002), most receptor kinases remain doubly orphan. The ligand and the signal transduction pathways they control are largely unknown.

The wall-associated kinases (WAKs) belong to this family of orphan receptors. The first WAKs described in *Arabidopsis thaliana* are encoded by five genes (*WAK1–WAK5*) forming a 30 kb gene cluster on chromosome 1 (He et al. 1996, He et al. 1999). This gene family has been extended recently to include several new members coding for WAK-like proteins (WAKLs). They are also grouped in clusters and are distributed among the five chromosomes of *Arabidopsis* (Verica et al. 2003). WAKs and WAKLs were also found in pea, tobacco and maize using anti-WAK antibodies (He et al. 1996, Gens et al. 2000).

WAK1-5 encode Ser/Thr kinases embedded in the plasma membrane. The intracellular domain of the five isoforms is relatively well conserved (86%) and contains the typical eukaryotic Ser/Thr kinase signature. The extracellular domain is not as well conserved (40–64%) and is unique to each WAK. Depending on the isoform, the extracellular domain contains several subdomains and shares some homology with proteins found in the extracellular matrix of mammalian cells. All isoforms, however, share an epidermal growth factor 2 (EGF2)-like and an EGF-Ca^{2+}-like domain near the transmembrane domain. The positions of the cysteine residues believed to be involved in the formation of disulfide bonds are conserved among the five WAKs. Some degeneration is noticed, however, in the spacing of the conserved residues and within the amino acid sequences laying in between (He et al. 1999, Verica and He 2002).

Most WAKs are expressed throughout the plant. *WAK1*, *2*, *3* and *5* are expressed in green organs; *WAK1* and *2* are also weakly expressed in flowers and siliques; and *WAK2* is also expressed in roots. *WAK4*, on the contrary, is only expressed in siliques. Antisense experiments showed that *WAK* expression is crucial for host–pathogen interactions (He et al. 1998), cell

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elongation, morphogenesis and plant development (Lally et al. 2001, Wagner and Kohorn 2001). Since WAKs are believed to play a crucial role at the cell wall–plasmalemma interface, it was postulated that the specific expression patterns of *WAK* genes at the cellular and tissue level reflect the diversity of interactions between the extracellular domain of WAKs and specific cell wall components (Kohorn 2001).

Among the five WAKs, WAK1 is the most studied receptor kinase. *WAK1* encodes a 68 kDa protein of 595 amino acids and is found in almost all tissues. Expression of *WAK1* can be induced by salicylic acid or by its homologue, 2,6-dichloroisonicotinic acid (INA). WAK1 also belongs to the PR protein family since its expression needs the NPR1/NIM1 positive regulator. The nature of the signalling link between WAK1 and NPR1/NIM1 is unknown, however. The expression of an antisense or a dominant-negative allele of *WAK1* showed that *WAK1* expression is crucial for plant survival in the presence of exogenous INA. Similarly, ectopic expression of *WAK1* protects plant tissue against lethal accumulation of salicylic acid during plant–pathogen interactions (He et al. 1998). *WAK1* is also up-regulated during systemic acquired resistance (Maleck et al. 2000), by methyljasmonate and ethylene (Schenk et al. 2000). Other stresses such as high temperatures or high salt concentrations have no effect on *WAK1* expression, whereas wounding reduces its expression (He et al. 1998). Expression of *WAK1* is also induced by aluminium treatment, leading to a delayed accumulation of the receptor kinase at the periphery of cortex cells within the elongation zone of the root apex. Interestingly, overexpression of *WAK1* in transgenic *Arabidopsis* plants increased aluminium tolerance (Sivaguru et al. 2003).

Two-hybrid and in vitro binding experiments using a recombinant subdomain (amino acids 178–334) of the extracellular region of WAK1 allowed the identification of a secreted glycine-rich protein (AtGRP3) that specifically interacts with WAK1. Moreover, exogenous GRP3 induced the transcription of *WAK1*, *AtGRP3* and *PR1* genes in *Arabidopsis*. It was also shown that the WAK1–GRP3 complex interacts with the intracellular KAPP phosphatase to form a 500 kDa complex (Park et al. 2001), which is similar to the complexes involved in the regulation of meristem identity (Stone et al. 1998, Trotochaud et al. 1999). The putative signalling role of this WAK1–GRP3–KAPP complex is largely unknown. One of the possible targets of this complex could be the chloroplast oxygen-evolving enhancer protein 2 (OEE2) which becomes phosphorylated in a WAK1/GRP3-dependent manner in *Arabidopsis* (Yang et al. 2003). The physiological significance of this interaction is unknown.

WAKs, and WAK1 in particular, are also connected to cell wall components. The exact nature of this link is still unclear. WAK1 remains strongly attached to the insoluble cell wall fraction. It can be released from the extracellular matrix either by a 4% SDS, 50 mM dithiothreitol (DTT) treatment and boiling (He et al. 1996), or by a pectinase treatment (Wagner and Kohorn 2001), suggesting that WAK1 might be covalently bound to pectin. The interaction between WAK1 and pectins was confirmed by using anti-WAK1 and anti-pectin JIM5 and JIM7 antibodies. Both antibodies recognized the same 68 kDa protein band in Western blots of cell wall proteins extracted from pectinase-treated cell walls (Wagner and Kohorn 2001).

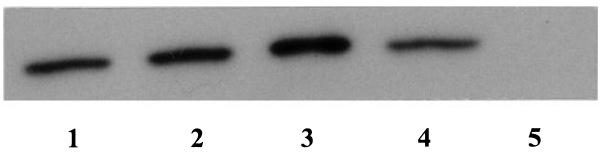

Fig. 1 Western blot of the purified recombinant extracellular subdomain $WAK_{67–254}$ in the presence of PGA. An equal amount (100 ng) of purified recombinant $WAK_{67–254}$ was incubated during 1 h at room temperature in the presence of PGA prepared in 0.5 mM Ca^{2+}/150 mM Na^{+} Tris buffer. After centrifugation, the supernatant was analysed by SDS–PAGE and Western blotting. Recombinant proteins were detected using an anti-Xpress antibody. Lane 1, $WAK_{67–254}$; lanes 2–5, $WAK_{67–254}$ + 0.3, 0.5, 2 or 10 μg of PGA, respectively.

In order to gain a better insight into the signalling role of WAK1 at the plasmalemma–cell wall interface, we investigated the interaction between a subdomain of the extracellular domain of WAK1 and polygalacturonic acid and oligogalacturonides in vitro. We show in this report, using enzyme-linked immunosorbent assays (ELISAs), that the extracellular domain of WAK1 is bound to a calcium-induced conformation in polygalacturonic acid, oligogalacturonides and pectins extracted from *Arabidopsis* cell walls. This interaction was prevented by methylesterification, calcium chelators and pectin depolymerization.

Results

Recombinant $WAK_{67–254}$ binds to PGA

A 564 bp DNA sequence encoding amino acids 67–254 which is unique to the extracellular domain of WAK1 (NCBI accession no. AJ009696) was cloned. It excludes the region encoding the EGF-like repeats common to all WAKs. This sequence, called $WAK_{67–254}$, was overexpressed in yeast, purified and used in the in vitro binding assays.

In order to confirm the binding of the recombinant subdomain of $WAK_{67–254}$ to pectin, we used a modified binding test described by Carpin et al. (2001) to demonstrate the interaction of recombinant isoperoxidases with PGA in the extracellular matrix of *Cucurbita pepo*. Briefly, the purified recombinant protein was incubated for 1 h at room temperature in the presence of PGA and then centrifuged to discard pelleting PGA–$WAK_{67–254}$ complexes. The resulting supernatant was analysed by SDS–PAGE and Western blotting using an anti-Xpress primary antibody. Fig. 1 shows that the recombinant $WAK_{67–254}$ subdomain disappeared from the supernatant when incubated in the presence of increasing amounts of PGA. This indicates that the recombinant extracellular subdomain $WAK_{67–254}$ interacts with PGA and becomes pelletable in a con-

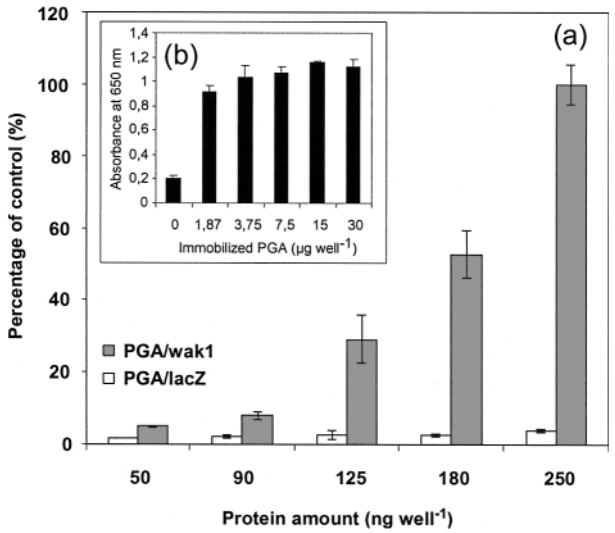

Fig. 2 (a) Direct ELISA test comparing the interaction of increasing amounts of recombinant $WAK_{67–254}$ with PGA and the interaction of increasing amounts of a recombinant β-galactosidase (lacZ) with PGA prepared in 0.5 mM Ca^{2+}/150 mM Na^{+} Tris buffer (20 µg of PGA $well^{-1}$). Results are expressed as a percentage of the response obtained in the presence of PGA. (b) Direct ELISA test showing typical absorbance values obtained with 250 ng of $WAK_{67–254}$ in the presence of increasing amounts of PGA coated within the microwells.

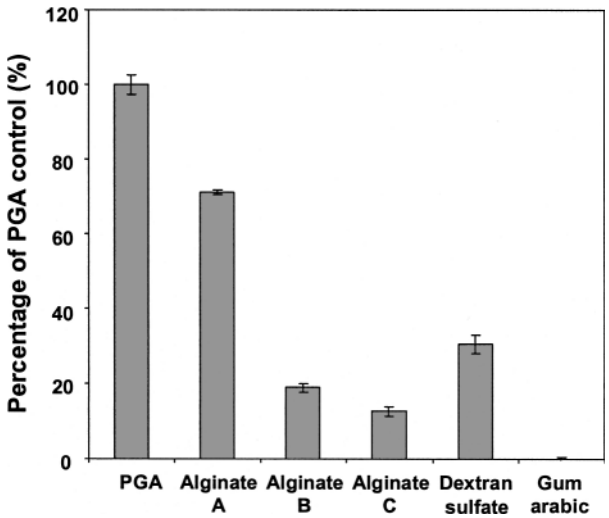

Fig. 3 Direct ELISA test comparing the interaction of recombinant $WAK_{67–254}$ (250 ng) with PGA and other polysaccharides prepared in 0.5 mM Ca^{2+}/150 mM Na^{+} Tris buffer (20 µg of polysaccharide $well^{-1}$). Results are expressed as a percentage of the response obtained in the presence of PGA.

centration-dependent way. This recombinant subdomain was analysed further in the following experiments.

Binding of the recombinant $WAK_{67–254}$ to PGA is specific

In order to characterize the interaction of $WAK_{67–254}$ with PGA, we used a modified direct ELISA binding assay in which PGA was coated onto the wells of a microplate as a polysaccharide trapper. PGA was prepared in ionic conditions (0.5 mM Ca^{2+}/150 mM Na^{+} Tris buffer, pH 8.2) promoting the formation of PGA dimers as described by Liners et al. (1989). After a blocking step, the recombinant proteins were added to the wells and incubated during 1 h at room temperature. Recombinant proteins interacting with PGA were detected with the anti-Xpress primary antibody. The recombinant extracellular domain $WAK_{67–254}$ bound to immobilized PGA, confirming the Western blot result. This interaction was compared with the binding of a recombinant β-galactosidase (lacZ) purified from yeast in the same conditions and containing the same tags as the recombinant $WAK_{67–254}$. LacZ did not bind to PGA, indicating that the presence of the purification and detection tags on the recombinant $WAK_{67–254}$ had no effect on its binding to PGA (Fig. 2a, b). Since similar results were obtained with purified and total yeast extracts containing $WAK_{67–254}$ (not shown), the following binding experiments were performed only in the presence of the purified $WAK_{67–254}$ peptide.

In a first step, we wanted to know if the binding of $WAK_{67–254}$ to PGA could be considered as a simple ionic interaction or as a more complex interaction combining ionic and structural requirements. The ELISA binding assay was thus used to evaluate the interaction of purified $WAK_{67–254}$ with other uncharged and charged polysaccharides coated into the wells (Fig. 3). $WAK_{67–254}$ did not bind to gum arabic, a high molecular weight branched and uncharged polysaccharide with a β(1→3) galactose backbone. $WAK_{67–254}$ bound partially to dextran sulfate (30% of PGA control), a high molecular weight branched α(1→6)-D-glucose polysulfated synthetic polysaccharide. We observed that $WAK_{67–254}$ bound alginates, negatively charged polysaccharides containing β(1→4)-L-mannuronic acid (ManA) and α(1→4)-D-guluronic acid (GulA) distributed as blocks of ManA, GulA and heteropolymeric alternating GulA–ManU sequences (Tombs and Harding 1998). The binding of $WAK_{67–254}$ to alginates was, however, always lower when compared with PGA and was dependent on the GulA content of the tested polysaccharides: $WAK_{67–254}$ bound alginate A, containing 70% GulA residues, significantly more than alginates B and C containing a maximum of 40% GulA residues.

Since alginates share similar structural, chemical and physical properties with PGA, their interaction with $WAK_{67–254}$ was investigated further. A competitive ELISA binding assay was used to measure $WAK_{67–254}$ binding to a competitor polysaccharide in solution. If binding occurs in solution (competition), $WAK_{67–254}$ would be inhibited by the competitor and would not be available to bind the PGA coating of the microwells. On the contrary, in the absence of competitor binding, $WAK_{67–254}$ would bind the PGA coating. We observed that the concentration of soluble PGA needed to prevent 50% binding of $WAK_{67–254}$ (250 ng) to immobilized PGA was about 15 µg ml^{-1} (Fig. 4). Here again, alginates behaved differently depend-

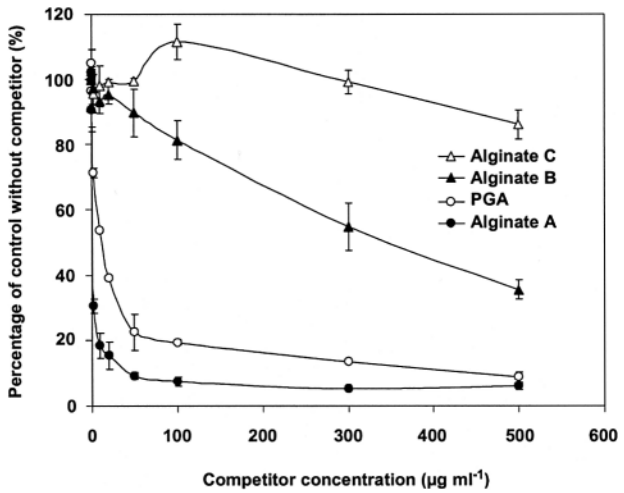

Fig. 4 Competition ELISA test comparing the interaction of recombinant $WAK_{67–254}$ with alginates and PGA. Competitor alginates or PGA were pre-incubated during 1 h at room temperature with $WAK_{67–254}$ (250 ng) in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer. The competition mix was added to the wells and incubated further within the PGA-coated microwells (20 µg $well^{-1}$). Results are expressed as a percentage of control without competitor.

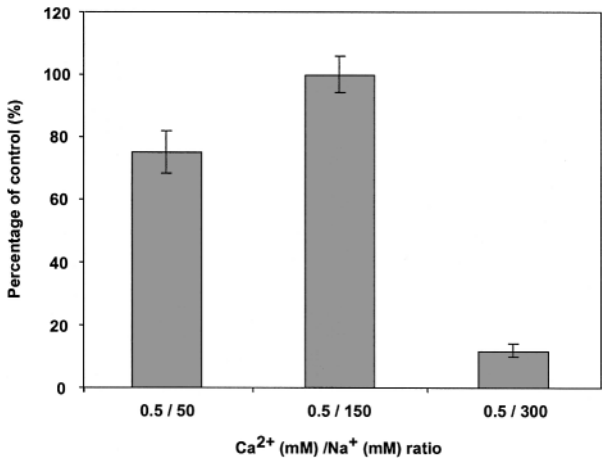

Fig. 5 Direct ELISA test comparing the interaction of recombinant $WAK_{67–254}$ (250 ng) with coated PGA prepared in Tris buffers with different Ca^{2+}/Na^+ ratios. Results are expressed as a percentage of the response obtained in the presence of PGA (20 µg $well^{-1}$) prepared in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer.

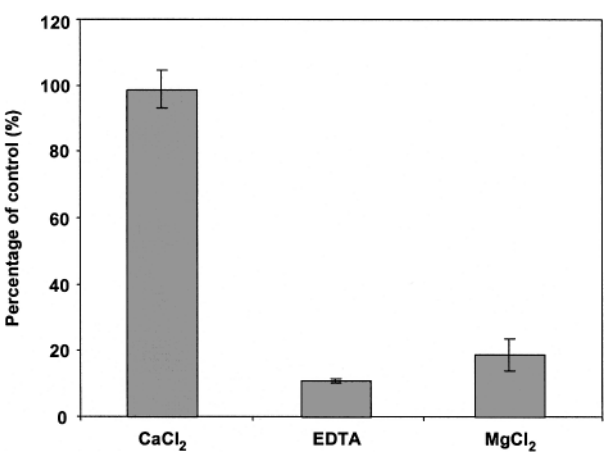

Fig. 6 Direct ELISA test comparing the interaction of recombinant $WAK_{67–254}$ (250 ng) with coated PGA prepared in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer ('$CaCl_2$'), in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer + 5 mM EDTA ('EDTA') or in 0.5 mM Mg^{2+}/150 mM Na^+ Tris buffer in which Ca^{2+} is replaced by Mg^{2+} ('$MgCl_2$'). Results are expressed as a percentage of the response obtained in the presence of PGA (20 µg $well^{-1}$) prepared in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer.

ing on their GulA content. Surprisingly, alginate A with a high GulA content, which are known to form strong inter-chain associations between poly(GulA) domains in the presence of calcium (Tombs and Harding 1998), bound to $WAK_{67–254}$ better than PGA. The binding of $WAK_{67–254}$ to alginate A in solution was probably overestimated by this competitive ELISA compared with the direct ELISA (Fig. 3) test in which alginates were directly coated onto the microwells. Solutions of alginate A appeared more viscous than PGA and other alginate (B and C) solutions, limiting the mobility of $WAK_{67–254}$ to the PGA coating. Alginates B and C, which form fewer and more flexible poly(GulA) inter-chain associations in the presence of calcium than alginate A (Tombs and Harding 1998), also bound to $WAK_{67–254}$. This interaction was, however, only measured in the presence of relatively high concentrations >300 µg ml^{-1} of alginates B and C.

Taken together, these results show that $WAK_{67–254}$ bound polyanions with different affinities. The dextran sulfate data also demonstrate that the presence of negative charges on a polysaccharide was not sufficient to explain $WAK_{67–254}$ binding, and that additional steric constraints certainly account for the specificity of the interaction with PGA. Since both PGA and poly(GulA) blocks in alginates adopt a similar 'egg-box' conformation in the presence of calcium (Davis et al. 2003), we investigated the importance of this conformation for the binding of $WAK_{67–254}$.

Binding of $WAK_{67–254}$ to pectin is conformation dependent

Since the conformation of PGA in solution depends largely on its ionic environment, we tested the binding of $WAK_{67–254}$ to PGA in the ionic conditions used by Liners et al. (1989) to control the formation of inter-chain bridges in the presence of calcium. We observed that $WAK_{67–254}$ bound to PGA in the 0.5 mM Ca^{2+}/50 mM Na^+ and 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffers, but not in the 0.5 mM Ca^{2+}/300 mM Na^+ Tris buffer (Fig. 5). These results suggest that $WAK_{67–254}$ bound to PGA only in conditions promoting the formation of calcium intermolecular bridges previously described as the egg-box model (Morris et al. 1982, Powell et al. 1982, Liners et al. 1989).

In order to confirm this hypothesis, we measured the binding of $WAK_{67–254}$ to PGA in the 0.5 mM Ca^{2+}/150 mM

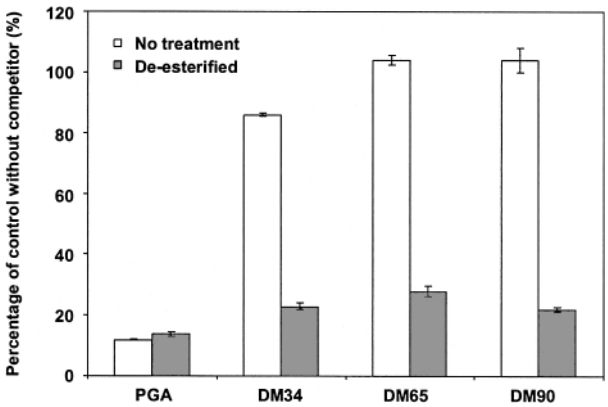

Fig. 7 Competition ELISA test comparing the interaction of recombinant $WAK_{67–254}$ (250 ng) with methylesterified pectins or NaOH-treated methylesterified pectins (50 μg ml^{-1}) before the addition of $WAK_{67–254}$. Competitor pectins with a degree of methylesterification of 34 (DM34), 65 (DM65) and 90% (DM90) were pre-incubated during 1 h at room temperature with $WAK_{67–254}$ in 0.5 mM Ca^{2+}/150 mM Na^{+} Tris buffer. The competition mix was incubated further in the presence of coated PGA (20 μg $well^{-1}$). Pre-incubation with PGA (50 μg ml^{-1}) was used as a positive competition control. Results are expressed as a percentage of control without competitor.

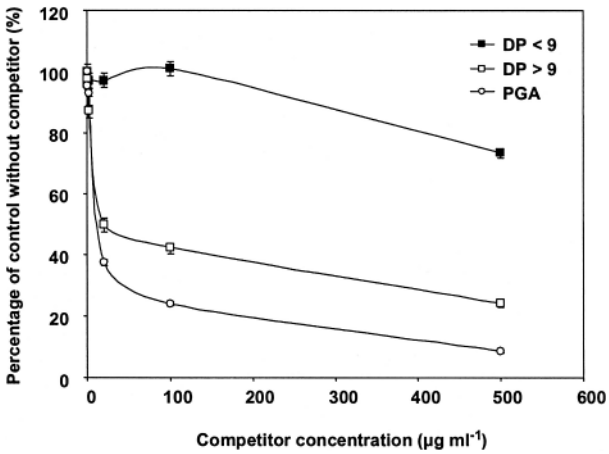

Fig. 8 Competition ELISA test comparing the interaction of recombinant $WAK_{67–254}$ (250 ng) with oligogalacturonides with a degree of polymerization (DP) ≥9 or <9. Competitor oligogalacturonides were pre-incubated during 1 h at room temperature with $WAK_{67–254}$ in 0.5 mM Ca^{2+}/150 mM Na^{+} Tris buffer. The competition mix was incubated further in the presence of coated PGA (20 μg $well^{-1}$). Results are expressed as a percentage of control without competitor.

Na^{+} buffer containing 5 mM EDTA or in the 0.5 mM Mg^{2+}/150 mM Na^{+} buffer in which calcium Ca^{2+} was replaced by Mg^{2+}. Fig. 6 shows that both treatments suppressed the binding of $WAK_{67–254}$ to PGA. As previously shown by Liners et al. (1989), EDTA chelates calcium ions and prevents the formation of calcium intermolecular bridges between PGA polymers. In these conditions, the binding of $WAK_{67–254}$ to isolated PGA polymers was also inhibited. Similarly, hydrated magnesium ions are larger than hydrated calcium ions and are unable to stabilize PGA dimers or multimers in solution (Liners et al. 1992). Therefore, they did not enhance the binding of $WAK_{67–254}$ to PGA.

A second indication supporting the hypothesis that binding of $WAK_{67–254}$ occurs on calcium-bridged PGA came from binding tests performed in the presence of methylesterified pectins. Methylesterification masks the carboxyl groups of PGA and therefore interferes with the formation of inter-chain calcium bridges as previously shown by Liners et al. (1992). We showed that pectins with a degree of methylesterification (DM) of 90 or 65% were not binding substrates for $WAK_{67–254}$ (Fig. 7). Some $WAK_{67–254}$ binding was observed, however, in the presence of pectins with a DM of 34%, which corresponds almost to the minimum DM compatible with the formation of stable inter-chain calcium bridges as shown by Liners et al. (1992). Moreover, chemical de-esterification of all tested methylesterified pectins before the incubation step with $WAK_{67–254}$ restored their ability to adopt a calcium-induced conformation and to bind $WAK_{67–254}$ again.

A third indication that $WAK_{67–254}$ binds a PGA conformation came from experiments performed in the presence of oligogalacturonides which can adopt a stable calcium-induced conformation if their degree of polymerization (DP) is ≥9 (Liners et al. 1992). As shown in Fig. 8, $WAK_{67–254}$ bound only to oligogalacturonides with a DP ≥9. The concentration of oligogalacturonides with a DP ≥9 needed to inhibit 50% of the binding of $WAK_{67–254}$ (250 ng) to PGA was 20 μg ml^{-1}. At higher concentrations, oligogalacturonides with a DP ≥9 were, however, unable to compete as efficiently as PGA for the recruitment of $WAK_{67–254}$. This difference in binding properties of $WAK_{67–254}$ to oligogalacturonides, compared with PGA, probably relies on the size of the minimum binding site recognized by $WAK_{67–254}$ on its target. The complex formed between $WAK_{67–254}$ and a single long polymer (PGA) is likely to be more stable than in the presence of a mixture of oligogalacturonides with a DP ≥9. These results suggest again that binding of $WAK_{67–254}$ occurs on calcium-bridged oligo- or polygalacturonides since oligomers with a DP ≥9 are able, unlike oligomers with a DP <9, to undergo a calcium-induced change of conformation in 0.5 mM Ca^{2+}/150 mM Na^{+} Tris buffer.

$WAK_{67–254}$ binds to pectins purified from Arabidopsis thaliana cell walls

A competitive ELISA binding assay was used to measure the binding of $WAK_{67–254}$ to four pectin fractions extracted from *A. thaliana* purified cell walls. Pectins were extracted successively in the presence of water (fraction F_1), oxalate (fraction F_2), HCl (fraction F_3) and NaOH (fraction F_4). Equal concentrations (15 μg of uronic acids ml^{-1}) of each pectin fraction (native or deesterified) were then incubated with $WAK_{67–254}$ to evaluate their competitor activity (Fig. 9a). We found that $WAK_{67–254}$ had little binding activity on native

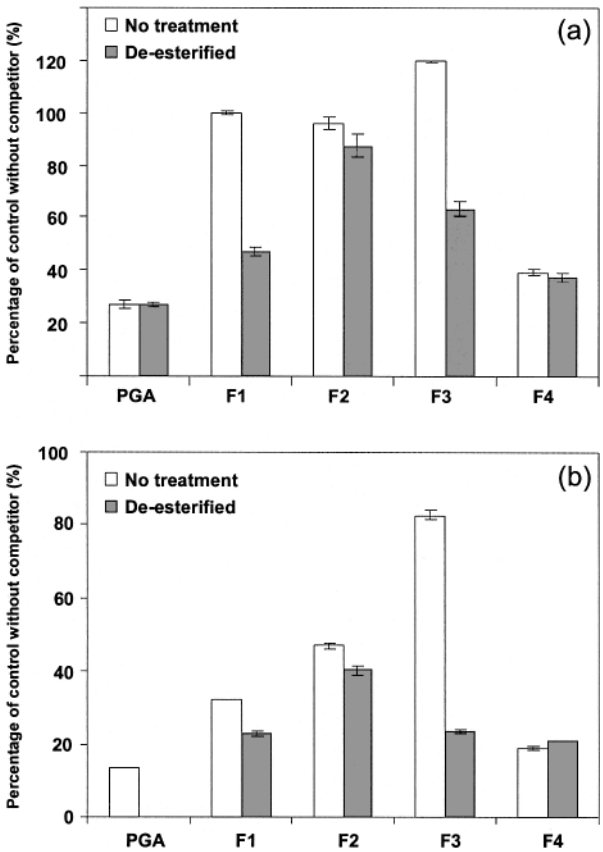

Fig. 9 (a) Competition ELISA test comparing the interaction of recombinant $WAK_{67–254}$ (250 ng) with *A. thaliana* pectins extracted from cell walls in water (F_1), in ammonium oxalate (F_2), in HCl (F_3) and in NaOH (F_4). Competitor pectin fractions (native or de-esterified, 15 μg ml^{-1}) were pre-incubated during 1 h at room temperature with $WAK_{67–254}$ in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer. The competition mix was incubated further in the presence of coated PGA (20 μg $well^{-1}$). (b) Competitive ELISA test of fractions F_1 to F_4 in the presence of the 2F4 monoclonal antibody. Competitor pectin fractions (native or de-esterified, 15 μg ml^{-1}) were pre-incubated during 1 h at room temperature with the antibody in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer. The competition mix was incubated further in the presence of coated PGA (20 μg $well^{-1}$). Pre-incubation with PGA (20 μg ml^{-1}) was used as a positive competition control. Results are expressed as a percentage of control without competitor.

Arabidopsis pectins unless treated with 0.05 M NaOH for de-esterification (except fraction F_4 that had been already extracted in the presence of 0.05 M NaOH), suggesting that *Arabidopsis* pectins are highly methylesterified. The absence of $WAK_{67–254}$ binding to the oxalate-extractable pectins of fraction F_2 was surprising since oxalate treatment is known to extract calcium-bound pectins from the cell wall (Schols et al. 1995). Fractions F_1–F_4 were therefore analysed further with the 2F4 monoclonal antibody that specifically recognizes the calcium-induced egg-box conformation in pectin (Liners et al. 1989). The competitive ELISA performed in the presence of the 2F4 antibody showed that native *Arabidopsis* pectins of fraction F_1 are partially methylesterified, contain the egg-box epitope (70% inhibition, Fig. 9b) but were unable to bind $WAK_{67–254}$ unless deesterified (Fig. 9a). This indicates that the binding of $WAK_{67–254}$ to pectin is more severely affected by methylesterification than the binding of the 2F4 antibody to its epitope (Liners et al. 1992). Interestingly, F_2 pectins inhibited the 2F4 response only by 50% and this inhibition was not affected by chemical de-esterification. These results indicate that egg-box epitopes in F_2 pectins are either less abundant (not all galacturonic acid residues are involved in egg-box formation) or less accessible to the 2F4 antibody than in pectins from the F_1, F_3 and F_4 fractions. This lower competitor activity of fraction F_2 in the 2F4 antibody assay probably explains the unsuccessful detection of the binding of $WAK_{67–254}$ to fraction F_2 (Fig. 9a). We also confirmed that native F_3 pectins are highly methylesterified (no competition), but do adopt an egg-box epitope after de-esterification and then become competitors of the 2F4 response (Fig. 9b). This response was consistent with the binding of $WAK_{67–254}$ to fraction F_3 (Fig. 9a). As expected from the $WAK_{67–254}$ binding ELISA test, pectins in fraction F_4 strongly inhibited the 2F4 response (Fig. 9b). These competitive ELISA tests confirm that $WAK_{67–254}$ binding on *Arabidopsis* pectins (i) is severely affected by methylesterification and (ii) occurs on a pectin domain that is very similar to the egg-box epitope recognized by the 2F4 antibody.

Discussion

The central signalling role of the plant cell extracellular matrix has already been suggested in many physiological responses such as plant cell growth, differentiation and defence against invading pathogens (Brownlee 2002). In animal cells, several plasma membrane proteins and integrins are involved in establishing a signalling continuum between the extracellular matrix, the cytosol and the cytoskeleton that coordinates cell differentiation and responses (Pozzi and Zent 2003). Despite many direct or indirect experiments designed to identify molecular or immunological homologues in plants, it became obvious that plant cells possess unique molecular mechanisms to communicate with its unique cell wall polysaccharides and proteins. The interaction of those cell wall components with the interior of the cell through specific transmembrane 'receptors' therefore remains largely unknown.

In this study, we focused on the interaction of pectin with the wall-associated Ser/Thr receptor kinase WAK1 involved in cell elongation, morphogenesis (Lally et al. 2001, Wagner and Kohorn 2001) and protection against pathogens (He et al. 1998). The link between WAK1 and the pectin network was first suggested by He et al. (1996) and Wagner and Kohorn (2001). They showed that only relatively harsh extraction methods or pectinase treatments were able to release WAK1 from purified cell walls, leading to the conclusion that pectins were covalently bound to WAK1. Whatever the exact nature of the link between WAK1 and pectin, it might be relevant for the

molecular dialogue at the cell wall–plasmalemma interface. Pectins are indeed major signalling components of the cell wall involved in a series of physiological responses including morphogenesis, cell differentiation and host–pathogen interactions (Ridley et al. 2001). We showed in this study that PGA binds to a subdomain of the extracellular domain of WAK1 that is different from the binding domain of AtGRP3 (Park et al. 2001). Interestingly, $WAK_{67–254}$ did not bind 65 or 90% methylesterified pectins, suggesting that the negative charges of the polyanion are somehow involved in the assembly of the PGA–$WAK_{67–254}$ complex. This behaviour was also observed with zucchini cell wall isoperoxidases that bind PGA, but not methylesterified pectins (Penel and Greppin 1994). Binding of $WAK_{67–254}$ to PGA was, however, not strictly dependent on the presence of negative charges since $WAK_{67–254}$ did not bind other polyanions in vitro with the same affinity.

Binding of $WAK_{67–254}$ to PGA was modulated by calcium ions and, more accurately, by the calcium/monovalent cation ratio. Binding could only be observed in ionic conditions promoting the formation of calcium-bridged PGA dimers and/or multimers (also known as 'egg-boxes'). Increasing the monovalent cation content, which inhibits the formation of intermolecular bridges between PGA polymers (Liners et al. 1989), also inhibits the interaction with $WAK_{67–254}$. On the other hand, decreasing the monovalent cation content, which induces the formation of PGA multimers (Liners et al. 1989), reduced by 25% the binding of $WAK_{67–254}$ to PGA. Total removal of calcium ions from the binding environment, replacement of Ca^{2+} ions by Mg^{2+} ions and methylesterification of pectin abolished the formation of PGA–$WAK_{67–254}$ complexes. This calcium-dependent interaction indicates that the binding sites at the surface of the extracellular domain of WAK1 do not simply match negative charges aligned on a single PGA polymer but match a conformation located on two or more PGA chains held together by calcium. The differential binding of $WAK_{67–254}$ to alginates is therefore particularly relevant since poly(GulA) blocks in alginates from brown seaweeds are almost the mirror image of PGA in higher plants. The probability that poly(GulA) blocks form egg-box junction zones in the presence of calcium, similar to those found in PGA, and recruit $WAK_{67–254}$ on this conformation, is therefore higher in alginates containing 70% GulA compared with alginates containing a maximum of 40% GulA. Even if WAK1 has not been identified in brown seaweeds, it is interesting to note here that several secreted proteins from asymmetrically growing *Fucus distichus* embryos share some homology with cell surface proteins, including WAKs, involved in cell morphogenesis in other eukaryotes (Belanger et al. 2003).

Even if we cannot exclude a direct influence of the size of the ligand on $WAK_{67–254}$ recruitment, the results obtained in the presence of oligogalacturonides with a DP ≥9 also support the hypothesis that WAK1 interacts with calcium-bridged PGA since $WAK_{67–254}$ bound only to pectin oligomers known to adopt a calcium-induced conformation as described by Liners et al. (1992). Finally, the binding of $WAK_{67–254}$ to *Arabidopsis* pectins could be clearly correlated with the ability of those pectins to be detected by the 2F4 monoclonal antibody which specifically recognized the egg-box conformation in pectin (Liners et al. 1992).

Our results thus suggest that binding of WAK1 occurs on a calcium-induced conformation of pectin and could be more labile than first mentioned by He et al. (1996). Our data are not incompatible with the results of the above-mentioned study, since the difficulty in extracting WAK1 from the cell wall could be attributed to the use of a 500 mM $CaCl_2$-containing buffer to resuspend purified cell walls. Considering the calcium-dependent gelling properties of pectin and the importance of calcium for the formation of WAK1–PGA complexes, these ionic conditions certainly stiffened the cell wall, therefore limiting WAK1 mobility and extractability. The existence of a non-covalent link between PGA and WAK1 might also be an explanation for the unsuccessful attempts to immunoprecipitate this complex with either anti-WAK or anti-pectin antibodies as reported by Wagner and Kohorn (2001). WAK1 binding to PGA could resemble the relatively strong interaction of some HRGPs (MacDougall et al. 2001) and isoperoxidases in zucchini and *Arabidopsis* (Carpin et al. 2001, Shah et al. 2004) with pectin through cationic amino acids. In the case of the zucchini isoperoxidase, homology modelling and site-directed mutagenesis allowed the identification of three clustered arginines, located away from the active site of the enzyme, directly involved in binding to calcium-bridged PGA (Carpin et al. 2001).

A non-covalent link between WAK1 and pectins is also not necessarily in contradiction to the extraction of WAK1 by pectinase treatments reported by Wagner and Kohorn (2001). As pectinase treatments simply depolymerize pectins in the cell wall, WAK1 could become extractable when the pectic ligand becomes too short to be bridged by calcium ions (DP <9). Subtle changes of cell wall architecture by pectinases during cell wall turnover, or more drastic changes during pathogen attack, could be sensed by WAK1 and become relevant in terms of cell signalling at the cell wall–plasmalemma interface. Moreover, the interaction of WAK1 with oligogalacturonides with a DP ≥9 could modulate the many physiological responses induced by pectin elicitors such as ion fluxes (Mathieu et al. 1991, Messiaen et al. 1993, Messiaen and Van Cutsem 1994, Lecourieux et al. 2002, Navazio et al. 2002), oxidative burst (Legendre et al. 1993), accumulation of methyljasmonate (Doares et al. 1995), activation of mitogen-activated protein kinases (Cazale et al. 1999, Droillard et al. 2000), protein phosphorylation (Droillard et al. 1997), transcription of several defence genes (Messiaen and Van Cutsem 1993), auxin-dependent morphogenesis (Bellincampi et al. 2000, Mauro et al. 2002, Spiro et al. 2002) and fruit ripening (Melotto et al. 1994).

The interaction of the pectic polyanion with cell wall- or plasmalemma-located basic proteins might also be at the origin of signalling processes. Circular dichroism studies demon-

strated that basic domains of carrot extensin and basic synthetic peptides were able to cross-link pectin polymers and induce conformational changes that affect their gelling and swelling behaviour in the presence of calcium. It was shown that both electrostatic and more specific interactions determined by the amino acid sequence of the peptides contribute to the observed new rheological properties (MacDougall et al. 2001). Since the subdomain of the extracellular domain of WAK1 used in this study also contains short basic amino acid sequences, the binding of WAK1 to pectins in the presence of calcium could result in in muro disturbances of the pectin network which in turn could generate signals within the cell wall.

Even if we do not know how WAK1 signals, binding to pectins or pectin oligomers might be crucial for the signalling role of the WAK1–GRP3–KAPP complex described in several physiological responses (Wagner and Kohorn 2001). Although it has been shown that oligogalacturonides bind to cell walls (Mathieu et al. 1998) and plasma membranes (Reymond et al. 1995), and are internalized in a saturable and temperature-dependent way by protoplasts (Horn et al. 1989), no direct link has been made between this binding, a putative oligogalacturonide receptor and a biological response induced by pectin oligomers. Considering the pleiotropic effects of oligogalacturonides on plant cells, WAK1 might belong to one of the perception mechanisms used by the protoplast to sense directly or indirectly the pectins deposited in the cell wall and to respond efficiently to environmental cues that alter pectin content, structure or biological activity. Enzymatic depolymerization of pectins, deposition of newly synthesized methylesterified pectins, de-esterification of pectins in muro, local or systemic changes of the cell wall ionic environment (e.g. through the opening of ion channels), proton pumping or abiotic stresses (heavy metals, high salt concentrations) could modulate the reported signalling role of WAK1 during cell differentiation (Lally et al. 2001, Wagner and Kohorn 2001) and plant–pathogen interactions (He et al. 1998).

Materials and Methods

Polysaccharide and oligogalacturonide preparation

Methylesterified pectin (DM 34, 65 and 90%) and PGA were from Sigma (U.S.A.). Alginate A from *Laminaria hyperborea* with 70% guluronic acid residues was from Sigma (U.S.A.). Alginates B and C from *Macrocystis pyrifera* with maximum 40% GulA residues were from Sigma (U.S.A.) and Fluka (Belgium), respectively.

Methylesterified pectins were de-esterified in the presence of 50 mM NaOH (pH 12) during 30 min at 4°C as described by Liners et al. (1992).

Oligogalacturonides with DP ≥9 and DP <9 were obtained by acid hydrolysis of PGA and HPLC purification as previously described by Messiaen and Van Cutsem (1994).

Oligo- and polysaccharides were prepared in Tris buffer (20 mM Tris–HCl, pH 8.2) supplemented with the appropriate mono- and divalent cations to be tested.

Cloning of the extracellular domain of WAK1 and transformation of Saccharomyces cerevisiae

Total RNA was isolated from 2-week-old *A. thaliana* seedlings (Col-0, N1092) by using Tripure (Roche Applied Science, Belgium) according to the supplier's instructions. First-strand cDNA was synthesized by using the First-Strand Synthesis Kit (Amersham Biosciences, Belgium) according to the supplier's instructions. A 564 bp DNA sequence encoding amino acids 67–254 from the extracellular domain of WAK1 (NCBI accession no. AJ009696) was amplified from first-strand cDNA by polymerase chain reaction (PCR) using *Taq* polymerase (Roche Applied Science, Belgium) and the following primers: 5′-CCCCTCGAGGAGACAAGTGCTGTTCCCA-3′ and 5′-CCCGGATCCAGGCCACATGTCTTAAGCGAC-3′ (Eurogentec, Belgium). This PCR product was ligated into the multiple cloning site of the yeast expression vector pYES2/NT C (Invitrogen, Belgium) designed to express a recombinant protein in fusion with N- and C-terminal poly(His) tags for purification and with an N-terminal Xpress epitope for immunodetection. *Saccharomyces cerevisiae* host cells (strain INVSc1, Invitrogen, Belgium) were transformed with the resulting plasmid using the lithium acetate transformation method according to the supplier's instructions. Recombinant yeast cells were maintained on SC-U medium (SC minimal medium lacking uracil, Invitrogen, Belgium) containing 2% raffinose.

Expression of recombinant protein

Recombinant yeast cells were pre-cultured in 50 ml of liquid SC-U medium containing 2% raffinose at 30°C with shaking. After 24 h, cells were diluted with the induction medium (SC-U medium containing 2% galactose and 1% raffinose) to obtain an OD_{600} of 0.4. After an additional 17 h culture at 30°C with shaking, induced cells were pelleted at 1,500×*g* for 5 min at 4°C. The cells were then washed with sterile water and centrifuged at 1,500×*g* for 5 min at 4°C. Yeast pellets were used directly for recombinant protein extraction or were frozen in liquid nitrogen and stored at −80°C until use. A pYES2/NT/lacZ vector was used as a positive control for protein expression and as negative control for the binding tests.

Total protein extraction in native conditions

Total proteins were extracted by grinding the cells in liquid nitrogen in the presence of 3.5 ml of non-denaturing lysis buffer [140 mM NaCl, 2.7 mM KCl, 20 mM Tris–HCl (pH 7.9), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} chymostatin, 1 μM pepstatin A, 100 μM leupeptin]. The cell lysate was centrifuged at 3,000×*g* for 15 min at 4°C and the resulting supernatant was used in the ELISA tests.

Purification of recombinant protein

Total proteins were extracted by grinding the cells in liquid nitrogen in the presence of 7 ml of denaturing guanidinium lysis buffer [6 M guanidine-HCl, 20 mM sodium phosphate buffer (pH 7.8), 500 mM NaCl, 1 mM PMSF, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} chymostatin, 1 μM pepstatin A, 100 μM leupeptin]. The resulting lysate was incubated for 10 min at room temperature under mild agitation and then centrifuged at 3,000×*g* for 15 min at 4°C. The recombinant fusion protein was purified from the supernatant on a nickel-chelating resin according to the supplier's instructions (Probond resine, Invitrogen, Belgium). The eluate containing the recombinant protein was dialysed sequentially overnight as described by Ferguson and Goodrich (2001). The final dialysis was performed in the appropriate buffer used in the ELISA tests. Binding activity of the dialysed and purified $WAK_{67–254}$ peptide to PGA was compared with the binding activity of the non-purified native $WAK_{67–254}$ peptide by ELISA before use in further experiments (positive renaturation control). Protein concentration was

determined with the NanoOrange protein quantification kit (Molecular Probes, U.S.A.).

Direct ELISA binding assay

Maxisorp microplates (VWR, Belgium) were pre-treated with polylysine-HBr (50 μg ml^{-1} in H_20, 50 μl $well^{-1}$, Sigma, U.S.A.) during 1 h at room temperature. The wells were washed once with 250 μl $well^{-1}$ of 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer (20 mM Tris–HCl, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 8.2) and coated overnight at 4°C with the trapper polysaccharide at the appropriate concentration in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer (50 μl $well^{-1}$) as described by Liners et al. (1989). Non-specific binding sites were blocked for 2 h at room temperature with 3% low fat dried milk dissolved in the 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer. After removal of excess blocking solution, the recombinant proteins were added and incubated for 2 h at room temperature. The wells were washed with 5×250 μl of 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer and further incubated during 1 h at room temperature with 1 μg ml^{-1} of anti-Xpress primary antibody (Invitrogen, Belgium) in the 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer containing 1% low fat dried milk (50 μl $well^{-1}$). After washing with 7×250 μl 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer, 50 μl of a 1/1,000 dilution of horseradish peroxidase (HRP)-SAM secondary antibody (Amersham Biosciences, Belgium) prepared in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer containing 1% low fat dried milk were added and incubated for 1 h at room temperature. After washing with 7×250 μl of 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer, the binding of the recombinant protein to the coated polysaccharide was visualized in the presence of the TMB substrate (Enhanced K Blue TMB substrate, Neogen, U.S.A.). Absorbance was measured after 20 min in the dark at 650 nm with a microplate reader (Elx800, Bio-TEK instruments, U.S.A.). Binding assays were done in triplicate for at least three protein extracts.

Competitive ELISA binding assay

When compared with the binding assay using direct ELISA, interaction between the recombinant protein and the polysaccharide occurs in solution. Excess or non-interacting recombinant proteins are detected by their binding on the coated trapper polysaccharide. Recombinant proteins were therefore pre-incubated for 1 h with different competitor oligo- or polysaccharides prepared at the appropriate dilution in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer. Plates were coated with PGA and blocked with 3% low fat dried milk dissolved in the 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer as described by Liners et al. (1989). The competition mixtures were then added to the wells and left for 2 h at room temperature. The following steps are strictly identical to the direct ELISA binding assay.

Competitive ELISA tests with the 2F4 monoclonal antibody were performed as described by Liners et al. (1989).

Western blot

Purified recombinant proteins were pre-incubated for 1 h at room temperature with increasing amounts of PGA prepared in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer (pH 8.2). After centrifugation at 8,500×*g*, the supernatants were analyzed by SDS–PAGE according to Laemmli (1970) on 12% polyacrylamide gels and electroblotted on a Hybond-P transfer membrane (Amersham Biosciences, Belgium) using a trans-blot semi-dry electrophoretic transfer cell (Biorad, Belgium). After blocking with 3% low fat dried milk prepared in TBS buffer (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4), the membrane was incubated for 1 h with 0.2 μg ml^{-1} anti-Xpress primary antibody (Invitrogen, Belgium) in TBS containing 1% low fat dried milk. The blot was washed three times with TBS and incubated for 1 h with HRP-SAM secondary antibody (Amersham Biosciences, Belgium) diluted 1/5,000 in TBS 1% low fat dried milk. Membranes were washed three times in TBS buffer and developed with the ECL Plus system (Amersham Biosciences, Belgium), and autoradiographed with BioMax Films (Kodak).

Pectin extraction from Arabidopsis thaliana cell walls

Ten-day-old *A. thaliana* (ecotype A-TL) suspension-cultured cells (20 g fresh weight) were ground in 100 ml absolute ethanol and transferred in 100 ml of boiling absolute ethanol during 20 min. After centrifugation (3,000×*g*, 5 min), the pellet was washed twice with 100 ml of aqueous ethanol (70%; v/v) and incubated overnight at 4°C with 100 ml of aqueous ethanol (70%; v/v). The insoluble cell wall material was then washed with absolute ethanol and acetone and allowed to dry at room temperature. Pectins were extracted successively from 300 mg of dried cell wall material in the presence of distilled water (fraction F_1) for 0.5 h at room temperature, 1% ammonium oxalate (fraction F_2) for 0.5 h at room temperature, 0.05 M HCl for 0.5 h at 85°C (fraction F_3) and 0.05 M NaOH for 0.5 h at 4°C (fraction F_4). Pectin fractions were neutralized, if necessary, as described by Rombouts and Thibault (1986). The uronic acid content of each fraction was determined with the meta-hydroxybiphenyl method according to Blumenkrantz and Asboe-Hansen (1973).

Acknowledgments

We wish to thank Professor P. Van Cutsem and Dr. F. Liners for their advices, valuable comments and for generously providing the 2F4 monoclonal antibody. We also thank C. Demazy and P. Cambier for technical assistance. This work was supported by the Fond National de la Recherche Scientifique (FRFC 2.4.501.00.F)

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(Received March 29, 2004; Accepted October 7, 2004)