

Egg box conformation of oligogalacturonides: The time-dependent stabilization of the elicitor-active conformation increases its biological activity

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Circular dichroism spectrometry was used on oligogalacturonides (OGAs) and showed the existence of a calcium/sodium-induced conformational state that is intermediate between single-isolated chains and calcium-associated multimer chains. This conformation is interpreted as being egg box dimers. Using the 2F4 monoclonal antibody that specifically binds such an egg box dimer conformation of pectin, the stability of OGA dimers was investigated over a period of 24 hours. The extent to which egg box dimers were recognized by the antibody was dependent on the temperature and duration of preincubation of the OGA. This suggests a “maturation” process of the egg-box structure that consists in a progressive increase in the length of the junction sequences between two chains that slide along each other in order to form a maximum number of calcium bridges and dimer ends. The maturation of egg boxes induced both a significant increase in their binding to wall-associated kinase 1 (WAK1) and an increased extracellular alkalization when applied to *Arabidopsis thaliana* cell suspensions. The chemical modification of the reducing end of the OGAs largely diminished their eliciting activity but did not hinder either dimerization or binding of these end-reduced egg boxes to WAK1. We conclude that there are at least two different perception systems for egg box dimers. One binds egg box junctions and the other binds egg box ends. The relevance of these results is discussed in terms of pectic signal perception and plant–pathogen interaction.

Keywords: *Arabidopsis*/egg box/elicitation/oligogalacturonides/pectin

Introduction

Homopolygalacturonic fragments released from the plant cell wall are well-known activators of biological responses involved in plant defense, growth, and development (Aldington and Fry 1993; Cote and Hahn 1994; Darvill et al. 1994; Van Cutsem and Messiaen 1994; Ridley et al. 2001; Baldan et al. 2003). While smaller pectin fragments show some bioactivity (Boudart et al. 1995; Simpson et al. 1998; Moerschbacher et al. 1999),

oligogalacturonides (OGAs) with a degree of polymerization (DP) between 9 and 15 have been shown to be most potent inducers of plant responses (Marfa et al. 1991; Mathieu et al. 1991; Bellincampi et al. 1993; Cote and Hahn 1994; Huang et al. 2007). Considering the diversity of biological activities and structure requirements, OGAs are very probably perceived by the plant cells through different mechanisms (Cote and Hahn 1994; Spiro et al. 1998).

The structural features and the ionic conditions necessary for OGA to be biologically most active in plants coincide with the requirements for the formation of a Ca²⁺-dependent “egg box” conformation. It has therefore been hypothesized that a putative perception system specifically recognizes this conformation (Farmer et al. 1990; Messiaen et al. 1993; Messiaen and Van Cutsem 1999).

In the egg box model (Morris et al. 1982), there is an initial dimerization step of two homogalacturonide chains in a 2₁ symmetry by cooperative bridging of parallel facing chains through Ca²⁺ ions. Cooperativity is possible because homogalacturonides are relatively rigid chains (Axelos and Thibault 1991) and the binding of a first calcium cation by two pectin chains facilitates their alignment with respect to each other, which in turn allows the easier binding of a next calcium ion, and so on along the sequence. The antiparallel orientation of the two chains seems to be the most favorable arrangement, and this initial dimer association is strongly stabilized by van der Waals and hydrogen bonding, in addition to electrostatic interactions (Braccini and Perez 2001). These authors distinguish between true egg box structure characteristics of polygaluronate regions of alginates and egg box-like structures observed in homogalacturonides. Here we use the term egg box to designate the calcium-induced association between chains of homogalacturonides of DP > 8.

Subsequent Ca²⁺-induced aggregation of these preformed egg box dimers in tetramers, hexamers, etc. can occur, but these subsequent associations of dimers display no particular specificity and seem to be merely governed by electrostatic interactions. These multimers are therefore easily disrupted by competing monovalent ions (like Na⁺ ions) while the initially formed chain dimers are not.

A monoclonal antibody (2F4) that specifically recognizes Ca²⁺-induced pectin dimers according to the egg box model has been described by Liners et al. (1989). These authors later showed that a minimum DP of 9 is critical for OGAs to form stable egg boxes that can resist thermal agitation at room temperature (Liners et al. 1992). In other words, at least five calcium ions are needed between two pectin chains to allow egg box dimer formation. This pectin dimer conformation is most stable in solutions with a range of Ca²⁺/Na⁺ ratios obtained with 0.5 mM Ca²⁺ and 50–300 mM Na⁺ (Liners et al. 1989); isolated chains largely predominate when calcium is absent and

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multimeric associations become prevalent when calcium is in excess. A similar optimum divalent/monovalent cation ratio was required to induce PAL activity in carrot cells treated with OGAs (Messiaen and Van Cutsem 1993b), corroborating that the bioactive pectin structure and the epitope of the 2F4 monoclonal antibody (MoAb) both belong to the same egg box conformation.

Recently, Decreux and Messiaen (2005) proposed that pectic egg boxes could ionically bind the extracellular domain of wall-associated kinases 1 (WAK1). Any condition that impaired the formation of egg boxes also impaired the interaction between WAK1 and homogalacturonides. WAKs belong to the huge family of 610 receptor-like kinases identified in the *Arabidopsis thaliana* genome (Shiu and Bleecker 2001). WAKs share two juxta-membrane degenerate epidermal growth factor (EGF)-like domains in their extracellular domain and a well-conserved intracellular Ser/Thr kinase domain. WAKs are linked directly to the pectin network in the plant cell wall and they are somehow implicated in cell expansion, wounding, and other stress responses such as plant responses to pathogen infection (Kohorn 2001). There are five WAKs in *Arabidopsis* and representatives are present in other angiosperm families. WAK1 and WAK2 are the most universally and abundantly expressed of the five tandemly arrayed genes. Kohorn (2007) showed recently that pectin could induce activation in protoplasts of several WAK2-dependent MAPKs and of vacuolar invertases.

In this report, we first used circular dichroism (CD) spectroscopy to confirm the existence of a high DP OGA conformation induced by the simultaneous presence of Ca^{2+} and Na^{+} and distinct from both isolated and multimer chain conformations. We then used the 2F4 MoAb to investigate the stability over time of this Ca^{2+} -bridged OGA dimers and the impact any change in stability of these dimers could have on plant cell responses to oligogalacturonides and on the link between WAK1 and pectin egg boxes. We conclude by suggesting a model for OGA perception by the plant cells.

Results

OGA preparation

Two pools of OGAs with defined DPs were generated by enzymatic hydrolysis of polygalacturonic acid (PGA) and separated by selective precipitation of the reaction products (Cabrera 2000). The elution profiles of the fraction analyzed by HPAEC-PAD confirmed the presence of monogalacturonic acid and OGA with DPs mainly up to 6, in the low DP fraction. In the high DP fraction, peaks corresponding to hepta- and octa-oligomers until heptadeca- α -(1,4)-D-oligogalacturonide were detected (data not shown).

Circular dichroism

By using circular dichroism spectrometry we observed that in the presence of calcium ions (Figure 1A), high DP OGA presented a significant shift of the carboxylate absorption band caused by the ion-driven conformational change of the polygalacturonans (Morris et al. 1982). The spectra of the low DP OGA did not show any significant wavelength shift, indicating that small OGA did not undergo any conformational change in the presence of calcium. We also tested the effect of monovalent versus divalent cations on high DP OGA (Figure 1B). In the presence of 50 mM Na^{+} ions no shift of the absorption

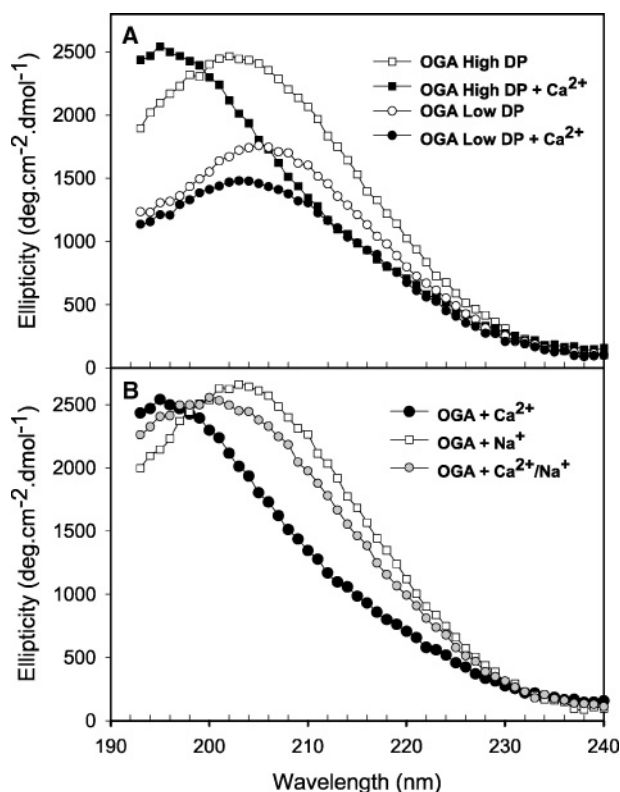


Fig. 1. Changes in the circular dichroism spectra of OGA in equilibrium with different cation chloride solutions. (A) CD spectra of low and high DP OGA either in water or Ca^{2+} solutions. (B) CD spectra of high DP OGA in Ca^{2+} , in Na^{+} , or in a mixture of both Ca^{2+} and Na^{+} chloride solutions. The average of 10 scans is presented for each experiment.

of the carboxylate group could be observed as compared to the spectra taken in water. In a solution containing 0.5 mM Ca^{2+} and 50 mM Na^{+} the low wavelength CD spectrum was shifted to a position intermediate between that obtained in the presence of calcium ions only and the one in the presence of sodium ions only. The observation of an isodichroic point at the intersection of the CD spectra indicates the existence of equilibrium between conformationally different forms. We interpret these spectra as a result of the existence of three OGA conformations: isolated chains in NaCl solutions, chain dimers (egg box dimers) in the presence of both Ca^{2+} and Na^{+} cations, and chain multimers in calcium solutions. This is perfectly consistent with results obtained earlier on PGA with an average DP of 25 (Morris et al. 1982).

Egg box maturation

The effect of calcium and sodium concentrations and of oligomer size on OGA and PGA recognition by 2F4 MoAbs has been studied before. Liners et al. (1992) showed that low DP OGAs were not recognized by the 2F4 antibodies, even at high concentration in the presence of the right $\text{Ca}^{2+}/\text{Na}^{+}$ ratio. High DP OGA and PGA that dimerized in the presence of the right calcium–sodium ratio were specifically bound by the antibodies and the absorbance of the inhibition ELISA test was inversely proportional to the concentration of pectin molecules used in the incubation solution.

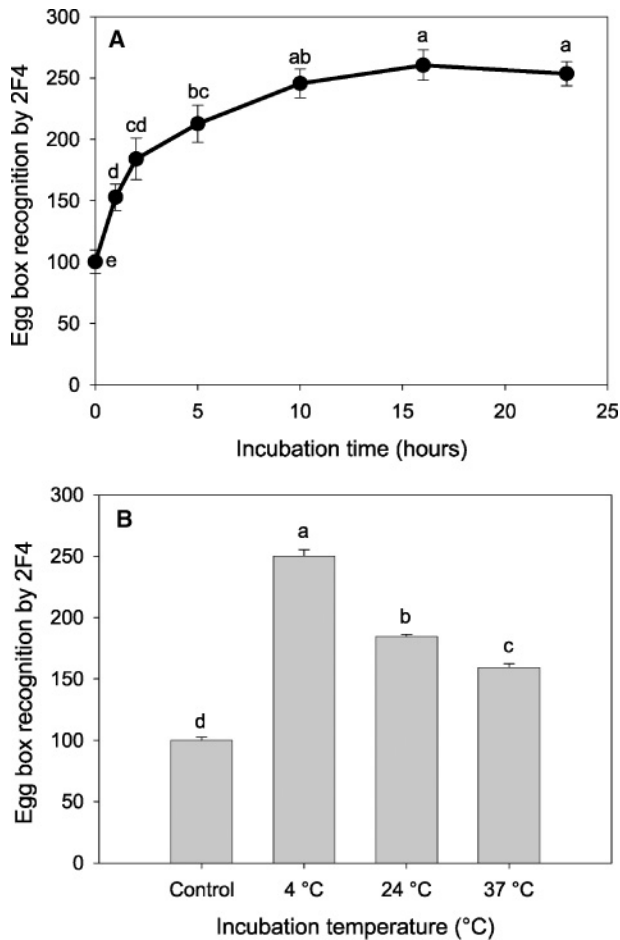


Fig. 2. Effect of preincubation time and temperature of OGA on the recognition by 2F4 MoAbs. High DP OGAs were incubated in a Ca/Na buffer at 4°C for different durations (A) or for 1 day at different temperatures (B). The presence of egg box dimers was evaluated in an ELISA test using the 2F4 MoAbs. Results are expressed as a percentage (\pm SE) of the response obtained for OGA solutions prepared before the test (control). Different letters among treatments indicate significant differences ($P \leq 0.05$ as determined by the Duncan test).

Since these earlier observations were confirmed by our CD results, we used this ELISA test to study the effect on the formation of egg box dimers of preincubating high DP OGA solutions for increasing lengths of time in an adequate Ca/Na buffer. As shown in Figure 2A, identical OGA concentrations were increasingly detected by the 2F4 antibodies as a function of preincubation time, indicating that egg box dimer formation was a kinetic process that needed time to reach equilibrium. We call this process egg box “maturation.” This egg box dimer maturation was also dependent on the preincubation temperature: the lower the preincubation temperature, the higher the rate of egg box maturation (Figure 2B). A similar behavior was observed for calcium-induced PGA dimerization (data not shown).

Effect of egg box maturation on pectic binding to WAK1

We hypothesized that WAK1 binding to OGA could increase following “maturation” of the egg boxes. To confirm this hypothesis, we used the competitive ELISA test of Decreux and Messiaen (2005) to measure the binding of WAK_{67–254} (the

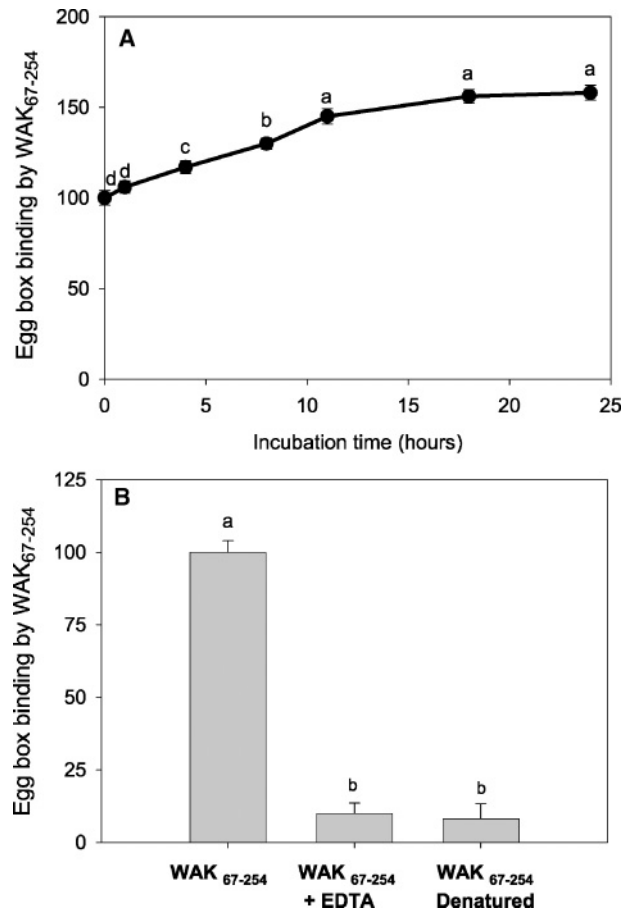


Fig. 3. ELISA test of the effect of egg box maturation on high DP OGA binding to recombinant WAK_{67–254}. OGA preincubated for increasing numbers of hours in a Ca/Na buffer were incubated for 1 h at room temperature with WAK_{67–254} before centrifugation. The supernatant containing unbound recombinant protein was dispensed in PGA-coated microwells and binding to the PGA was detected using an anti-Xpress antibody (A). Heat denatured WAK_{67–254} and a treatment using a Ca/Na buffer containing 5 mM EDTA were used as negative control (B). Results are expressed as a percentage (\pm SE) of the control response obtained for OGA solutions prepared before the test. Different letters among treatments indicate significant differences ($P \leq 0.05$ as determined by the Duncan test).

extracellular domain of WAK1 corresponding to amino acids 67–254) to OGA preincubated in the Ca/Na buffer for different times at 4°C. We indeed observed a significant increase of the binding of OGA to WAK_{67–254} as a function of OGA preincubation time (Figure 3A). The aging of OGA that induced more egg box detection by the 2F4 MoAb also resulted in an increased binding to WAK1 extracellular subdomain.

As previously shown (Decreux and Messiaen 2005), EDTA chelated calcium ions thereby preventing pectin egg box formation and binding to the WAK fragment (Figure 3B). Heat denaturation of recombinant WAK_{67–254} also abolished its binding to OGA egg boxes.

Effect of egg box maturation on early defense responses of *Arabidopsis thaliana* cell suspensions

Alkalinization, K⁺ efflux, and hydrogen peroxide production are well-known early responses of plant cell suspensions

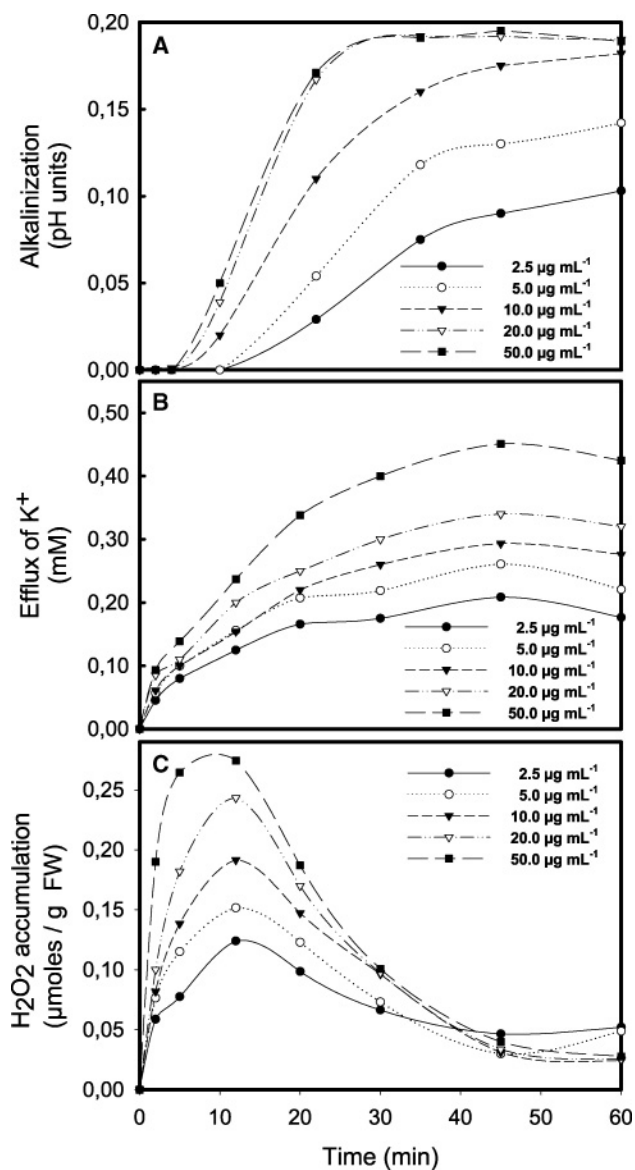


Fig. 4. Time course for alkalization (A), K^+ efflux (B), and H_2O_2 accumulation (C) in the incubation medium of *Arabidopsis* cell suspensions treated with high DP OGA. Cells were washed and equilibrated in a Ca/Na bioassay solution at pH 5.7. Cell density was adjusted at 100 mg FW/mL for all samples. At time 0, the incubation medium was removed and the cells resuspended in the medium containing OGA at the indicated final concentrations. Values of the control solutions to which no OGA were added were subtracted for all data. Data reflect the mean of at least three independent experiments.

challenged with elicitors and are key events in the transduction pathway leading to various defense mechanisms (Alvarez et al. 1998). In an attempt to study the physiological relevance of this egg box maturation process, the induction of early defense responses by freshly prepared and “mature” OGA egg boxes was studied in *Arabidopsis* cell suspensions. Figure 4 shows the kinetics of alkalization, K^+ efflux, and H_2O_2 accumulation induced by freshly prepared OGA in the presence of the adequate ionic conditions needed to maintain the dimeric egg box conformation. OGA induced a rapid and transient alkalization

of the cell culture medium (Figure 4A) and this alkalization reached a plateau with 20 $\mu\text{g/mL}$ OGA. Since the level of the plateau differed slightly from one experiment to the other, we show here representative results of at least three independent experiments. The time course of K^+ efflux from OGA-treated cells is illustrated in Figure 4B. The amplitude of the response was concentration dependent but no plateau could be observed within the OGA range evaluated (0–50 $\mu\text{g/mL}$). The cell suspensions also produced H_2O_2 transiently within a few minutes after the addition of OGAs (Figure 4C). The amplitude and pattern of the effects measured are perfectly consistent with previous reports (Mathieu et al. 1998; Spiro et al. 1998; Romani et al. 2004) considering the differences due to the plant model and cell density used.

Measuring these early responses after the addition of OGA solutions preincubated for different lengths of time did not show any significant effect on K^+ efflux and H_2O_2 accumulation (data not shown); we therefore focused on extracellular alkalization.

OGAs preincubated for different times were used at different concentrations in at least six independent experiments together with a range of concentrations of freshly prepared oligomers. It appeared that the magnitude and kinetics of the extracellular alkalization were clearly dependent on egg box maturation (Figure 5): mature egg boxes (1- and 2-day-old egg boxes) triggered a significantly higher alkalization of the culture medium than freshly prepared solutions at similar concentrations.

Effect of reducing end modification of oligogalacturonides

Spiro et al. (1998) report that the modification of the reducing end of OGA reduces their biological activity as compared with the corresponding underivatized OGA. They suggest that it could be due to a shift in the equilibrium of OGA away from a physiologically active conformation such as the egg box. Here we tested the ability of OGA with modified reducing ends (OGA-R) to induce early defense responses in *A. thaliana* cell suspensions in comparison to unmodified OGA (Figure 6).

In agreement with results obtained on tobacco tissue cultures (Spiro et al. 1998) we observed a significant loss of the ability of OGA-R to induce alkalization and H_2O_2 accumulation in the *Arabidopsis* culture medium as compared to unmodified OGA (Figure 6A and C) but K^+ efflux from the cells was much less affected (Figure 6B). These results suggest that the structural requirements for OGA to induce K^+ efflux from *Arabidopsis* cell suspensions were different from what was needed to induce medium alkalization and H_2O_2 accumulation.

The ability of OGA-R to adopt the egg box conformation and to bind WAK_{67–254} was also tested. End-modified oligomers formed as many egg box dimers than unmodified OGA, showing that this reducing end modification did not hinder the ability of OGA to adopt the egg box conformation recognized by the 2F4 (Figure 7). OGA-R egg box dimers also bound to WAK1 to a similar extent with a $98 \pm 3\%$ binding as compared to unmodified OGA.

Discussion

The existence of calcium-induced dimers of high DP OGA has long been taken for granted, considering the work of previous authors on polygalacturonides (Morris et al. 1982). Before

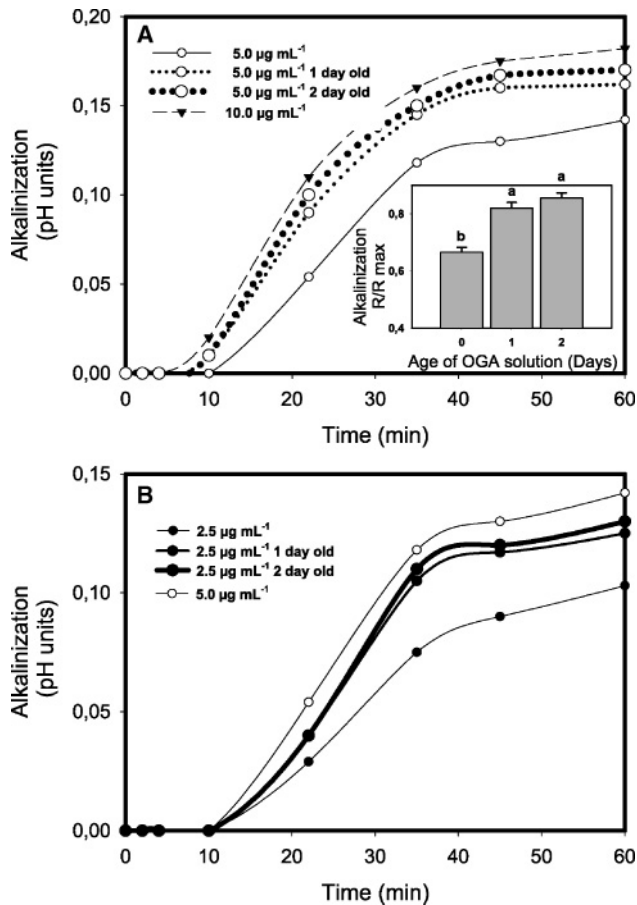


Fig. 5. Effect of egg box maturation on the alkalization of *Arabidopsis* cell suspensions treated with high DP OGA. The cells were washed and equilibrated in a Ca/Na bioassay solution at pH 5.7. Cell density was 100 mg FW/mL. At time 0, the incubation medium was removed and the cells resuspended in OGA containing the medium of different ages at the indicated final concentrations. Values of the control solutions to which no OGA were added were subtracted from all data. Data are representative of the mean of at least six independent experiments. In the inset (A), each bar represents the mean alkalization (\pm SE) measured after 45 min of treatment with 5 μ g/mL OGAs of different ages. The data were expressed as a response of each sample divided by the response induced by the saturating concentration of freshly prepared OGA in that experiment (R/R_{max}). Different letters among treatments indicate significant differences ($P \leq 0.05$ as determined by the Duncan test).

elaborating any further on the role and importance of dimerized OGA on the physiology of plant cells, the existence of such egg box dimers was investigated using circular dichroism spectroscopy. It appeared clearly that high DP OGA underwent a conformational transition in the presence of calcium, which was not observed with low DP OGA (Figure 1A). The size-dependent change of OGA conformation detected in the presence of calcium by the 2F4 antibody (Liners et al. 1992) is therefore confirmed by CD spectrometry. Even more, the spectra of high DP OGA in a mixture of calcium and sodium cations were intermediate between the spectra of the same OGA in calcium-alone and sodium-alone solutions (Figure 1B). This clearly proves the existence of a conformation of OGA that is intermediate between the isolated chains and the calcium-induced multimers. We call this conformation egg box dimer

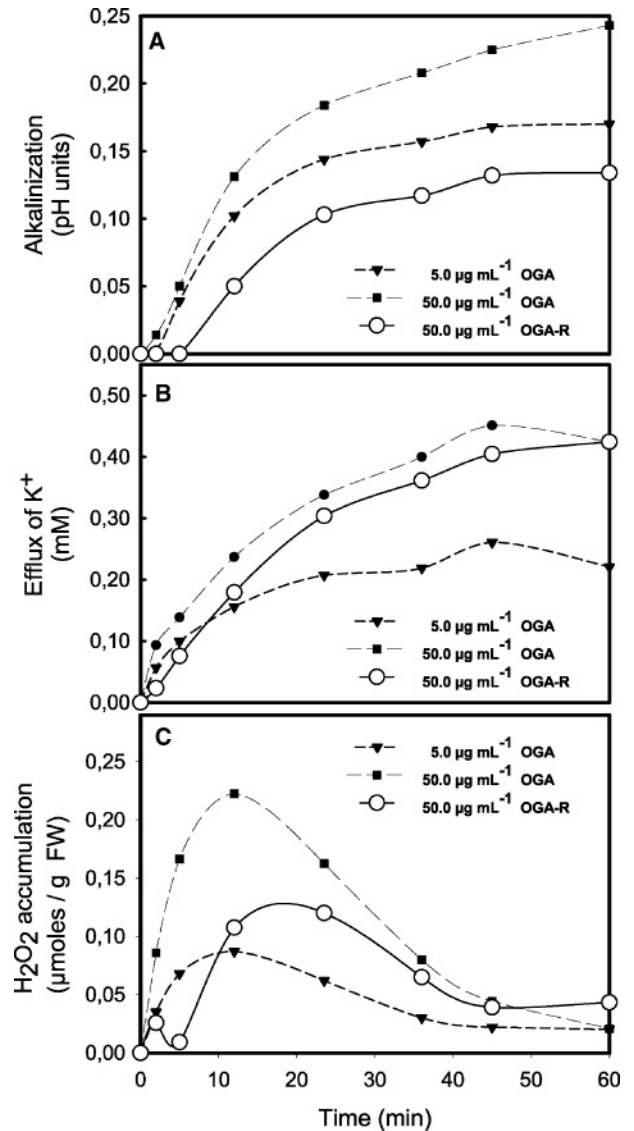


Fig. 6. Time course for alkalization (A), K^+ efflux (B) and H_2O_2 accumulation (C) in the incubation medium of *Arabidopsis* cell suspensions treated with high DP OGA-R. The cells were washed and equilibrated in the Ca/Na bioassay solution at pH 5.7. Cell density was 100 mg FW/mL. At time 0, the incubation medium was removed and the cells resuspended in the medium containing OGA-R at the indicated final concentrations. Values of the control solutions to which no OGA-R were added were subtracted from all data. Data reflect the mean of at least two independent experiments.

even if it differs slightly from the egg boxes originally defined for alginates (Braccini and Perez 2001).

Various studies tend to support the hypothesis that the structural requirements for OGA with $DP \geq 9$ to adopt the calcium-induced egg box conformation determine most of their structure–function relationship (Mathieu et al. 1991; Messiaen et al. 1993; Messiaen and Van Cutsem 1993a,b, 1994, 1999; Penel et al. 1999). In this work, we found that recognition by 2F4 antibodies of OGA dimerized through Ca^{2+} bridges according to the egg box model increased with the age of the solutions (Figure 2A). Our interpretation of these results needs topological and thermodynamic considerations.

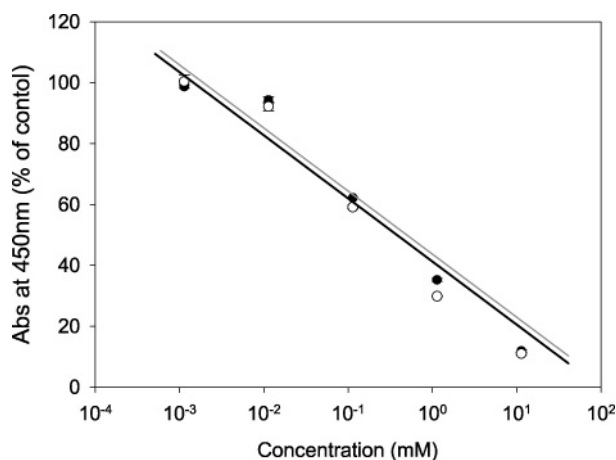


Fig. 7. Calibration curves of intact OGA (●) or chemically reduced OGA-R (○) egg box dimers in an ELISA-sandwich test with the 2F4 MoAbs. The MoAbs were incubated with different concentrations of oligogalacturonates; the resulting mixtures were centrifugated and the supernatants dispensed in antimouse Ig-coated microwells. The mean values ($n = 3$) are presented.

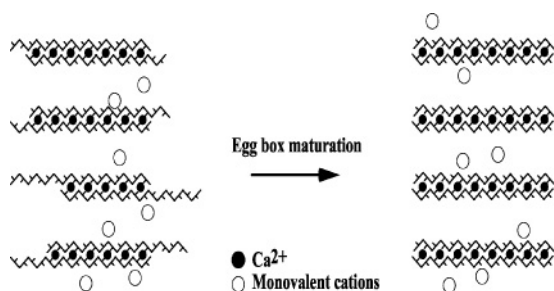


Fig. 8. Schematic representation of the model for egg box maturation. Initial dimerization through at least five calcium ions occurs randomly between more or less coaligned chains. The length of junction zones progressively increases with time to finally form perfectly coinciding dimers. For the sake of simplicity, only one DP of OGAs was considered here.

We start by considering that the probability of binding a first calcium ion between two chains perfectly aligned to each other from end to end is very low. Instead, we propose a more entropically plausible system in which pairs of pectin chains would first bind through at least five calcium bridges over partially overlapping ends (Figure 8). Different lengths of calcium bridging between pectin chains would coexist initially, each dimer differing in stability with longer and shorter ones. These partial dimers are obviously thermodynamically less stable than chains fully associated all along their lengths. However, calcium bridges are weak bonds that continuously associate and dissociate in a solution and the time pectin chains spend in each state is a function of the system temperature. It is therefore possible that egg box dimers reassociate over a longer distance after sliding along each other in their relaxed state. This would progressively bring the pectin chains to the less entropically but more thermochemically favorable state in which they coalign over their entire length, allowing a maximum number of calcium bridges. The “maturation” of the egg boxes would create more binding sites for the 2F4 antibodies, leading to a progressive increase with time of the recognition in the ELISA tests. Despite an adequate divalent/monovalent cation ratio used to in-

duce pectin egg box dimerization, the process described here is clearly not instantaneous and could need up to hours to achieve equilibrium, depending on the temperature.

This process of egg box maturation as detected by the ELISA test was corroborated by its impact on OGA’s biological activity as an inducer of alkalization in *A. thaliana* cell suspensions (Figure 5) and on the WAK1–OGA egg box interaction (Figure 3).

The extracellular alkalization is one of the earliest responses of suspension-cultured cells to elicitors, leading to the activation of late defense responses. Alkalization results from the inhibition of the plasma membrane proton ATPases (PMA) via a signaling pathway that involves calcium ions and a calcium-dependent protein kinase (Schaller and Oecking 1999). In plants, PMA is the main electrogenic pump that generates the proton motive force across the plasma membrane and PMA inhibition reduces ATP consumption at a time when pathogen-challenged cells switch from primary to secondary metabolism. In this bioassay, egg box maturation had no significant effect on K^+ efflux and H_2O_2 production, suggesting that different transduction pathways triggered by the same OGA elicitor independently control proton pumps, K^+ channels, and hydrogen peroxide production. These pathways may however share a common intracellular second messenger acting downstream of the perception of OGA elicitors. Changes in free cytosolic and nuclear calcium concentration are known to modulate numerous defense responses and cross-talks between parallel signaling pathways (Lecourieux et al. 2005, 2006). In *Nicotiana plumbaginifolia* cells, OGA and cryptogein both induced a typical calcium signature originating from a calcium influx followed by a calcium mobilization from internal stores. Both calcium signatures were, to some extent, modulated by a calcium-induced H_2O_2 synthesis and activated MAP kinases differentially (Lecourieux et al. 2002).

Endopolygalacturonases (PGs) are among the first enzymes secreted by phytopathogenic fungi upon plant infection (Annis and Goodwin 1997). They generate OGA by hydrolyzing pectin and the final success of the pathogen depends on its ability to invade plant tissues before host cells can mobilize efficient defense reactions. OGA production plays a critical role in the host-pathogen interaction: once OGAs are the right size, they dimerize, bind putative receptors on the plasma membrane which triggers defense responses, among which the production of extracellular polygalacturonase inhibiting proteins (PGIP) that specifically recognize and inhibit pathogenic PGs (Di Matteo et al. 2003). PG inhibition by PGIP delays OGA hydrolysis by 24 h (Cervone et al. 1989; D’Ovidio et al. 2004). Our results show that egg box formation by OGA progressively increases with time and that it needs about 10 h for OGA to form fully associated egg boxes. The physiological significance of PGIP would therefore be to delay OGA hydrolysis enough for the oligomers not only to accumulate but also to mature into more bioactive egg boxes.

According to our maturation model (Figure 8), the length of the junction sequences increases with time, but the number of terminal residue pairing through calcium bridges (egg box ends) also increases. To check the importance of egg box ends on the bioactivity of OGA, we tested the eliciting activity of a pool of high DP OGA whose reducing ends had been chemically reduced by sodium borohydride (OGA-R).

While modifying the reducing end of OGA did not hinder egg box formation (Figure 7) nor binding by WAK₆₇₋₂₅₄, it significantly decreased their biological activity as inducers of extracellular alkalization and peroxide production. We conclude that the observed reduction in the biological activity of the OGA-R in *Arabidopsis* cell suspensions was probably due to the loss of egg box ends that presumably bind to the perception system that transduces OGA elicitation and was not due to the destruction of the egg box conformation itself.

The extracellular domain of WAK1 interacts with at least two components of the wall matrix: a glycine-rich protein (AtGRP-3) (Park et al. 2001) and pectin (Wagner and Kohorn 2001). Decreux and Messiaen (2005) showed that the extracellular domain of the transmembrane receptor-like WAK1 could ionically bind calcium-associated homogalacturonans. They also established that conditions inhibiting the formation of calcium bridges between pectic chains (i.e., EDTA treatment, magnesium substitution for calcium, pectin depolymerisation, and methylesterification) also inhibited the binding of WAK₆₇₋₂₅₄ recombinant extracellular subdomain to pectin while heat denatured WAK₆₇₋₂₅₄ and recombinant β -galactosidase from *E. coli* did not bind pectin.

Decreux et al. (2006) 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. Mutated forms of WAK1 allowed them to identify and confirm at least five specific amino acids involved in the interaction with calcium-associated pectins. They also evaluated three truncated recombinant WAKs with different binding activities (from 70% to 0% of the binding activity of WAK₆₇₋₂₅₄) that confirmed the importance of these specific amino acids for the binding of calcium-associated pectin (Decreux et al. 2006). We show here that the increase in 2F4 recognition of egg boxes caused by the maturation process was sufficient to significantly increase binding of the egg boxes to WAK₆₇₋₂₅₄. On the other hand, egg box binding to WAK₆₇₋₂₅₄ was not affected by modifications to the reducing end of the oligosaccharides.

The signaling role, if any, of the WAK1-AtGRP-3-pectin complex is still largely unknown. Since the modification of the ends of the egg box dimers strongly decreased medium alkalization and hydrogen peroxide production in *Arabidopsis* cells but did not hinder egg box binding to WAK1, we conclude that the WAK1-egg box interaction is little or not associated with the activation of these responses. In support to this observation, it has been shown that signaling pathways involving MAPKs and extracellular pH changes operate in parallel and do not belong to the same linear pathway (Higgins et al. 2007).

Taking all results together, we propose that there are at least two different ways egg box dimers interact or are perceived by the plant cells (Figure 9). Perception system 1 is able to bind OGA in a size- and conformation-dependent way. The egg box junctions are the ligand of this perception system 1. Perception system 2 also binds OGA in a size- and conformation-dependent way but the ends of the egg boxes constitute the ligand. Many of the early defense responses induced by OGA in *Arabidopsis* cells would depend on this type 2 perception system. In other words, the modification of the reducing end of the OGA does not hinder egg box formation but it prevents egg box binding to the type 2 perception system.

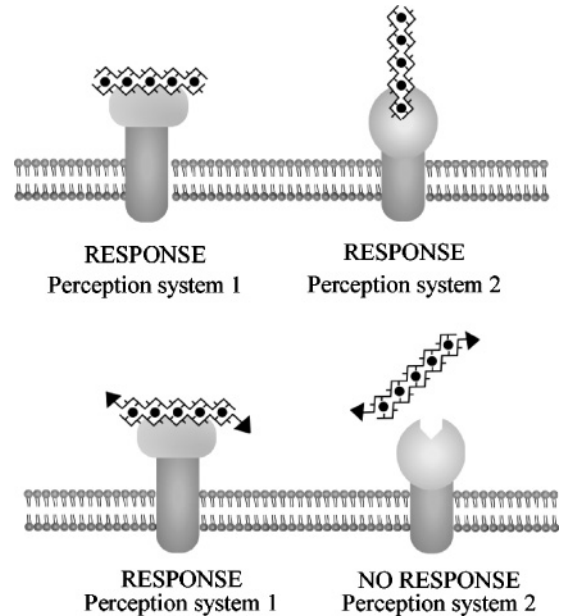


Fig. 9. Model of plant perception systems for the recognition of OGA egg box dimers by putative membrane receptors. Perception system 1 specifically recognizes egg box junctions while perception system 2 binds egg box ends. OGA modified to their reducing ends (triangle) bind system 1 but not system 2.

Beside diffusion problems, this perception system 2 could explain why large pectin egg boxes (DP > 16) possess little or no biological activity. First because at similar mass concentrations, the proportion of egg box ends in long pectin molecules is lower than in OGA. Second, because we expect long overlapping molecules to form concatenating dimers nearly devoid of any egg box ends.

This model is probably a simplified scheme of a more complex physicochemical mechanism associated with the perception of pectin molecules by the plant cell.

Materials and methods

Preparation of oligogalacturonides

Pectic oligomers were obtained by enzymatic hydrolysis of polygalacturonic acid. The PGA solution (2% (w/v), pH 6.0, 90 mL) was hydrolyzed with 10 mL of (1:3000) Pectinex Ultra Spl solution for 60 min. After the solution had been boiled for 10 min, the pectic oligosaccharides were selectively fractionated as described by Cabrera (2000).

High DP OGAs with the C-1 of their reducing end chemically reduced to a primary alcohol (OGA-R) were prepared essentially according to the procedure described by Spiro et al. (1998). In short, OGA-R was generated by treating the OGAs (40 mg in 8 mL of 1 N NH₄OH) for 16 h at 4°C with NaBH₄ (50 mg). The reaction was terminated by the addition of 5 volumes of 10% (v/v) acetic acid in methanol, which destroyed any remaining NaBH₄. This solution was kept for 2 h at -20°C and the resulting precipitate was collected by centrifugation. The supernatant was discarded. The pellet was washed twice with methanol (20 mL). The final pellet was dried under a stream of air at 25°C. The residue was dissolved in water (2 mL) and stored at -20°C.

Circular dichroism

Conformational characteristics were investigated by means of CD using a Jasco J-815 spectropolarimeter. The CD spectra were collected in the wavelength range of 190–250 nm at room temperature using a 1 mm path length cell. The reported spectra are the means of 10 scans each. The concentration of oligogalacturonides was 1 mg/mL in aqueous solutions at pH 5.7.

Egg box detection by ELISA

Microwells (NUNC High Binding Capacity microplates MAX-ISORP) were pretreated with polylysine-HBr (50 µg/mL, SIGMA, CA) during 1 h at room temperature. The wells were washed once with 250 µL of 50 mM MES buffer, pH 5.7, containing 0.5 mM CaCl₂ and 50 mM NaCl (Ca/Na buffer) and coated overnight at 4°C with 200 µg/mL PGA in the same buffer. Nonspecific binding was blocked by incubating the wells for 2 h at 37°C with 250 µL of 3% low fat dried milk (30 mg/mL) dissolved in the Ca/Na buffer. After the removal of the excess blocking solution, competitive solutions were added to the wells.

Preparation of competitive solutions and incubation with antibodies: competitive solutions were prepared by mixing 100 µL of 2F4 supernatant diluted 177 times in the Ca/Na buffer containing 1% (w/v) low fat dried milk and OGA of different ages and incubated for 30 min at 25°C. These oligosaccharides–antibody mixtures were then centrifuged for 10 min at 7500 × *g* before dispensing the supernatants in PGA-coated microwells and incubated for 60 min at 37°C.

The microplates were then washed eight times with the Ca/Na buffer containing 0.1% (w/v) Tween 20 before the addition of 50 µL of horseradish peroxidase-labeled sheep anti-mouse immunoglobulin (1:5000 in the Ca/Na buffer containing 1% (w/v) low fat dried milk) for 60 min at 37°C. After the second washing cycle, the binding of the antibodies was revealed by 100 µL of an enhanced K-blue TMB substrate (Enhanced K Blue TMB substrate, Neogen, MI) incubated for 20 min in the dark at room temperature. The process was stopped by the addition of 50 µL of 1 N HCl. The absorbance of the solution was measured after 15 min with a microplate reader (El × 800, Bio-TEK instruments, VT) at 450 nm.

ELISA assay of oligogalacturonides-WAK1 binding

The binding of the extracellular domain of WAK1, corresponding to amino acids 67–254 (WAK_{67–254}), with calcium-induced OGAs dimers was determined essentially according to Decreux and Messiaen (2005). Maxisorp microplates (VWR, Belgium) were pretreated with poly-L-lysine-HBr (50 µg/mL in H₂O, 50 µL/well, Sigma) for 1 h at room temperature. The wells were washed once with 250 µL/well of the Ca/Na buffer and coated overnight at 4°C with PGA at the appropriate concentration in the same buffer (50 µL/well) as described by Liners et al. (1989). Nonspecific binding sites were blocked for 2 h at room temperature with 3% (w/v) low fat dried milk dissolved in the Ca/Na buffer. Meanwhile, WAK_{67–254} recombinant proteins were incubated for 1 h with different competitor OGA solutions prepared at the appropriate dilution in the Ca/Na buffer and centrifuged for 10 min at 7500 × *g*. The supernatants were transferred to the PGA-coated microwells and incubated for 2 h at room temperature. The wells were washed eight times with a 250 µL Ca/Na buffer and further incubated for 1 h at room temperature with 50 µL of 1 µg/mL anti-Xpress primary antibody (Invitrogen,

Belgium) in the Ca/Na buffer containing 1% (w/v) low fat dried milk. After washing eight times with the 250 µL Ca/Na buffer, 50 µL of a 1:1000 dilution of the HRP-SAM secondary antibody (Amersham Biosciences, Belgium) prepared in the Ca/Na buffer containing 1% (w/v) low fat dried milk was added and incubated for 1 h at room temperature. After washing eight times with the 250 µL Ca/Na buffer, WAK_{67–254} binding to PGA was revealed by a 20 min incubation in the dark in the presence of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Enhanced K Blue TMB substrate, Neogen). Absorbance was measured at 650 nm with a microplate reader (El × 800, Bio-TEK Instruments). Binding assays were done in triplicate.

Bioassay

Suspension-cultured cells derived from leaves of *A. thaliana* strain L-MM1 ecotype *Landsberg erecta* were grown in the Murashige and Skoog medium (4.43 g/L) with sucrose (30 g/L), 0.5 µg/mL NAA, 0.05 µg/mL Kinetin, pH 5.7. Cultures were maintained under a 16/8 h light/dark photoperiod, at 25°C, on a rotary shaker at 100 rpm. Cells were diluted 10-fold in a fresh medium every 7 days.

Seven-day-old cells were filtrated on Miracloth (Calbiochem, Merck, UK), washed, and equilibrated in a K⁺-free medium containing 10 mM sucrose, 0.5 mM Ca²⁺, 50 mM Na⁺, and 0.5 mM MES adjusted to pH 5.7 with tris-(hydroxymethyl)-aminomethane (Ca/Na bioassay solution), for 30 min at room temperature with gentle shaking. This washing was repeated four times at 30 min intervals. After the last washing, the cells were incubated in the same Ca/Na bioassay solution for an additional 120 min before use.

Lyophilized OGAs were dissolved and allowed to “mature” for different lengths of time at 4°C in the Ca/Na bioassay solution. Aliquots of washed cells (100 mg fresh weight (FW)/mL) were placed in glass vials and the incubation medium was changed for equal volume of OGA solutions using a Pasteur pipette and agitated on a rotatory shaker at 150 rpm. The extracellular pH, K⁺, and H₂O₂ concentrations were determined in aliquots of the incubation medium obtained by rapid filtration of the cells through Miracloth.

pH was monitored with a Hamilton Biotrode pH electrode and K⁺ concentrations determined in 1 N HCl using an atomic absorption spectrophotometer (PU 9200X, Pye-Unicam, Cambridge, UK). The H₂O₂ concentration was measured in the extracellular medium using the Amplex Red hydrogen peroxide/Peroxidase Assay Kit (MOLECULAR PROBES) according to the supplier's instructions.

Statistical analysis

Statistical analysis was performed using STATISTICA 7 software. Data were subjected to a one-way ANOVA analysis and Duncan honestly significant difference for the comparison of means. Unless stated otherwise, means ± SE are reported.

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Conflict of interest statement

None declared.

Abbreviations

AtGRP, glycine-rich extracellular protein; CD, circular dichroism; DP, degree of polymerization; ELISA, enzyme-linked immunosorbent assay; MAPKs, mitogen-activated protein kinases; MES, 2-(*N*-morpholino)ethanesulfonic acid; OGA, oligogalacturonides; OGA-R, reduced oligogalacturonides; PGIP, polygalacturonase inhibiting proteins; PMA, plasma membrane proton ATPases; TMB, tetramethylbenzidine; WAK, wall-associated kinases.

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