Available online at www.sciencedirect.com
SCIENCE DIRECT
Phytochemistry 67 (2006) 1068–1079
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

In vitro characterization of the homogalacturonan-binding domain of the wall-associated kinase WAK1 using site-directed mutagenesis

Annabelle Decreux [a], Annick Thomas [b], Benoît Spies [b], Robert Brasseur [b], Pierre Van Cutsem [a], Johan Messiaen [a,*]

[a] *FUNDP, Unité de Recherche en Biologie Cellulaire et Moléculaire Végétale, 61 rue de Bruxelles, 5000 Namur, Belgium*
[b] *Centre de Biophysique Moléculaire Numérique, Faculté Agronomique, 2 Passage des Déportés, 5030 Gembloux, Belgium*

Received 17 January 2006; received in revised form 6 March 2006
Available online 24 April 2006

Abstract

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 wall in *Arabidopsis thaliana* (L.) HEYNH. In a previous paper [Decreux, A., Messiaen, J., 2005. Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. Plant Cell Physiol. 46, 268–278], we showed that a recombinant peptide expressed in yeast corresponding to amino acids 67–254 of the extracellular domain of WAK1 specifically interacts with commercial non-methylesterified homogalacturonic acid, purified homogalacturonans from *Arabidopsis* and oligogalacturonides in a calcium-induced conformation. In this report, we used a receptor binding domain sequence-based prediction method to identify four putative binding sites in the extracellular domain of WAK1, in which cationic amino acids were selected for substitution by site-directed mutagenesis. Interaction studies between mutated forms of WAK1 and homogalacturonans allowed us to identify and confirm at least five specific amino acids involved in the interaction with homogalacturonan dimers and multimers. The presence of this homogalacturonan-binding domain within the extracellular domain of WAK1 is discussed in terms of cell wall architecture and signal transduction.

Keywords: *Arabidopsis thaliana*; Brassicaeae; Receptor binding domain analysis; Receptor–ligand interaction analysis; WAK1; Cell wall; Pectin; Wall-associated kinase

1. Introduction

Wall-associated kinases (WAK) belong to the huge family of 610 receptor-like kinases (RLKs) identified in the *Arabidopsis thaliana* (L.) HEYNH. genome, corresponding to 2.5% of the total number of protein coding sequences (Shiu and Bleecker, 2001). Most RLKs have in common an extracellular putative sensing domain, a transmembrane domain and an intra-cellular putative signalling domain. They can be grouped in 21 structural sub-classes based mainly on the sub-domain organization of their extracellular domain (Shiu and Bleecker, 2001). WAKs, which constitute one sub-class on their own, all share two juxtamembrane degenerate epidermal growth factor (EGF)-like domains in their extracellular domain and a well conserved intracellular Ser/Thr kinase domain. The original *WAK* gene family was recently extended with 22 genes coding for WAK-like proteins (WAKLs), some of them truncated and coding only for the extracellular domain (Verica and He, 2002). WAK and WAKL proteins are considered to be involved either in morphogenesis and/or in host–pathogen interactions (He et al., 1998; Lally et al., 2001; Wagner and Kohorn, 2001; Verica et al., 2003).

The best characterized WAK is WAK1, a 78.8-kDa protein of 713 amino acids found in almost all green tissues of *A. thaliana* (He et al., 1999). The extracellular domain of WAK1 interacts at least with two components of the

* Corresponding author. Tel.: +32 81 724366; fax: +32 81 724382.
E-mail address: johan.messiaen@fundp.ac.be (J. Messiaen).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.03.009

extracellular matrix: a glycine-rich protein (AtGRP-3) and pectin. AtGRP-3 interacts, through its cysteine-rich C-terminus, with the EGF-like containing peptide corresponding to amino acids 178–334 of the extracellular domain of WAK1. The WAK1–AtGRP-3 duo is part of a membrane-associated 500-kDa complex containing the KAPP phosphatase (Park et al., 2001), a type 2C protein phosphatase also involved in the negative regulation of the CLAVATA/WUSCHEL signalling loop within the *A. thaliana* meristem (Trotochaud et al., 1999).

The interaction of WAK1 with cell wall pectins was first reported by Wagner and Kohorn (2001). The use of harsh extraction conditions or heavy pectinase treatments to release WAK1 from the cell walls led to the conclusion that WAK1 was covalently bound to pectin (He et al., 1996; Wagner and Kohorn, 2001). More recently, Decreux and Messiaen (2005) proposed that WAK1 could be ionically bound to calcium-associated homogalacturonans, forming a tuneable complex during cell wall turn-over or plant–microbe interactions. It was indeed shown that WAK1 binds to *Arabidopsis* native non-methylesterified homogalacturonans in conditions that are compatible with the formation of calcium-induced intermolecular bridges between individual homogalacturonic chains. All conditions that impaired the formation of these calcium bridges, i.e., depolymerization, the presence of side chains (rhamnogalacturonan I), the use of calcium chelators and the methylesterification of the galacturonic acid residues (Liners et al., 1989), also impaired the interaction between WAK1 and homogalacturonans (Decreux and Messiaen, 2005). WAK1 also binds to oligogalacturonides with a degree of polymerization $\geqslant 9$ in a calcium-induced conformation, oligosaccharides known to act as elicitor of defence and morphogenetic responses in higher plants (Messiaen et al., 1993; Messiaen and Van Cutsem, 1993, 1994; Bellincampi et al., 2000; Mauro et al., 2002).

The signalling role, if any, of the WAK1–AtGRP-3–pectin complex is still largely unknown. It has been shown that upon in vivo pectinase treatment, phosphorylated WAK1 is released from the cell wall, but no signalling role has been connected so far to that phosphorylation event (Anderson et al., 2001). Independently, two-dimensional gel electrophoresis of AtGRP-3-treated *A. thaliana* protoplasts and two-hybrid experiments revealed that the kinase domain of WAK1 binds and phosphorylates the oxygen-evolving enhancer protein 2 (Yang et al., 2003).

In order to further characterize the interaction of WAK1 with homogalacturonans, we present in this report an in silico analysis of the extracellular domain of WAK1, combined to in vitro WAK1–homogalacturonan interaction studies, that could help to better understand the signalling role of this polysaccharide at the cell wall-plasmalemma interface in *A. thaliana*. We used a receptor binding domain analysis (Gallet et al., 2000) combined to hydrophobic cluster analysis (Gaboriaud et al., 1987) and network secondary structure consensus analysis (Combet et al., 2000) of the extracellular domain of WAK1 to locate putative sub-domains involved in the interaction with homogalacturonans. Combining site-directed mutagenesis of specific amino acids and interaction studies, we could identify and confirm the amino acids involved in the recruitment of homogalacturonans on the extracellular domain of WAK1.

2. Results

2.1. Purification of recombinant proteins

Recombinant tagged proteins were purified on a nickel-chelating resin according to the manufacturer. The absence of protein aggregates was verified by SDS–PAGE-Coomassie staining and western blotting using the anti-Xpress primary antibody. Fig. 1 presents typical SDS–PAGE and western blot profiles of wild-type and mutant forms of some recombinant proteins used in the following interaction studies. The binding behaviour of each dialysed and purified peptide to homogalacturonan was checked to be always identical to that of the corresponding native non-purified recombinant peptide. Protein samples failing to fulfill that condition were discarded.

2.2. Homogalacturonans bind to a specific sub-domain of the extracellular domain of WAK1

In a previous study, we showed that a specific region of the extracellular domain of WAK1, corresponding

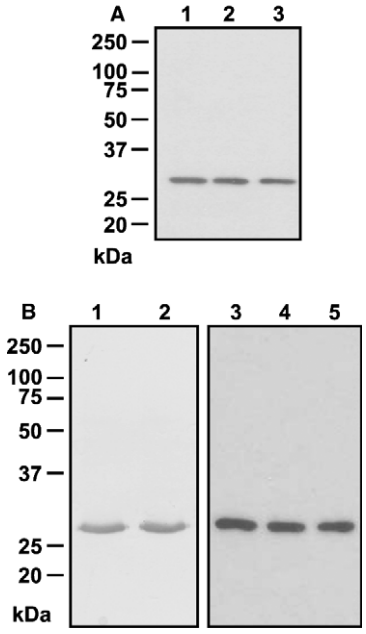

Fig. 1. (A) SDS–PAGE profile (Coomassie staining) of purified recombinant wild-type WAK_{67-254}(1), WAK_{67-254} R166Q (2) and WAK_{67-254} K101T/K102T/R166Q (3) (5 μg protein $well^{-1}$). (B) Western blot (anti-Xpress primary antibody) of purified recombinant wild-type WAK_{67-254} before (1) and after (2) centrifugation showing the absence of protein aggregates, and of mutants WAK_{67-254} R166Q (3), WAK_{67-254} R91Q/R166Q (4) and WAK_{67-254} K101T/K102T/R166Q (5) (250 ng protein $well^{-1}$).

to amino acids 67–254 (called $WAK_{67–254}$), binds calcium-induced homogalacturonan dimers and multimers (Decreux and Messiaen, 2005). In order to locate more accurately peptide segments involved in the interaction of WAK1 with homogalacturonans, the coding sequence of the extracellular domain of WAK1 (NCBI Accession No. AJ009696) was subdivided arbitrarily into three segments encoding, respectively, amino acids 2–111, 112–221 and 222–332. The 222–332 segment contains the two EGF-like domains found in all five WAKs (He et al., 1999). These three sequences, called, respectively, $WAK_{2–111}$, $WAK_{112–221}$, and $WAK_{222–332}$ were cloned and over-expressed in yeast. The binding of the purified recombinant proteins to homogalacturonan was measured in an in vitro binding assay as previously described (Decreux and Messiaen, 2005) and compared to the binding of $WAK_{67–254}$. We used therefore a modified ELISA binding assay in which PGA (a commercial source of homogalacturonan) was coated into 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 calcium-induced PGA dimers as described by Liners et al. (1989). After a blocking step, the purified recombinant proteins were added to the wells (250 ng $well^{-1}$) and incubated for 2 h at room temperature. Recombinant proteins interacting with PGA were detected with an anti-Xpress primary antibody. Optimum binding to PGA was always observed in the presence of $WAK_{67–254}$ (Fig. 2). Among the three "truncated" recombinant proteins, $WAK_{2–111}$ interacted most strongly with PGA (almost 70% of the binding activity of $WAK_{67–254}$ to PGA). The binding activity of $WAK_{112–221}$ to PGA was reduced to 10% of that of $WAK_{67–254}$ to PGA, and $WAK_{222–332}$ did not bind PGA. Within the technical limits of the ELISA method used and the solubility of homogalacturonans, the interaction between $WAK_{67–254}$ and homogalacturonans was detected between pH 5 and pH 8 (Fig. 2). Extreme non physiological pHs were not considered.

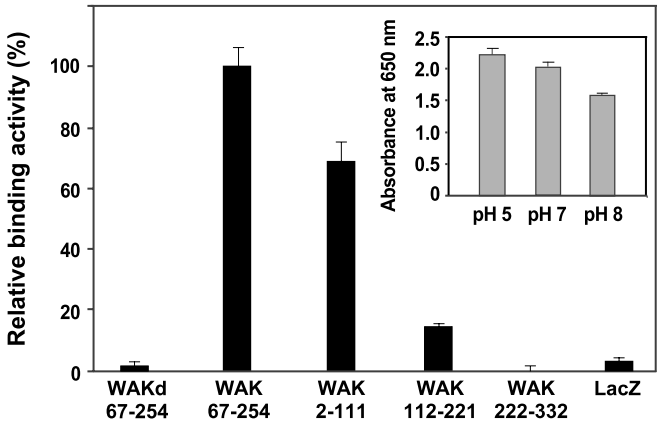

Fig. 2. ELISA test comparing the interaction of the three purified recombinant sub-domains $WAK_{2–111}$, $WAK_{112–221}$ and $WAK_{222–332}$ (250 ng $well^{-1}$) with PGA prepared in 1 mN Ca^{2+}/150 mN Na^{+} Tris buffer (20 μg PGA $well^{-1}$). Results are expressed as a percentage of the response obtained in the presence of $WAK_{67–254}$ (100%). Heat denatured recombinant $WAK_{67–254}$ ($WAKd_{67–254}$) and recombinant β-galactosidase (LacZ) from *E. coli* was used as negative control. *Insert*. Effect of pH on the interaction between $WAK_{67–254}$ and homogalacturonans. Error bars indicate SE ($n = 3$).

2.3. Receptor binding domain and hydrophobic cluster analysis

Since the extracellular domain of WAK1 shares little sequence homology with other plant receptors, in silico modelling starting from a known crystalline structure to locate putative binding domains for homogalacturonans was impossible. In order to identify putative amino acids of WAK1 involved in homogalacturonan binding, the amino acid sequence of the extracellular domain of WAK1 was submitted to receptor binding domain analysis, a sequence-based prediction method of interacting domains in proteins, and combined to hydrophobic cluster analysis and network secondary structure analysis.

Hydrophobic cluster analysis (HCA) is a high-performance sequence analysis method used to identify similar folding zones in sequences which otherwise have little in common (Gaboriaud et al., 1987; Lemesle-Varloot et al., 1990). It allows also the detection of amphipathic sites and secondary structure types (Woodcock et al., 1992). This method is based on a unique 2-D representation of a protein sequence as an alpha helix around an imaginary cylinder with 3.6 amino acids per rotation, or 100° between each. This cylinder is cut lengthwise then unrolled and flattened out. The resulting diagram is duplicated in order to realign the amino acids with the neighbours they had in the intact cylinder and the contiguous hydrophobic residues are outlined and shaded. HCA of the extracellular domain of WAK1 predicted the presence of many vertical amino acid clusters corresponding probably to β-sheets (Fig. 3A). Since some hydrophobic clusters were difficult to interpret, the amino acid sequence of the extracellular domain of WAK1 was submitted to network secondary structure consensus analysis platform (NPS@) using eight different interconnected prediction softwares developed by Combet et al. (2000). The predicted consensus secondary structure of the extracellular domain of WAK1 showed that at least 21% of the amino acid residues are involved in extended β-strands, whereas 8% of the amino acids were predicted to be involved in three α-helices (the N-terminal signal peptide inclusive) (Fig. 3A).

Using the 5, 7 or 9-residue analysis window, stretches of the extracellular domain of the WAK1 sequence exhibiting low mean hydrophobicity ($\{H\} \leqslant 0.5$) and variable mean hydrophobic moments ($0.3 \leqslant \{\mu_H\} \leqslant 0.8$) were predicted as interacting domains by the RBD method. The analysis of the extracellular domain of WAK1 with the 5-residue analysis window revealed several putative RBDs (Fig. 3B). All predicted RBDs appeared to be located outside the β-strand hydrophobic clusters as depicted by the HCA and NPS@ plots. Some of the RBDs showed up to be more relevant either because their size

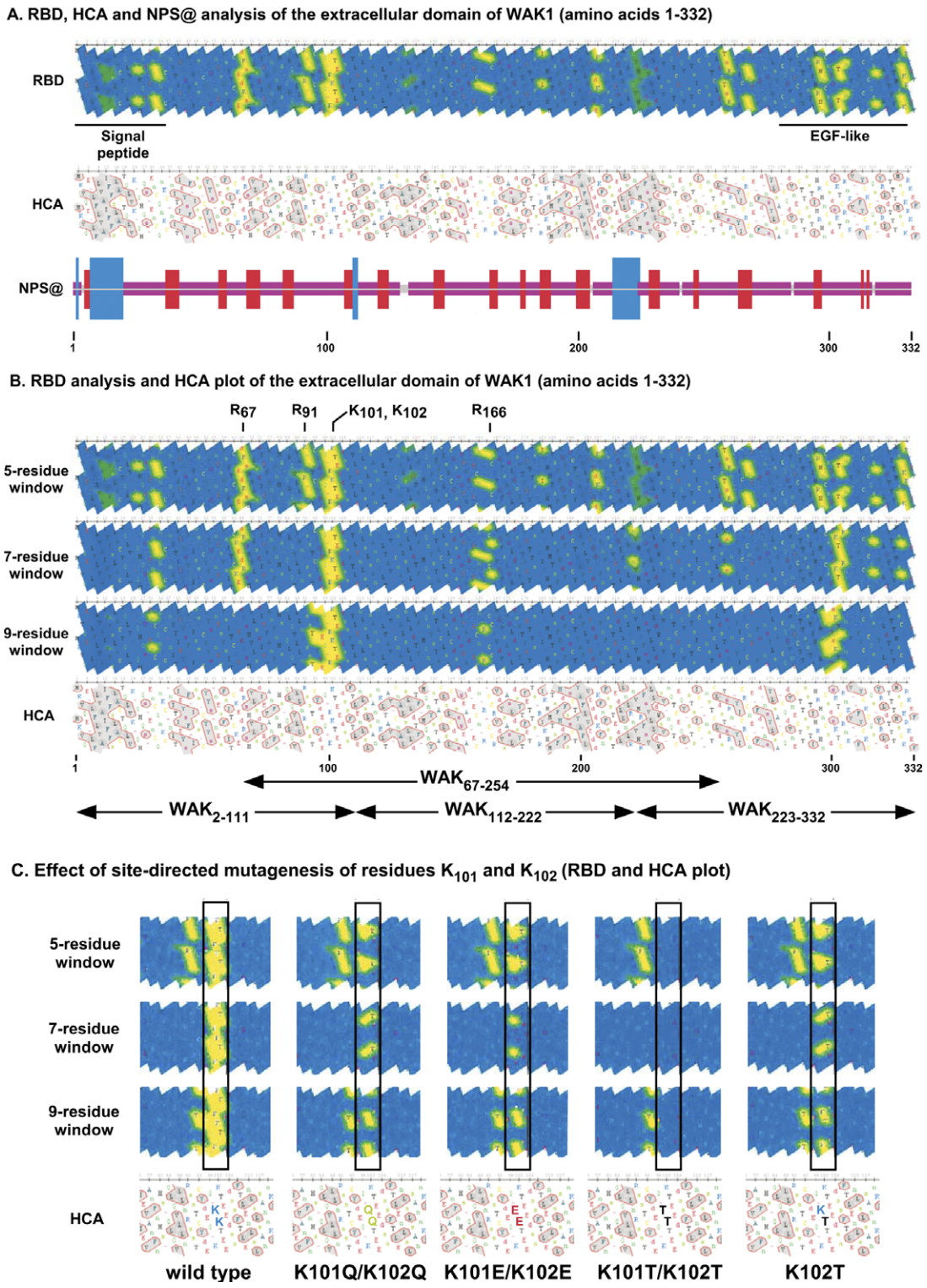

Fig. 3. (A) Receptor binding domain (RBD) analysis, hydrophobic cluster analysis (HCA) and NPS@ secondary structure consensus of the extracellular domain of WAK1 (amino acids 1–332). RBD plot: Accessible residues and residues predicted to be located in interacting domains of the protein are, respectively, depicted in green and yellow; residues located outside the predicted interacting domains are represented in blue. HCA plot: Contiguous hydrophobic residues are outlined and shaded; vertical and horizontal clusters correspond, respectively, to β-sheets and α helices. NPS@ plot: β-sheets and α helices are depicted as red and blue vertical bars, respectively. Amino acid positions are numbered. (B) RBD analysis and HCA plot of the extracellular domain (amino acids 1–332) of WAK1 using the 5-, 7- and 9-residue analysis window, and showing the $WAK_{2–111}$, $WAK_{112–222}$, $WAK_{223–332}$ and $WAK_{67–254}$ peptide segments. The colour legend is the same as in A. C. RBD analysis and HCA plot of wild-type $WAK_{67–254}$ and effect of several mutations on the RBD status of the K101/K102 residues (shown in bold in the HCA plot) shown on amino acid segment 74–122. RBD results are presented for the 5-, 7- and 9 residue analysis window. All shown mutations do not alter the predicted protein structure as depicted by the HCA plot. The K101T/K102T mutation was the only one to change the RBD status of the K101/K102 residues. The colour legend is the same as in A.

increased or because their RBD status was unchanged when the analysis window was increased to 7 or 9 residues. Four RBDs were clearly predicted within $WAK_{67–254}$: amino acid segments 65–70, 90–95, 100–105 and 160–170. As shown by the 9-residue analysis window data, segments 90–95 and 100–105 probably belong to one single interaction domain since they fused to form a more extended RBD (90–105). Segment 65–70, however, was not longer predicted to be a RBD with the same analysis window. Therefore and because of its location at the N-terminal extreme border of $WAK_{67–254}$, domain 65–70 was not analyzed any further in a first approach. The other RBDs predicted outside the $WAK_{67–254}$ segment were not analyzed in this study. Segment 30–34 was excluded because of its proximity with the recognition site of the cleavable signal peptide, and segment 299–304 because of its location within the EGF-domain shown to be involved in the binding of AtGRP-3 (Park et al., 2001).

In the following experiments, we focused on domains 90–105 and 160–170 in which we located cationic amino acids that were already shown to be involved in the interaction of homogalacturonans with isoperoxidases from zucchini and *Arabidopsis* (Carpin et al., 2001; Shah et al., 2004). Two arginines (R_{91} and R_{166}) and two neighbouring lysines (K_{101} and K_{102}) were selected within or just near the RBDs as good candidate cationic residues for site-directed mutagenesis.

2.4. Choice of mutations and production of $WAK_{67–254}$ mutants

In order to suppress these positive charges and verify their implication in homogalacturonan binding, the arginine (R_{91} and R_{166}) and lysine (K_{101} and K_{102}) residues were, respectively, mutated to glutamine (Q) and to threonine (T) using site-directed mutagenesis. These specific amino acid substitutions were selected in order to fulfill two specific criteria: (1) these mutations must not alter the predicted secondary structure of the extracellular domain of WAK1; and (2) the resulting mutated peptide stretches containing the R91Q, R166Q, K101T and K102T mutations must no longer be recognized as putative RBDs (Gallet et al., 2000; De Loof et al., 1986; Eisenberg et al., 1982, 1984). For each candidate residue, several substitutions were tested and their effect visualized on RBD plots. Fig. 3C shows the effect of four mutations on the RBD status of K101/K102: K101Q/K102Q, K101E/K102E, K101T/K102T and K102T. All shown mutations preserved the predicted secondary structure (see HCA plot), but the double K101T/K102T mutation was the only mutation to change the RBD status of the K101/K102 residues. The effect of the K101T/K102T and R166Q mutations on the mean hydrophobicity and mean hydrophobic moment of each residue of peptides E_{98}–E_{105} and G_{161}–A_{171} are shown in Fig. 4 and compared to the wild-type peptide. Both series of mutations resulted in a shift of the peptide properties out of the RBD area of the Eisenberg's graph. Similar substitutions and predictions were performed for the R_{91} mutations (not shown). Seven mutants (R91Q, K101T/K102T, R166Q, R91Q/R166Q, R166Q/K101T/K102T, R91Q/K101T/K102T and R91Q/R166Q/K101T/K102T) were obtained by site-directed mutagenesis and sequenced. Recombinant mutant proteins were over-expressed in yeast, purified as already described (Fig. 1) and tested for their ability to interact with homogalacturonans.

2.5. Binding of homogalacturonan to wild type $WAK_{67–254}$ in the presence of different Ca^{2+}/Na^{+} ratios

Since ionic conditions strongly affect the binding of WAK1 to homogalacturonans (Decreux and Messiaen, 2005), we developed an ELISA binding test in which the binding activity of each purified mutated peptide (250 ng) was measured in different Ca^{2+}/Na^{+} ratios to better evaluate their binding activity on different homogalacturonan conformations in solution, in comparison to the wild-type $WAK_{67–254}$ peptide. Practically, PGA (20 μg $well^{-1}$) was prepared in different Ca^{2+}/Na^{+} ratios in order to control

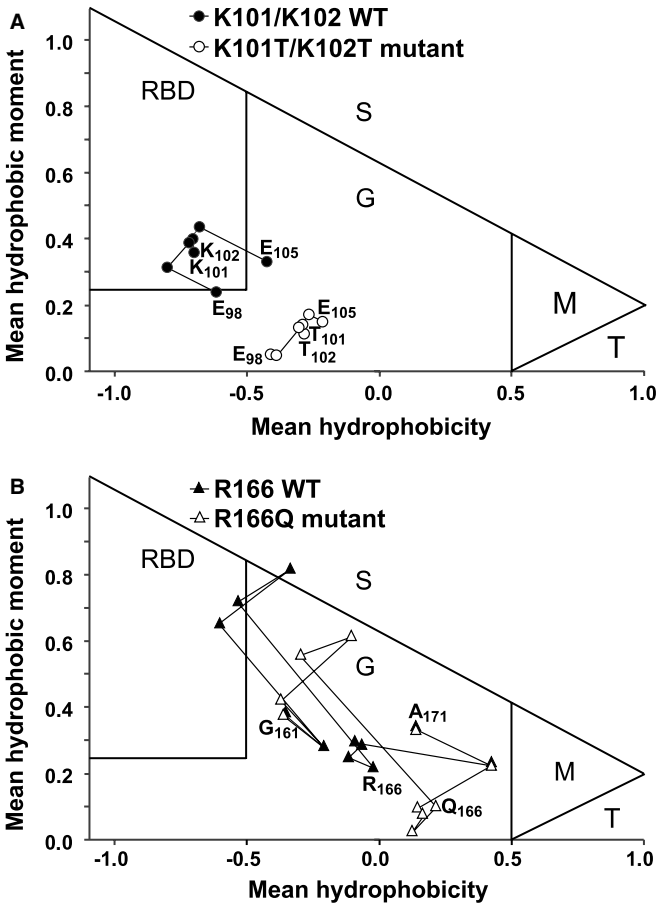

Fig. 4. Plots of mean hydrophobicity versus mean hydrophobic moment of peptide segments E_{98}-E_{105} (A) and G_{161}-A_{171} (B) in the wild-type $WAK_{67–254}$ and the K101T/K102T $WAK_{67–254}$ (A) and R166Q $WAK_{67–254}$ (B) mutants using the 7 amino acid residue analysis window. Globular (G), surface (S), membrane (M) and transmembrane (T) areas were defined according to Eisenberg et al. (1982); the receptor binding domain (RBD) area was defined according to Gallet et al. (2000).

the conformation of the immobilized polysaccharide chains. A Ca^{2+} (mN)/Na^{+} (mN) ratio of 1/150 was shown to be optimum for the formation of PGA dimers. Higher ratio results in the formation of multimers, whereas a lower ratio prevents calcium-induced intermolecular bridges (Morris et al., 1982; Liners et al., 1989). The binding of $WAK_{67–254}$ to PGA was detected as previously described (Decreux and Messiaen, 2005) and further compared to the recognition of PGA by the 2F4 monoclonal antibody that specifically recognises calcium-associated homogalacturonan dimers (Liners et al., 1989). The binding of $WAK_{67–254}$ to PGA was maximum for a Ca^{2+}/Na^{+} ratio equal to 0.5 mM $CaCl_2$/150 mM NaCl (Fig. 5) which corresponds to the optimum ratio for PGA to adopt a dimeric calcium-induced conformation (Morris et al., 1982; Liners et al., 1989) as depicted by the 2F4 response (Liners et al., 1989). The binding of $WAK_{67–254}$ to PGA was still important in the presence of higher Ca^{2+}/Na^{+} ratios indicating that $WAK_{67–254}$, unlike the 2F4 monoclonal antibody, also interacts with calcium-induced PGA multimers (Morris et al., 1982; Powell et al., 1982; Liners et al., 1992). At lower Ca^{2+}/Na^{+} ratios, the binding of $WAK_{67–254}$ was impaired, indicating either that $WAK_{67–254}$ did not bind monomeric PGA chains or that $WAK_{67–254}$ did undergo some unfolding in the presence of high salt concentrations.

2.6. Binding of homogalacturonan to mutant $WAK_{67–254}$ in the presence of different Ca^{2+}/Na^{+} ratios

The effect of the above mentioned mutations on homogalacturonan recruitment was compared to the behaviour of $WAK_{67–254}$ which exhibited the strongest binding activity to PGA (Fig. 2). In order to avoid any small difference in protein concentration between protein samples, interaction profiles were normalized. Practically, interaction between PGA and each recombinant protein was expressed as a percentage of a maximum of interaction corresponding to the "1/30 ratio" interaction value arbitrarily set to 100%.

Two single mutations, R91Q and R166Q, did not impair the binding of $WAK_{67–254}$ to PGA whatever the PGA conformation (Fig. 6A). Interestingly, the simultaneous double mutations R91Q/R166Q or K101T/K102T decreased the binding of $WAK_{67–254}$ to dimeric PGA by almost 50% compared to the wild-type $WAK_{67–254}$ (Fig. 6B). The binding of the R91Q/R166Q or the K101T/K102T mutant to multimeric PGA (Ca^{2+}/Na^{+} ratio 1/50) was however unaffected. In the R91Q/K101T/K102T mutant, the binding to PGA dimers was curiously improved compared to the K101T/K102T mutant, but still reduced (30%) compared to the binding of the wild-type $WAK_{67–254}$. The binding of the K101T/K102T/R166Q or the R91Q/K101T/K102T/R166Q mutant to dimeric PGA was however almost abolished in the presence of the 1/150 buffer. Here again, the binding of these mutant proteins to multimeric PGA (Ca^{2+}/Na^{+} ratio 1/50) was less affected. These data

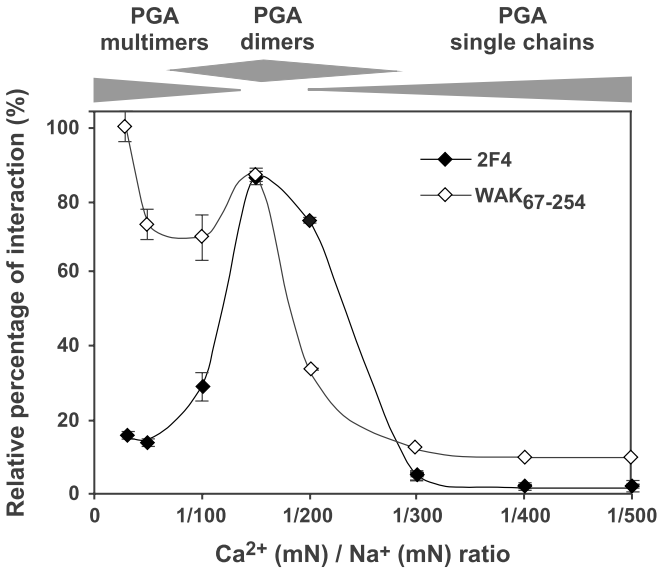

Fig. 5. ELISA test comparing the interaction of PGA (20 μg PGA $well^{-1}$) with recombinant $WAK_{67–254}$ (250 ng $well^{-1}$) or the 2F4 monoclonal antibody directed against calcium-associated homogalacturonan dimers. PGA was prepared in different Ca^{2+} (mN)/Na^{+} (mN) ratios in order to control its single chain, dimeric or multimeric conformation. Results are expressed as a percentage of the response obtained in the presence of $WAK_{67–254}$, or 2F4, tested in 1 mN Ca^{2+}/150 mN Na^{+} Tris buffer (100%). Error bars indicate SE ($n = 3$).

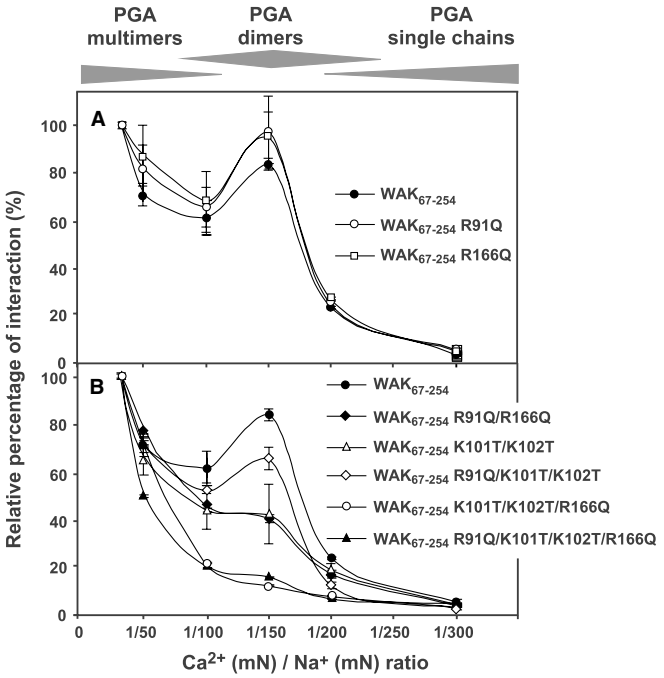

Fig. 6. ELISA binding test comparing the interaction of PGA (20 μg PGA $well^{-1}$) with recombinant wild-type and mutant $WAK_{67–254}$ (250 ng $well^{-1}$). PGA was prepared in different Ca^{2+} (mN)/Na^{+} (mN) ratios in order to control its single chain, dimeric or multimeric conformation. (A) Mutations without effect on PGA binding: $WAK_{67–254}$ R91Q and $WAK_{67–254}$ R166Q. (B) Mutations affecting PGA binding: $WAK_{67–254}$ R91Q/R166Q, $WAK_{67–254}$ K101T/K102T, $WAK_{67–254}$R91Q/K101T/K102T, $WAK_{67–254}$ K101T/K102T/R166Q and $WAK_{67–254}$ R91Q/K101T/K102T/R166Q. Interaction profiles were normalized by expressing the results as a percentage of a maximum of interaction corresponding to the "1/30 ratio" interaction value arbitrarily set to 100%.

indicate that, at least, K_{101}, K_{102} and R_{166} when mutated together impair the binding of $WAK_{67–254}$ to PGA dimers and, to a lesser extent, to PGA multimers. The successive loss of three positive charges is thus sufficient to induce a progressive disruption of the WAK1–PGA dimer complex in our test. The role of residue R_{91} in PGA binding is more difficult to ascertain. Compared to the K101T/K102T/R166Q mutant, the loss of a fourth positive charge had apparently no impact on the binding of the R91Q/K101T/K102T/R166Q mutant to PGA dimers, but resulted in a significant loss of binding activity (20–30%) to PGA multimers (Ca^{2+}/Na^{+} ratio 1/50) (Fig. 6B). This complex binding behaviour suggests that the cumulative loss of positive charges is probably not sufficient to explain all aspects of the interaction between WAK1 and homogalacturonans.

2.7. Binding of homogalacturonan to wild-type and mutant $WAK_{2–332}$ in the presence of different Ca^{2+}/Na^{+} ratios

Since RBD analysis data also predicted a putative RBD between residues 65–70 (Fig. 3A), we decided to measure the effect of the addition of the R67Q mutation on the binding to PGA of the full-length extracellular domain of WAK1 (called $WAK_{2–332}$). The R67Q mutation was combined to the K101T/K102T mutations which reduced the binding of $WAK_{67–254}$ to PGA dimers by 50% (Fig. 6B). The same criteria as mentioned above were used to justify the choice to switch R_{67} to Q (Fig. 7A). The corresponding DNA sequence encoding amino acids 2–332 was PCR amplified, cloned and over-expressed in yeast. $WAK_{2–332}$ K101T/K102T and $WAK_{2–332}$ R67Q/K101T/K102T mutants were obtained by site-directed mutagenesis of the pYESNTC-$WAK_{2–332}$ and the following complementary primers: 5′-ACCTGTAAGGAAGATCA(ag)GCCACA TGTCTTAAGC-3′ for the R67Q mutation and 5′-CTACGACGAGCAAGGAAC(a)AAC(a)AACTGAGGAGGACAG-3′ for the K101T-K102T double mutation. Recombinant mutant proteins were over-expressed in yeast, purified and tested for their ability to bind to PGA. The binding of $WAK_{2–332}$ to PGA was first compared to that of $WAK_{67–254}$ (Fig. 7B), and further to that of the $WAK_{2–332}$ K101T/K102T and $WAK_{2–332}$ R67Q/K101T/K102T mutants (Fig. 7C).

The binding profile of $WAK_{2–332}$ to PGA was slightly different from that of $WAK_{67–254}$ (Fig. 7B). First, the binding of $WAK_{2–332}$ to PGA was improved compared to $WAK_{67–254}$ whatever the conformation of PGA. Second, the binding of $WAK_{2–332}$ to PGA multimers seemed to be more variable as depicted by the high standard deviation between the 1/50 and 1/150 Ca^{2+}/Na^{+} ratios (results from 3 independent experiments). This reflects probably the complexity of the interaction of the full-length extracellular domain within a Ca^{2+}/Na^{+} ratio range coinciding with a conformational transition between PGA dimers (Ca^{2+}/Na^{+} ratio 1/150) and multimers (Ca^{2+}/Na^{+} ratio 1/50), the two binding substrates of WAK1. The K101T/K102T mutations had a different effect on the binding of

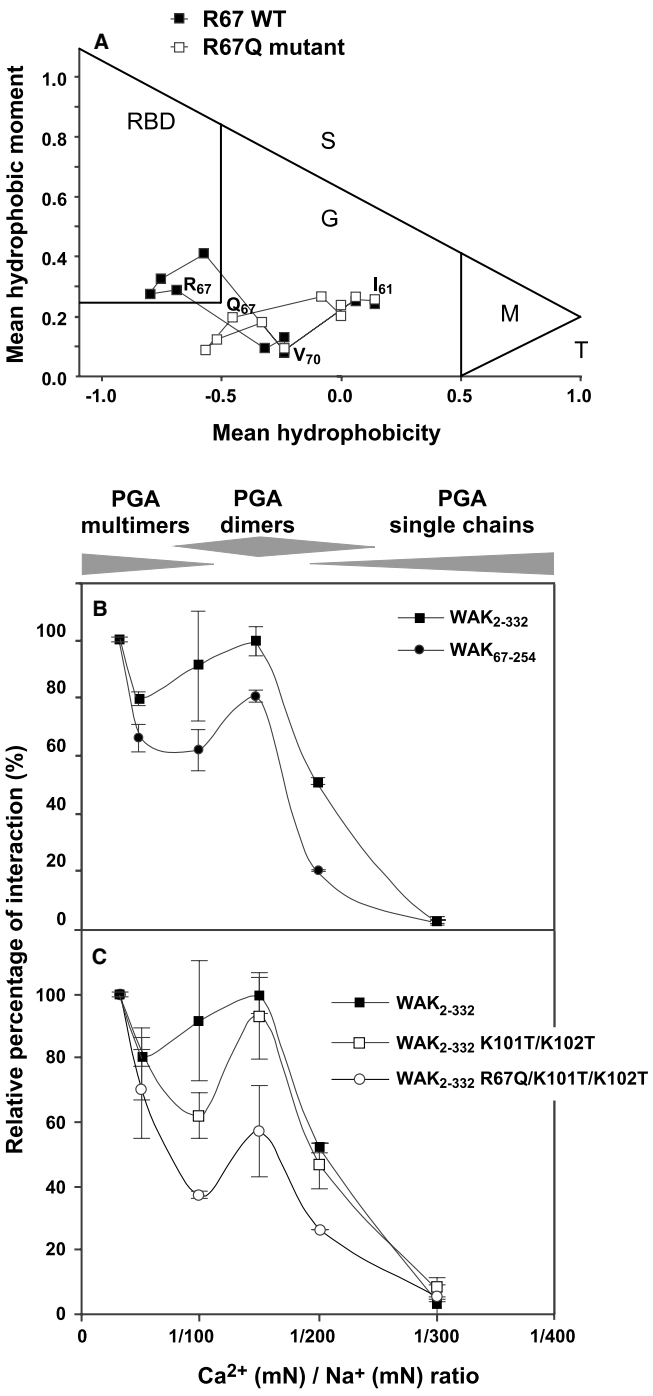

Fig. 7. (A) Plots of mean hydrophobicity versus mean hydrophobic moment of peptide segments I_{61}–V_{70} in the wild-type and the R67Q/K101T/K102T $WAK_{2–332}$ mutant. Globular (G), surface (S), membrane (M) and transmembrane (T) areas were defined according to Eisenberg et al. (1982); the receptor binding domain (RBD) area was defined according to Gallet et al. (2000). (B) ELISA binding test comparing the interaction of PGA (20 μg PGA $well^{-1}$) with $WAK_{67–254}$ or $WAK_{2–332}$ (250 ng $well^{-1}$). PGA was prepared in different Ca^{2+} (mN)/Na^{+} (mN) ratios in order to control its single chain, dimeric or multimeric conformation. (C) ELISA test comparing the interaction of PGA (20 μg PGA $well^{-1}$) with wild-type, K101T/K102T $WAK_{2–332}$ and R67Q/K101T/K102T $WAK_{2–332}$ mutants (250 ng $well^{-1}$). Interaction profiles were normalized by expressing the results as a percentage of a maximum of interaction corresponding to the "1/30 ratio" interaction value arbitrarily set to 100% (B and C). Error bars indicate SE ($n = 3$).

$WAK_{2–332}$ to pectin than on that of $WAK_{67–254}$. The K101T/K102T mutations had almost no effect on the binding of $WAK_{2–332}$ to PGA dimers and multimers, indicating that the loss of two positive charges is not sufficient to inhibit the binding of the full-length extracellular domain of WAK1 to PGA (Fig. 7C). The addition of the R67Q mutation to the K101T/K102T mutations reduced the binding of $WAK_{2–332}$ to PGA dimers by 40%, without affecting its binding to PGA multimers (Ca^{2+}/Na^{+} ratio 1/50). These data suggest that residue R_{67} of the extracellular domain of WAK1 is also involved in the binding to homogalacturonans adopting a calcium-induced conformation.

3. Discussion

Using truncated versions of the full-length extracellular domain of WAK1, we found that most of the homogalacturonan-binding activity of WAK1 is located near the N-terminal end of the extracellular domain of WAK1, between amino acid residues 2–111 (70% of the binding activity of $WAK_{67–254}$). Using that approach, no significant homogalacturonan-binding activity was detected neither in segment 112–221 nor in segment 222–332 when tested separately. RBD analysis of the extracellular domain of $WAK_{67–254}$ allowed us to identify four interacting domains. Within those domains, five basic amino acids (R_{67}, R_{91}, K_{101}, K_{102} and R_{166}) that could be involved in the interaction with negatively charged homogalacturonans were selected for substitution by site-directed mutagenesis. The binding of mutated versions of $WAK_{67–254}$ to calcium-associated homogalacturonan correlated well with the homogalacturonan-binding activity of sub-domains $WAK_{2–111}$, $WAK_{112–221}$ and $WAK_{222–332}$ (Fig. 2). Four out of five amino acid residues (R_{67}, R_{91}, K_{101} and K_{102}) that impaired the binding of homogalacturonan dimers to $WAK_{67–254}$ once mutated are indeed located within sub-domain $WAK_{2–111}$. Although $WAK_{112–221}$ contained residue R_{166} that was shown to be involved in homogalacturonan binding using the site-directed mutagenesis approach, the binding of $WAK_{112–221}$ to homogalacturonans was reduced to less than 20% of that of $WAK_{67–254}$ (Fig. 2). This suggests that residue R_{166} belongs to a RBD which alone is not sufficient to bind homogalacturonans significantly, a conclusion supported by the absence of impact of the single R166Q mutation on the binding of $WAK_{67–254}$ to homogalacturonan dimmers (Fig. 6A). However, the importance of residue R_{166} in homogalacturonan binding was clearly highlighted when mutation R166Q was combined with the K101T/K102T mutations (Fig. 6B). The absence of interaction of this triple mutant with homogalacturonan dimers suggests an additive and probably cooperative effect of each residue in the binding of $WAK_{67–254}$. This probably explains why peptide $WAK_{67–254}$, which contains five positively charged residues involved in homogalacturonan binding, always binds better to PGA than peptide $WAK_{2–111}$ which contains only four of those residues (Fig. 2).

How those specific basic amino acids interact with negatively charged carboxylic groups within homogalacturonan dimers and multimers is not known. However, our binding data obtained in the presence of mutated versions of WAK1 clearly indicate that even a small reduction of the number of positive charges located within the RBD domains of WAK1 results in a weaker interaction. Since this reduction of interaction is more drastic in the presence of homogalacturonan dimers than with multimers, it is not excluded that additional interaction sites could be involved during the interaction with multimers. In terms of cell wall architecture, as already suggested in our previous study (Decreux and Messiaen, 2005), changes of the degree of methylesterification of native pectin (i.e., the number of negative charges unavailable on the homogalacturonic ligand to interact with WAK1) could therefore drastically affect the strength of the WAK1–pectin interaction along plant development. Since the average degree of methylesterification of newly synthesized pectin is generally high (Liners and Van Cutsem, 1992; Ridley et al., 2001) and is modified during time by the block-wise action of pectin methylesterases (Liners et al., 1992; Willats et al., 2001), one could suspect the WAK1–homogalacturonan interaction to be become stronger during cell differentiation. The presence of homogalacturonan stretches long enough to adopt a calcium-induced conformation, and to potentially interact with WAK1, have been recently detected in *Arabidopsis* stem cell walls (Manfield et al., 2005). Moreover, if we assume that the calcium to monovalent cation ratio within non-challenged cell walls is probably high, the progressively de-esterified homogalacturonans are more likely to adopt a multimeric conformation and become a potential strong binding substrate for WAK1, if accessible or present. On the contrary, during infection by pectolytic pathogens, ion leakage from damaged cells, or induced ion fluxes, decrease the calcium to monovalent cation ratio allowing pathogen released homogalacturonan oligomers to adopt the calcium-induced dimer conformation also known to bind WAK1 (Decreux and Messiaen, 2005) and to elicit several defense responses in plants (Messiaen and Van Cutsem, 1994).

Although RBD domains were predicted in sub-domain $WAK_{222–332}$ (Fig. 3) that partially covers the AtGRP-3 binding-peptide (amino acid residues 178–334; Park et al., 2001), $WAK_{222–332}$ did not bind to calcium-bound homogalacturonans (Fig. 2). We can however not rule out any indirect role of the two EGF-like domains on WAK1–homogalacturonan interaction because the binding of the full-length extracellular domain ($WAK_{2–332}$) to homogalacturonans was always stronger than that of $WAK_{67–254}$ alone.

The homogalacturonan-binding domain of $WAK_{67–254}$ also contains, besides the basic amino acid residues involved in homogalacturonan binding, 11 conserved cysteine residues that might be relevant for WAK1–homogalacturonan interaction. Even if we have no evidence of their implication in homogalacturonan binding by WAK1, it has been shown

that a basic cysteine-rich protein, known as the stigma/stylar cysteine-rich adhesin (SCA), binds pectin and controls the adhesion of pollen cells to the surface of the stylar transmitting tract epidermis in lily (Mollet et al., 2000). The binding of SCA itself to pectin did not involve the cysteine residues directly, but was mediated by ionic interactions (like the pectin–WAK1 interaction; Decreux and Messiaen, 2005) and was clearly pH dependent. Moreover, both SCA and WAK1 were shown to interact with alginates, an other polyanionic polysaccharide known to adopt a similar calcium-induced conformation (Mollet et al., 2000; Decreux and Messiaen, 2005). The cysteine-rich domain of SCA was proposed to form a carbohydrate-reception pocket also found in some plant lectins (Peumans et al., 2003), or in some receptors such as the mannose receptor of hepatic endothelial cells involved in the binding of oligosaccharides ending with 4-sulphated *N*-acetylglucosamine (GalNAc-4-SO_4) (Fiete et al., 1998). The conserved cysteines of the homogalacturonan-binding domain of WAK1 could similarly be involved in the consolidation of a 3D structure exposing the arginine and lysine residues in order to interact with a calcium-induced conformation adopted by homogalacturonic regions within pectin.

In other known homogalacturonan-binding proteins for which structural data is available, such as for pectolytic enzymes (Scavetta et al., 1999; Niture et al., 2001; Johansson et al., 2002) and some isoperoxidases (Carpin et al., 2001; Shah et al., 2004), arginine and/or lysine residues were also predicted to play a critical role during their interaction with homogalacturonans. Since pectolytic enzymes and isoperoxidases are structurally not related, it seems plausible that homogalacturonan-binding domains can have different topologies, while sharing cationic amino acid residues such as arginines and lysines in a critical position or configuration. In absence of crystal structure for the extracellular domain of WAK1, the presence of 3D conformations similar to those found in other homogalacturonan-binding proteins remains thus speculative. If we consider now the other members of the WAK family, we found that the extracellular domain of WAK2 (but not other WAKs) has a RBD signature very similar to that of WAK1. The RBD domains are indeed found in conserved positions and they contain arginine and lysine residues in similar positions (Decreux, 2006), suggesting that WAK2 could be a good candidate to test for its interaction with homogalacturonans.

In conclusion, we successfully used receptor domain binding analysis and ELISA interaction studies to locate and confirm critical residues in the extracellular domain of WAK1 involved in the binding of homogalacturonans under a calcium-induced conformation. Since the disruption of the expression of WAKs using anti-sense constructs or negative dominant alleles was shown to drastically affect cell expansion and the ability of *Arabidopsis* plants to respond to invading pathogens (He et al., 1998; Wagner and Kohorn, 2001), our data could be relevant to design specific, and less severe, mutant forms of WAK1 exhibiting differential binding to homogalacturonans and test their effects on cell elongation and defence responses in transgenic plants.

4. Experimental

4.1. Plant growth conditions

A. thaliana seedlings (Col-0, N1092) were grown in a controlled growth chamber with a day/night temperature of 23 °C/21 °C and a 16-h/8-h light/dark regime (150 μmol m^{-2} s^{-1}) in axenic liquid cultures in Murashige and Skoog medium (including vitamins) supplemented with 30 g l^{-1} sucrose (pH 5.8).

4.2. Cloning and transformation of Saccharomyces cerevisiae

Total RNA was isolated from two-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. DNA sequences encoding sub-domains of the extracellular domain of WAK1 (NCBI Accession No. AJ009696) were amplified from first-strand cDNA by PCR using Taq polymerase (Roche Applied Science, Belgium) and the following primers (Eurogentec, Belgium): 5′-CCCGCGGCCGC-AGGTGCAGGAGGGTT-3′ and 5′-CCCCTCGAG-CAGTGTAAA AGAACTGTCCTCC-3′ for $WAK_{2–111}$; 5′-CCCCTCGAGATTTCGCAGATTCAGAAGAT-3′ and 5′-CCCGCGGCCGCAAAATTTATCTCTTTCCGC-3′ for $WAK_{112–221}$; 5′-CCCCTCGAGGAGACAAGTGCT-GTTCCCA-3′ and 5′-CCCGGATCCAGGCCA CAT-GTCTTAAGCGAC-3′ for $WAK_{67–254}$ 5′-CCCGCGGCC-GCTCATGAGGTTCCCT GTGTTA-3′ and 5′-CCCCTC GAGAAACTCTTTACGCTTGCAGCTC-3′ for $WAK_{222.332}$; and 5′-CCCGCGGCCGCAGGTGCAGGAGGGTT-3′ and 5′-CCCCTCGAGAAACTC TTTACGCTTGCAG-CTC-3′ for $WAK_{2.332}$. These PCR products were ligated into the multiple cloning site of the yeast expression vector pYES2/NTC (Invitrogen, Belgium) designed to express a recombinant protein in fusion with N- and C-terminal poly-His tags for purification and with a N-terminal Xpress epitope for immunodetection. *S. cerevisiae* host cells (strain INVScl, 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% (w/v) raffinose.

4.3. Sequence analysis and site-directed mutagenesis

Hydrophobic cluster analysis (HCA) was performed according to Gaboriaud et al. (1987). Network protein

sequence analysis (NPS@) using eight different interconnected prediction softwares was performed as described by Combet et al. (2000) and available at the following website: http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html. Receptor binding domain (RBD) analysis was performed as described by Gallet et al. (2000). This method uses a simple algorithm for predicting residues involved in interaction sites by analysing hydrophobicity distribution within a protein sequence. This method has been tested successfully (1) to compare experimental and computed predictions of interaction sites of known interacting partners including protein–protein and protein–DNA interactions, (2) to predict point mutations that disrupt the interaction between two partners without altering their structure or conformation (Gallet et al., 2000). Site-directed mutagenesis of the pYES2/NTC-$WAK_{67–254}$ and pYES2/NTC-$WAK_{2–332}$ plasmids was performed using the QuikChange Site-Directed kit following the manufacturer's protocol (Stratagene, Belgium) and the following complementary primers: 5′-CTACGACGAGCAAGGAAC(a)AAC(a)AACT GAGGAGGACAG-3′ for the K101T-K102T double mutation, 5′-CAAGTTCTGCTTAATCA(g)ATCCTCTACTTGCTACG-3′ for the R91Q mutation and 5′-GGAGAATGTAATGGTCA(ag)AGGTTGCTGCAGAGTC-3′ for the R166Q mutation. Mutated sequences were validated by sequencing on a capillary sequencer (CEQ™8000 genetic analysis system, Beckman Coulter, Belgium) using the CEQ Dye terminator Cycle sequencing Quick Start Kit (Beckman Coulter, Belgium). Mutated plasmids were used to transform *S. cerevisiae* host cells (strain INVScl, Invitrogen, Belgium) as described above.

4.4. Expression of recombinant protein

Recombinant yeast cells were pre-cultured in 50 ml liquid SC-U medium containing 2% (w/v) raffinose at 30 °C with shaking. After 24 h, cells were diluted with the induction medium (SC-U medium containing 2% (w/v) galactose and 1% raffinose (w/v)) to obtain an OD_{600} of 0.4. After an additional 17 h culture at 30 °C with shaking, induced cells were pelleted at 1500*g* for 5 min at 4 °C. The cells were then washed with sterile water and centrifuged at 1500*g* for 5 min at 4 °C. Yeast pellets were directly used for recombinant protein extraction or were frozen in liquid nitrogen and stored at −80 °C until use.

4.5. Purification of recombinant proteins

Total proteins were extracted by grinding yeast cells in liquid nitrogen in the presence of 7 ml denaturing guanidinium lysis buffer (6 M guanidine-HCl, 20 mM sodium phosphate buffer (pH 7.8), 500 mM NaCl, 1 mM phenylmethylsulphonylfluoride, 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 3000*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 resin, Invitrogen, Belgium). The recombinant proteins were renatured by sequential dialysis as described by Ferguson and Goodrich (2001). The final dialysis was performed in the appropriate buffer used in the ELISA binding tests and stored at −80 °C in the presence of Triton X-100 1% (w/v) and glycerol 15% (w/v) before use. The absence of protein aggregates was verified by SDS–PAGE-Coomassie staining and western blotting using the anti-Xpress primary antibody (Invitrogen, Belgium). The binding behaviour of each dialysed and purified peptide to PGA was checked to be always identical to that of the corresponding native non-purified recombinant peptide (positive renaturation control). Protein samples failing to fulfill these two criteria were discarded. Protein concentration was determined with the NanoOrange protein quantification kit (Molecular Probes, USA).

4.6. Homogalacturonan–WAK1 ELISA binding assay

All ELISA binding tests were performed in the presence of a commercial source of homogalacturonans known as polygalacturonic acid (PGA, Sigma, USA). The sugar content of this homogalacturonan preparation is 92.3% galacturonic acid (<5% methylesterification), 3.22% arabinose, 2.58% galactose, 0.90% rhamnose, 0.59% xylose and 0.41% glucose. The average degree of polymerization of PGA was estimated to 50 by viscosimetry analysis (Cabrera G., personal communication).

Maxisorp microplates (VWR, Belgium) were pretreated with polylysine-HBr (50 μg ml^{-1} in H_2O, 50 μl $well^{-1}$, Sigma, USA) 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 PGA 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% (w/v) of 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 (250 ng $well^{-1}$) 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% (w/v) low fat dried milk (50 μl $well^{-1}$). After washing with 7 × 250 μl of 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer, 50 μl of a 1/1000 dilution of HRP-SAM secondary antibody (Amersham Biosciences, Belgium) prepared in 0.5 mM Ca^{2+}/150 mM Na^+ Tris buffer containing 1% (w/v) 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 with the coated polysaccharide was visualized in the presence of the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Enhanced K Blue TMB substrate, Neogen, USA). Absorbance was measured after 20 min in the dark at 650 nm with a microplate reader (El × 800, Bio-TEK instruments, USA). Binding assays were done in triplicate for, at least, three protein extracts.

Acknowledgements

We thank Dr. F. Liners for critical discussions and N. Dauchot for sequencing. The work of A.D. and J.M. was supported by the National Funds for Scientific Research of Belgium (FRFC 2.4.501.00. F). The work of B.S. was supported by a grant from the Funds for Industrial and Agricultural Research (FRIA). R.B. is Research Director at the National Funds for Scientific Research of Belgium (FNRS). A.T. is Research Director at INSERM (France).

References

Anderson, C.M., Wagner, T.A., Perret, M., He, Z.H., He, D., Kohorn, B.D., 2001. WAKs: cell wall-associated kinases linking the cytoplasm to the extracellular matrix. Plant Mol. Biol. 47, 197–206.

Bellincampi, D., Dipierro, N., Salvi, G., Cervone, F., De Lorenzo, G., 2000. Extracellular H_2O_2 induced by oligogalacturonides is not involved in the inhibition of the auxin-regulated rolB gene expression in tobacco leaf explants. Plant Physiol. 122, 1379–1385.

Carpin, S., Crevecoeur, M., de Meyer, M., Simon, P., Greppin, H., Penel, C., 2001. Identification of a Ca(2+)-pectate binding site on an apoplastic peroxidase. Plant Cell 13, 511–520.

Combet, C., Blanchet, C., Geourjon, C., Deleage, G., 2000. NPS@: network protein sequence analysis. TIBS 25, 147–150.

Decreux, A., 2006. Study of the receptor-like kinase WAK1 from Arabidopsis thaliana. Highlighting and Characterization of the Interaction of WAK1 Extracellular Domain with Pectin. Presses Universitaires de Namur, Namur, Belgium.

Decreux, A., Messiaen, J., 2005. Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. Plant Cell Physiol. 46, 268–278.

De Loof, H., Rosseneu, M., Brasseur, R., Ruysschaert, J.M., 1986. Use of hydrophobicity profiles to predict receptor binding domains on apolipoprotein E and the low density lipoprotein apolipoprotein B-E receptor. Proc. Natl. Acad. Sci. USA 83, 2295–2299.

Eisenberg, D., Weiss, R.M., Terwilliger, T.C., 1982. The helical hydrophobic moment: a measure of the amphiphilicity of a helix. Nature 299, 371–374.

Eisenberg, D., Schwarz, E., Komaromy, M., Wall, R., 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. 179, 125–142.

Ferguson, H.A., Goodrich, J.A., 2001. Expression and purification of recombinant human c-Fos/c-Jun that is highly active in DNA binding and transcriptional activation in vivo. Nucleic Acid Res. 29, e98.

Fiete, D.J., Beranek, M.C., Baenziger, J.U., 1998. A cysteine-rich domain of the "mannose" receptor mediates GalNAc-4-SO4 binding. Proc. Natl. Acad. Sci. USA 95, 2089–2093.

Gaboriaud, C., Bissery, V., Benchetrit, T., Mornon, J.P., 1987. Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. FEES Lett. 224, 149–155.

Gallet, X., Charloteaux, B., Thomas, A., Brasseur, R., 2000. A fast method to predict protein interaction sites from sequences. J. Mol. Biol. 302, 917–926.

He, Z.H., Cheeseman, I., He, D., Kohorn, B.D., 1999. A cluster of five cell wall-associated receptor kinase genes, Wakl-5, are expressed in specific organs of *Arabidopsis*. Plant Mol. Biol. 39, 1189–1196.

He, Z.H., Fujiki, M., Kohorn, B.D., 1996. A cell wall-associated, receptor-like protein kinase. J. Biol. Chem. 271, 19789–19793.

He, Z.H., He, D., Kohorn, B.D., 1998. Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response. Plant J. 14, 55–63.

Johansson, K., El-Ahmad, M., Friemanna, R., Jornvall, H., Markovic, O., Eklund, H., 2002. Crystal structure of plant pectin methylesterase. FEES Lett. 514, 243–249.

Lally, D., Ingmire, P., Tong, H.Y., He, Z.H., 2001. Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. Plant Cell 13, 1317–1331.

Lemesle-Varloot, L., Henrissat, B., Gaboriaud, C., Bissery, V., Morgat, A., Mornon, J.P., 1990. Hydrophobic cluster analysis: procedures to derive structural and functional information from 2-D-representation of protein sequences. Biochimie 72, 555–574.

Liners, F., Letesson, J.J., Didembourg, C., Van Cutsem, P., 1989. Monoclonal antibodies against pectin – recognition of a conformation induced by calcium. Plant Physiol. 91, 1419–1424.

Liners, F., Thibault, J.F., Van Cutsem, P., 1992. Influence of the degree of polymerization of oligogalacturonates and of esterification pattern of pectin on their recognition by monoclonal antibodies. Plant Physiol. 99, 1099–1104.

Liners, F., Van Cutsem, P., 1992. Distribution of pectic polysaccharides throughout wall of suspension-cultured carrot cells. Protoplasma 170, 10–21.

Manfield, I.W., Bernal, A.J., Moller, I., McCartney, L., Riess, N.P., Knox, J.P., Willats, W.G.T., 2005. Re-engineering of the PAM1 phage display monoclonal antibody to produce a soluble, versatile anti-homogalacturonan scFv. Plant Sci. 169, 1090–1095.

Mauro, M.L., De Lorenzo, G., Costantino, P., Bellincampi, D., 2002. Oligogalacturonides inhibit the induction of late but not of early auxin-responsive genes in tobacco. Planta 215, 494–501.

Messiaen, J., Read, N.D., Van Cutsem, P., Trewavas, A.J., 1993. Cell wall elicitors increase cytosolic free Ca^{2+} in plant cells. J. Cell Sci. 104, 365–371.

Messiaen, J., Van Cutsem, P., 1993. Defense gene transcription in carrot cells treated with oligogalacturonides. Plant Cell Physiol. 34, 1117–1123.

Messiaen, J., Van Cutsem, P., 1994. Pectic signal transduction in carrot cells: membrane, cytosolic and nuclear responses induced by oligogalacturonides. Plant Cell Physiol. 35, 677–689.

Mollet, J.C., Park, S.Y., Nothnagel, E.A., Lord, E.M., 2000. A lily stylar pectin is necessary for pollen tube adhesion to an in vitro stylar matrix. Plant Cell 12, 1737–1749.

Morris, E.R., Powell, D.A., Gidley, M.J., Rees, D.A., 1982. Conformations and interactions of pectins. I. Polymorphism between gel and solid states of calcium polygalacturonate. J. Mol. Biol. 155, 507–516.

Niture, S.K., Pant, A., Kumar, A.R., 2001. Active site characterization of the single endo- polygalacturonase produced by *Fusarium moniliforme* NCIM 1276. Eur. J. Biochem. 268, 832–840.

Park, A.R., Cho, S.K., Yun, U.J., Jin, M.Y., Lee, S.H., Sachetto-Martins, G., Park, O.K., 2001. Interaction of the *Arabidopsis* receptor protein kinase Wakl with a glycine-rich protein, AtGRP-3. J. Biol. Chem. 276, 26688–26693.

Peumans, W.J., Rouge, P., van Damme, E.J.M., 2003. The tomato lectin consists of two homologous chitin-binding modules separated by an extensin-like linker. Biochem. J. 376, 717–724.

Powell, D.A., Morris, E.R., Gidley, M.J., Rees, D.A., 1982. Conformations and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gels. J. Mol. Biol. 155, 517–531.

Ridley, B.L., O'Neill, M.A., Mohnen, D., 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57, 929–967.

Scavetta, R.D., Herron, S.R., Hotchkiss, A.T., Kita, N., Keen, N.T., Benen, J.A.E., Kester, H.C.M., Visser, J., Jurnak, F., 1999. Structure of a plant cell wall fragment complexed to pectate lyase C. Plant Cell 11, 1081–1092.

Shah, K., Penel, C., Gagnon, J., Dunand, C., 2004. Purification and identification of a Ca^{2+}-pectate binding peroxidase from *Arabidopsis* leaves. Phytochemistry 65, 307–312.

Shiu, S.H., Bleecker, A.B., 2001. Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. Proc. Natl. Acad. Sci. USA 98, 10763–10768.

Trotochaud, A.E., Hao, T., Wu, G., Yang, Z., Clark, S.E., 1999. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. Plant Cell 11, 393–406.

Verica, J.A., Chae, L., Tong, H., Ingmire, P., He, Z.H., 2003. Tissue-specific and develop-mentally regulated expression of a cluster of tandemly arrayed cell wall-associated kinase-like kinase genes in *Arabidopsis*. Plant Physiol. 133, 1732–1746.

Verica, J.A., He, Z.H., 2002. The cell wall-associated kinase (WAK) and WAK-like kinase gene family. Plant Physiol. 129, 455–459.

Wagner, T.A., Kohorn, B.D., 2001. Wall-associated kinases are expressed throughout plant development and are required for cell expansion. Plant Cell 13, 303–318.

Willats, W.G.T., Orfila, C., Limberg, G., Buchholt, H.C., van Alebeek, G.J.W.M., Vor-agen, A.G.J., Marcus, S.E., Christensen, T.M.I.E., Mikkelsen, J.D., Murray, B.S., Knox, J.P., 2001. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls – implications for pectin methyl esterase action, matrix properties, and cell adhesion. J. Biol. Chem. 273, 19404–19413.

Woodcock, S., Mornon, J.P., Henrissat, B., 1992. Detection of secondary structure elements in proteins by hydrophobic cluster analysis. Protein Eng. 5, 629–635.

Yang, E.J., Oh, Y.A., Lee, E.S., Park, A.R., Cho, S.K., Yoo, Y.J., Park, O.K., 2003. Oxygen-evolving enhancer protein 2 is phosphorylated by glycine-rich protein 3/wall-associated kinase 1 in *Arabidopsis*. Biochem. Biophys. Res. Commun. 305, 862–868.