Chitosan oligosaccharides modulate the supramolecular conformation and the biological activity of oligogalacturonides in *Arabidopsis*

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Plant cell walls undergo remodeling during growth and development and are the first target of many invading pathogens. Acidic pectin (homogalacturonan) binds calcium and forms chain dimers called egg boxes and even multimers at higher calcium ion concentrations. Chitosan, the deacetylated form of chitin produced by fungi when invading plant tissues, is a cationic polymer that can interact with negatively charged pectin. The interaction between chitosan oligomers (COS) and pectic egg boxes was investigated using 2F4, a monoclonal antibody specific for calcium-associated dimers of pectin. Depending on the size of the pectic molecules, the COS to pectin ratio, the degree of polymerization and the degree of acetylation of COS in the mixture, the calcium-induced egg box conformation of oligogalacturonides (OGA) was strongly stabilized or destroyed. The biological activity of COS-stabilized egg boxes was assayed on *Arabidopsis* cell suspensions. COS–OGA egg boxes strongly enhanced extracellular alkalinization and decreased potassium fluxes compared to control COS and OGA alone. Furthermore, OGA rescued *Arabidopsis* from cell death induced by higher concentrations of deacetylated COS. The stabilized COS–OGA egg boxes could constitute a combined emergency signal that informs plant cells on both cell wall degradation and pathogen presence, triggering a much stronger response than individual components alone.

Introduction

Pectin constitutes a dense molecular network that accounts for much of the physico-chemical properties of the primary plant cell wall. Cross-linking of the pectic molecules by Ca$^{2+}$ ions according to the so-called “egg-box” model (Grant et al. 1973) plays an important role in the properties of the pectin network which strongly affects the physiological significance of the pectic polysaccharides in plant cell walls.

Upon plant invasion, most pathogens secrete pectolytic enzymes that degrade wall pectin into bioactive alpha-1,4-linked oligogalacturonides (OGA). These OGA function as elicitors of defense responses and regulators of developmental responses in plants (Aldington and Fry 1993; Cote and Hahn 1994; Van Cutsem and Messina 1994; Ridley et al. 2001). The structural requirements for OGA with degree of polymerization (DP) ≥ 9 to adopt the calcium-induced egg box conformation determine most of their bioactivity (Mathieu et al. 1991; Messina et al. 1993; Messina and Van Cutsem 1993a, 1993b, 1994, 1999). This egg box conformation can be modulated by polycations such as the polyanines spermidine and spermine that prevent polygalacturonidic acid (PGA) from adopting the Ca$^{2+}$-induced supramolecular conformation, block the transduction of the pectic signal and consequently their elicitor activity (Messina and Van Cutsem 1999).

No pectin receptor has been described up to now, but WAK1, a transmembrane receptor-like wall-associated kinase, contains a cytoplasmic Ser/Thr kinase domain and an extracellular domain in contact with the pectin fraction of the cell wall. The extracellular domain of WAK1 from *Arabidopsis thaliana* binds to PGA, OGA and pectins extracted from *A. thaliana* cells (Decreux and Messina 2005; Decreux et al. 2006).

Recently, we reported that OGA dimerization through Ca$^{2+}$ bridges increases with the age of the solution (Cabrera et al. 2008). We call this process egg box maturation and it consists of a progressive increase in length of the junction sequences between two calcium-associated chains that slide along each other in order to form a maximum number of calcium bridges and dimer ends. We could show that maturation of egg boxes induced a significant increase of both their binding to the extracellular domain of WAK1 and their bioactivity as inducer of extracellular alkalinization of *A. thaliana* suspension cells.

The cell wall of fungi and several oomycetes contains chitin (Werner et al. 2002, Kamoun 2003). Plants attacked by fungi respond by secreting pathogenesis-related proteins among which several chitinases. N-Acetylchitooligosaccharides and chitosan oligosaccharides, its de-N-acetylated derivative, have been shown to elicit defense responses in various plants (Hahn 1996). Chitin fragments bind to rice CEBiP, a transmembrane protein with two extracellular LysM motifs that lacks an intracellular signaling domain.
(Kaku et al. 2006). CERK1, a LysM RK may act with CE-BiP to transduce chitin signaling (Miya et al. 2007). Chitosan oligosaccharides that result from chitin deacetylation and hydrolysis elicit defense responses mostly in dicots (Shibuya and Minami 2001). The bioactivity of polycationic chitosans has been associated to their interaction with negatively charged phospholipids (Kauss et al. 1989). However, both deacetylated (Vasconsuelo et al. 2003) and acetylated (Wan et al. 2004) elicitors seem to activate a common mitogen-activated protein kinase cascade. The oligosaccharide concentration needed in plant bioassays also seems to differ between elicitors and depends on the plant model used. The chitosan concentrations required are usually much higher than those necessary for chitin oligosaccharides to elicit similar responses (Yamaguchi et al. 2000).

Polycationic chitosans can interact in solution with polyanionic pectins to form polyelectrolyte complexes, coacervates, networks and multilayers. These attractive interactions between polymers have been extensively studied (Marudova et al. 2004; Murata et al. 2005; Chou et al. 2006) and used in technological applications (Meshali and Gabr 1993; Hoagland 1996; Macleod et al. 1999; Jang et al. 2003). However, the interactions between chitosan oligosaccharides and oligopectic egg boxes have never been explored.

In this study, we investigate the effect of chitooligosaccharides on the egg box conformation of poly- and oligogalacturonan molecules present in the immunoprecipitation solution. The relative egg box concentration in the immunoprecipitation solution can be detected using the 2F4 antibody that specifically recognizes Ca2+-induced pectin dimers. This antibody can be used to study the interaction between COS and egg boxes in solution.

Results

Oligosaccharides preparation and characterization

Two defined OGA fractions of low DPs (mainly up to six) and high DPs (containing hepta- and octa-oligomers up to heptadecapeptide) were obtained by enzymatic hydrolysis of PGA and isolated by selective precipitation. Two sets of deacetylated chitooligomers of low DPs (up to six) and high DPs (between five and nine) were obtained by acid and enzymatic hydrolysis of chitosan, respectively. Acetylated chitosan oligomers (COS) were prepared by chemical reacetylation. All COS sets were characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Cabrera and Van Cutsem 2005). Since the degree of acetylation (DA) is an average value and reacetylation COS are polydisperse in terms of acetylated residues, we use the terms “deacetylated” COS for essentially fully deacetylated chitooligomers; “partially acetylated” COS for chitooligomers with DA between 15 and 25 and “highly acetylated” COS for chitooligomers with DA ~50%. Chitin oligosaccharides had DA higher than 60.

Enzyme-linked immunosorbent assay (ELISA) procedure

The conformational changes of the pectic molecules induced by calcium ions have been traditionally studied using equilibrium dialysis, circular dichroism (CD) (Morris et al. 1982; Powell et al. 1982) and IR spectroscopy (Jarvis and Apperley 1995). In general, these methods require relatively high concentration of the pectic oligo/polysaccharides. A valuable alternative is the ELISA procedure described by Liners et al. (1989), in which Ca2+-induced dimers of pectin can be quantitatively detected using the 2F4 antibody that specifically recognizes this conformation. Any modification of the pectic egg box conformation can thus be detected by the antibody. To study the interaction between COS and pectic egg boxes, we used the monoclonal antibody (MoAb) 2F4 that specifically recognizes Ca2+-induced pectin dimers. The first experiments were performed using an immunoprecipitation followed by an ELISA test of the remaining 2F4 in the supernatant as described by Liners et al. (1992). In this procedure, the MoAb is mixed and preincubated with the assayed compounds (OGA plus COS), and after centrifugation, the supernatant containing untreated 2F4 MoAbs but also untreated COS are transferred to PGA-coated microwells and bound 2F4 MoAbs detected by a horseradish peroxidase (HRP)-labeled secondary antibody.

Before using this procedure, we carried out a control test in which COS were directly added to PGA-coated microwells (Figure 1). We observed that fully deacetylated high DP COS inhibited binding of 2F4 MoAbs to PGA adsorbed in the microwells. Clearly, COS bound the immobilized, negatively charged PGA, thereby preventing access of the 2F4 antibody to its epitope. This ruled out the use of this test to study the interaction between COS and egg boxes in solution.

Another immunoprecipitation and ELISA test was then set up in which PGA coating of the microwells was substituted for GaMIg coating, a goat antimouse immunoglobulin to capture the untreated 2F4 IgG1 from the supernatant. In this GaMIg ELISA test, preincubation of 2F4 MoAbs with increasing COS concentrations did not interfere with the subsequent capture of 2F4 MoAbs by the antimouse immunoglobulin (Figure 1B), confirming that COS did not interact with 2F4.

A calibration curve for low and high DP OGA and PGA detection by 2F4 MoAbs is presented in Supplemental Figure 1. This test allowed quantification of PGA and OGA egg boxes over at least four orders of magnitude. As already known (Liners et al. 1992) and confirmed by CD (Cabrera et al. 2008), low DP OGA were not able to adopt the egg box conformation recognized by the 2F4 antibodies, even at high concentration. High DP OGA and PGA, dimerized in presence of suitable calcium–sodium ratios, were specifically bound by the 2F4 antibodies, and the absorbance of the inhibition ELISA test was inversely proportional to the concentration of pectin molecules present in the immunoprecipitation solution. The regression lines of Supplemental Figure 1 were used to determine the relative egg box concentrations in the immunoprecipitation — ELISA assays. Alginites (Supplemental Figure 1), methylesterified high DP OGA and methylesterified PGA were not recognized by the 2F4. In presence of ethylene-diaminetetraacetic acid, a calcium chelator, no egg box could be detected whatever the pectin tested (data not shown). This allowed using this immunoprecipitation — GaMIg ELISA test as a specific quantitative measurement of calcium-induced egg box dimers of pectin molecules.

Chitooligosaccharides interact with Ca2+-induced dimers of pectic molecules

PGA or OGA in Ca/Na ELISA buffer were incubated with partially acetylated COS, and the concentration of pectic egg box dimers in the mixture was determined using the immunoprecipit-
atication — GaMIg ELISA procedure (Figure 2). Concentrations were calculated using calibration curves (Supplemental Figure 1) and expressed as percentages of controls without COS. In presence of partially acetylated COS, a modification in the recognition of the Ca$^{2+}$-induced PGA conformation detected by the 2F4 MoAbs was observed (Figure 2A). When partially acetylated high DP COS were present at a COS/PGA molar monomer unit ratio about 0.5, the detected amount of pectic egg box dimers was more than seven times higher than that of PGA alone. Low DP COS showed a similar although smaller effect at higher COS/PGA molar monomer unit ratio. For both long and short COS fractions, increasing the COS/PGA molar monomer unit ratio finally completely suppressed PGA recognition by the 2F4 antibody. Glucosamine monomers at any concentration did not interfere with PGA recognition by the 2F4 antibody (not shown).

The same partially acetylated COS had similar effects on the egg box conformation of OGA with DP > 8 (Figure 2B). However, the stabilization of the egg box conformation by COS occurred at lower molar monomer unit ratios for OGA than for PGA. Whatever the COS to OGA ratio, no egg boxes were detected in low DP OGA and methylesterified high DP OGA solutions when they were either alone or mixed with COS (data not shown). Polycations such as histone, poly-l-lysine and polyethyleneimine (PEI) readily destroyed the egg boxes (Sup-
Supplemental Figure 2) at concentrations at least 10 times lower in the case of OGA as compared to PGA.

The specific effect of COS acetylation on the egg box conformation of PGA and OGA was studied using high DP COS with average DAs of 0%, 15%–25% and 50% (Figure 3). In agreement with Figure 2, the pectic egg boxes were best recognized by the 2F4 antibody in presence of partially acetylated COS. Preincubation of the pectic molecules with highly acetylated COS (i.e. chitin oligomers) did not modify the egg box concentration detected by the 2F4 MoAbs, while deacetylated COS completely suppressed egg box dimer formation above a molar monomer unit ratio of 0.5 in the case of PGA, which is consistent with Figure 1A. High molar monomer unit ratios of partially acetylated high DP COS also progressively abolished the egg box stabilization. The ratio required to dismantle the egg boxes increased with the DA of the chitooligosaccharide assayed (data not shown). Partially acetylated COS did not induce alginates to form any egg boxes detectable by the 2F4 MoAbs (data not shown).

To summarize, addition of COS either stabilized or destroyed the calcium-induced egg box conformation of the pectic substances, depending on the DA and the DP of COS and on the ratio between COS and pectic molecules in the mixture.

Fig. 3. Effect of the degree of acetylation of chitosan oligosaccharides on egg box dimer formation by polygalacturonides and high DP OGA. Egg boxes were detected by 2F4 MoAbs in an immunoprecipitation — GaMlg ELISA test, their concentration determined with the help of calibration curves (Supplemental Figure 1) and expressed as percentages of egg boxes formed by control PGA or OGA without COS. Only partially acetylated COS induced a pronounced stabilization of the egg box dimers up to an optimum COS/PGA or COS/OGA ratio beyond which egg boxes were destabilized. Largely acetylated COS (○) did not interfere with PGA and OGA at all while completely deacetylated COS (▲) destroyed the egg boxes. Only partially acetylated COS (●) could stabilize the egg boxes. (A) PGA (0.2 mg L⁻¹) egg box detection in presence of increasing concentrations of high DP COS deacetylated (▲), partially acetylated (●) and highly acetylated (○). (B) High DP OGA (4.0 mg L⁻¹) egg box detection in presence of increasing concentrations of high DP COS deacetylated (▲), partially acetylated (●) and highly acetylated (○).

Fig. 4. Effect of size and acetylation of chitosan oligosaccharides on medium alkalinization and K⁺ efflux in Arabidopsis cell suspensions. Washed cells were resuspended in 20 mg L⁻¹ COS solution for 30 min. Values of the control solutions without elicitor were subtracted from all data, and the response (R) induced by each treatment was expressed as a fraction of R_COS DA ~0, the response with COS fragments of DA ~0. Data reflect the mean of at least two independent experiments. Different letters among treatments indicate significant differences (P ≤ 0.05). (A) Medium alkalinization. High but not low DP COS induced medium alkalinization. (B) Potassium efflux. Only deacetylated or partially acetylated high DP COS could induce potassium efflux. The pattern of medium alkalinization response differs from K⁺ efflux, suggesting two separate perception and transduction pathways.
COS–OGA interaction modifies early responses of *A. thaliana* cell suspensions to both elicitors

The biological relevance of the interaction between COS and OGA was studied by measuring the induction of early membrane responses by both oligosaccharides alone and their combinations in *A. thaliana* suspension cells.

Medium alkalinization and K⁺ efflux are two of the earliest responses of suspension-cultured cells to elicitors. High DP OGA in calcium-induced egg box conformation have been shown to increase significantly both cellular responses. A rapid and transient alkalinization reaches a plateau at 20 µg mL⁻¹ OGA while K⁺ efflux is also dose dependent, but no plateau is observed within the OGA concentration range tested (0–50 µg mL⁻¹). While K⁺ efflux is not affected by OGA egg box maturation, the extracellular alkalinization increases significantly (Cabrera et al. 2008).

Depending on size and DA, COS induced a strong alkalinization of the extracellular medium and K⁺ efflux out of *A. thaliana* suspension cells (Figure 4). High DP COS with a DA ~25 was the most potent elicitor of alkalinization (Figure 4A) while fully deacetylated COS induced the strongest K⁺ efflux. No K⁺ efflux could be detected when the DA of COS was higher than 25% (Figure 4B). In presence of low DP COS, neither alkalinization nor K⁺ efflux was detected.

These early membrane responses were then used to test the bioactivity of mixtures of both OGA and partially acetylated high DP COS that formed strongly stabilized egg boxes. First, the effect of the COS/OGA molar monomer unit ratio on the bioactivity was tested (Figure 5). In this experiment, OGA concentration was 40 mg L⁻¹ and COS concentration was increased. While increasing COS concentration alone monotonously increased both medium alkalinization and K⁺ efflux, the COS + OGA mixture had contrasting effects. When COS fragments were present at concentrations up to nearly twice the OGA concentration, they clearly increased medium alkalinization over what was observed with COS alone (Figure 5A). K⁺ efflux on the other hand was always lower in mixtures when COS concentration was higher than or equal to OGA concentration. When COS concentration in the mixture was lower than that of OGA (i.e. 40 mg L⁻¹), K⁺ efflux was not really different from the treatment with OGA alone (Figure 5B). Highest medium alkalinization and lowest K⁺ efflux occurred with a mixture of 40 mg L⁻¹ OGA and 30 mg L⁻¹ COS.

*Arabidopsis* cell suspensions were then treated with this optimum COS–OGA mixture, and the time course of medium alkalinization and K⁺ efflux was evaluated (Figure 6). After 2 h, the alkalinization induced by the mixture was higher than the simple additive effects of COS and OGA alone (Figure 6A). In absence of the ionic conditions required to induce egg box dimers (Figure 6C), such an effect of the mixture on alkalinization could not be observed. While COS-treated cells exhibited high K⁺ efflux, the presence of OGA in combination with COS completely abolished the effect of COS (Figure 6B). The high DP OGA had the same effect on K⁺ efflux induced by COS in absence of the appropriate Ca/Na ionic solution (Figure 6D).

OGA inhibit *Arabidopsis* cell death induced by COS

Cabrera et al. (2006) showed that deacetylated high DP COS at concentrations higher than 100 mg L⁻¹ induce cell death of *Arabidopsis* cell suspensions within 24 h after treatment and that acetylation of these chitosan oligosaccharides suppressed or decreased their toxicity. In Figure 7A, the viability of suspension cells was evaluated 24 h after treatment with acetylated and deacetylated high DP COS at 300 mg L⁻¹ alone or in combination with OGA. Acetylated high DP COS, monogalacturonic acid and the pectic molecules alone did not affect cell viability. Deacetylated high DP COS (DA ~0, 300 mg L⁻¹) killed the cells but their combination with low or high DP OGA significantly prevented cell death, probably because OGA titrated out polycationic COS, preventing them from reaching their cell targets. Cell viability decreased with increasing concentrations of deacetylated high DP COS but was rescued in...
presence of OGA (Figure 7B): higher COS concentrations were needed to kill the cells when OGA were present.

Discussion
Since chitosans are polycations and pectins are polyanions, we tested the interaction between OGA and COS oligomers to see whether chitosan fragments could modulate the eliciting properties of pectic oligomers.

COS–pectic egg box interaction
Partly acetylated COS (DA 15–25%) strongly increased the formation of egg box dimers of PGA and of high DP OGA up to an optimum that was dependent on the size of both COS and pectic molecules (Figure 2) while glucosamine monomer had no effect (not shown). Since COS did not interfere with 2F4 MoAbs (Figure 1B), this means that COS first stabilized the egg box conformation while higher COS concentrations destroyed it, probably by displacing Ca$^{2+}$ ions from the pectic anions. The higher the DP of COS, the lower the concentration needed to observe the stabilizing effect. Similarly, the higher stability of PGA dimers also required more COS for altering their conformation (Figure 2A) as compared to OGA (Figure 2B).

COS acetylation
Highly cationic COS (DA ~0%) readily destroyed the pectic egg boxes (Figure 3) as did other polycations such as polyamines (11), histones, poly-L-lysine and PEI (Supplemental Figure 2). Only partially acetylated COS (DA ~25) stabilized the egg box structures while even a substantial concentration of weakly charged COS (DA 50%) could not hinder pectic egg box formation.

At the usual cell wall pH of 5.5, completely deacetylated chitosans bear a positive charge on each glucosamine resi-
due and form single helicoidal stiff chains stretched by electrostatic repulsions. In this zigzag structure, chitosan chains take a 2-fold helical conformation (Okuyama et al. 1997) which means that chitosans have positive charges alternating on each side of the chain at 1.03 nm between neighboring charges on the same side of the chain (Sikorski et al. 2005), exactly the same way homogalacturonans have negative charges alternating at a similar distance on each side of its 2-fold zigzag structure. Positive charges of chitosans can then interact with identically spaced negative charges of the pectin chains to form OGA–COS complexes (Figure 8A) and the longer the chains the higher the binding affinity.

Totally deacetylated chitosans, like any other polycation, are strongly attracted by polyanionic pectin and could completely displace calcium ions from within pectin egg box dimers (Figure 8A). The resulting pectin–COS complexes are not recognized by the 2F4 antibodies. Because of cooperativity of egg box formation, longer pectic chains bind calcium ions more strongly, and consequently higher COS concentrations are required to dismantle egg box dimers.

Partly acetylated oligochitosans bearing fewer positive charges per unit length could not displace calcium ions so easily from within egg box dimers for steric and coulombic reasons but they stabilized the association. A model for partly acetylated COS–OGA interaction can be proposed (Figure 8B). Two oligopectate chains associate cooperatively through calcium ions to form an egg box dimer, leaving external charges free to bind monovalent cations in solution. Due to hydrogen bonding, the two chains are not coplanar but form an angle with each other (Braccini and Perez 2001). This geometry could allow one partly acetylated COS to bind to the external faces of the egg box without being able to displace the calcium ions from within this egg box. The 2F4 antibody
would still bind the complex on the calcium-binding side of pectin chains.

The geometry of this ternary Ca$^{2+}$–COS–OGA complex also involves kinetic considerations. The partly and irregularly acetylated COS do not bind as strongly and stably with pectin as completely deacetylated chitosans and could therefore have more rapid kinetics of binding and dissociating. Partially acetylated COS bind probably only transiently to the “outside” charged groups of pectic dimers, competing with monovalent cations, but nevertheless strongly enough to increase the effective concentration of egg boxes for interaction with antibody or cells.

**Effect of COS–OGA egg boxes on Arabidopsis cells**

Beside production of an oxidative burst, suspension-cultured plant cells challenged by elicitors undergo perturbations of their cellular ionic balance with K$^+$ and Cl$^-$ efflux, Ca$^{2+}$ influx,
external alkalinization and cellular acidification. These early events are associated with changes in plasma membrane properties that play the role of a switch between the elicitor recognition and the pathogen defense-signaling pathways (Hagendoorn et al. 1991; Amborabe et al. 2003). Partly acetylated COS combined with OGA egg boxes induced a prolonged and sustained alkalinization of the culture medium well above levels attained by either COS or OGA alone (Figure 6A). This synergy was dependent on the COS/OGA ratio (Figure 5A) in a similar way as partly acetylated COS-stabilized OGA egg boxes in ELISA tests (Figure 2B). In absence of calcium, this synergistic effect was not observed (Figure 6C). It is therefore very tempting to interpret this stronger alkalinization as the result of partly acetylated COS binding and stabilizing OGA egg boxes that in turn triggered more intense signaling.

\[ K^+ \text{ efflux and } H^+ \text{ influx are not coupled} \]

\[ K^+ \text{ efflux in plant cells induces water loss and cell shrinkage commonly observed during cell death involved in the hyper- sensitive response (Garcia-Brugger et al. 2006). Completely deacetylated chitosans that induced the strongest } K^+ \text{ efflux (Figure 4B) also killed } Arabidopsis \text{ cells while reacetylation of COS suppressed both } K^+ \text{ efflux and cell death (Figure 7), but not medium alkalinization (Figure 4). Adding OGA to partially acetylated COS increased medium alkalinization and decreased } K^+ \text{ efflux (Figure 6A and B) in a calcium-de- pendent manner. The COS/OGA ratio that induced maximal alkalinization for minimal } K^+ \text{ efflux was found to be 30 mg L}^{-1} \text{ COS for 40 mg L}^{-1} \text{ OGA (Figure 5). This ratio is higher than the optimum detected by the ELISA test of Figure 2B and could be explained by binding of a fraction of COS to the cell wall pectin.} \]

The toxicity of deacetylated COS can be suppressed by either OGA addition or COS reacetylation (Figure 7). We explain this cell rescue by a simple titration of COS positive charges by the pectic anions or by chemical neutralization of amine groups by acetyl.

**Biological implications**

Several fungi start producing chitin deacetylase when they establish intimate contact with the tissue of their host plants (Hadwiger and Beckman 1980; Siegrist and Kauss 1990; Deising and Siegrist 1995), and deacetylated chitosans but not chitin are present on the surface of their infection structures growing within the invaded plant tissues (El Gueddari et al. 2002). Depolymerization of deacetylated chitosan can be carried out, at least in vitro, by a plethora of enzymes including lipases, glucanases, cellulases, hemicellulases and pectinases (Cabrera and Van Cutsem 2005, Kim and Rajapakse 2005).

How are COS of different DAs differentially perceived by plant cells? Considering that suspension cells respond to COS within minutes, the perception mechanism must be located in the plasma membrane. LysM RLK1 that binds completely acetylated COS (chitin oligomers) is essential for chitin signaling in plants and is involved in chitin-mediated innate immunity in plants (Wan et al. 2008). However, concerning COS of DA <35, to our knowledge, no receptor has ever been described, but it is often considered that the polycationic nature of chitosan may lead to membrane disturbance through its interaction with negatively charged phospholipids (Kauss et al. 1989; Shibuya and Minami 2001; Zuppini et al. 2004).

Taking together the results reported here, a tentative picture describing biological implications of COS–pectin egg box interaction can be drawn: strongly cationic COS target anionic pectin of the plant cell wall. This is in agreement with an earlier report in which chitosan is shown to displace calcium ions from isolated cell walls of Glycine max suspension cultures (Young and Kauss 1983). The disruption of calcium-bound pectin of the plant cell wall by deacetylated chitosan could then be perceived and interpreted by plant cells as a distress signal commanding the strongest possible response including cell death.

On the other hand, the presence of free OGA in the egg box conformation is sensed by plant cells (Van Cutsem and Messiäen 1994). This constitutes a warning signal indicating a depolymerization of the cell wall that could be due to the presence of a pathogen but also to a normal turnover of the cell wall such as during xylem differentiation or organ abscission. The simultaneous presence of partly acetylated COS that strongly stabilize OGA egg boxes would dramatically reinforce this warning signal, informing the cell upon active invasion by a fungal pathogen. However, increasing concentrations of even partly acetylated COS would finally displace calcium from any pectin and profoundly affect cell wall integrity: COS would titrate all egg boxes out and even single chain pectin of the walls would start binding excess COS. This could be detected by cell wall integrity sensors (Hematy, Cheerk and Somerville 2009), triggering a strong concentration-dependent increase of } K^+ \text{ efflux. High concentrations of COS, whether partly acetylated or not, alone or in presence of OGA finally kill plant cells.} \]

In other words, COS-stabilized OGA egg boxes could represent an emergency signal for plant cells while excess COS could be a hypersensitivity signal in the vicinity of extensive fungal penetration.

**Materials and methods**

**Chemicals**

Chitin from lobster was supplied by Mario Muñoz Pharmaceutical Laboratories (Hacendado 1, Ciudad Habana, Cuba) and used to prepare chitosan under heterogeneous conditions following the methodology described by Cabrera et al. (2000). PGA (sodium salt, Sigma, p-3850), histone from calf thymus (H9250) and poly- L-lysine hydrobromide (P-1399 mol wt 150,000–300,000) were from Sigma-Aldrich (St Louis, MO).

**Preparation and characterization of oligosaccharides**

Chitooligosaccharides (COS) were obtained and characterized as previously described (Cabrera et al. 2006). High DP COS were reacetylated as described by Hirano and Yamaguchi (1976). All COS samples were analyzed by MALDI-TOF mass spectrometry (Cabrera and Van Cutsem 2005).

OGA were obtained by enzymatic hydrolysis of PGA, and the pectic oligosaccharides were selectively fractionated in low DP and high DP OGA and characterized by high pH anion-exchange chromatography with pulsed amperometric detection analysis as described by Cabrera (2000).
Preparation of the mixtures of pectic molecules and chitooligosaccharides

PGA, OGA and COS stock solutions (1–10 mg/mL) were prepared in Milli-Q grade water. PGA and OGA stock solutions were diluted to the working concentration in 50 mM acetate buffer pH 5.7 containing 0.5 mM Ca²⁺ and 150 mM final Na⁺ concentration. The PGA or OGA–COS mixtures were prepared by adding COS to the pectic solutions followed by vortexing. The ratio between COS and pectic molecules was expressed as molar monomer unit ratio (COS/OGA or COS/PGA).

Egg box detection by ELISA

PGA Coating of Microwells. Fifty microliters of poly-L-lysine hydrobromide (0.05 mg mL⁻¹ in deionized water) was dispensed into wells of NUNC High Binding Capacity microplates (MAXISORP) and incubated for 1 h at room temperature. Identical volumes of a 200 mg L⁻¹ PGA sample in 50 mM acetate buffer pH 5.7 containing 0.5 mM Ca²⁺ and 150 mM Na⁺ (Ca/Na ELISA) were dispensed and left overnight at 4°C. Nonspecific binding was blocked by incubating the wells for 2 h at 37°C with 250 μL of 30 mg mL⁻¹ ELISA-grade bovine serum albumin (3% BSA) prepared in the same buffer.

Incubation with COS. After removal of the excess albumin, 50 μL of high DP fully deacetylated COS solutions was added to the wells and incubated for 30 min. The microplates were washed with the Ca/Na buffer before incubation with 50 μL of 2F4 MoAbs diluted 354 times in the Ca/Na buffer for 60 min at 37°C.

Washing and Detection. The microplates were washed eight times with the Ca/Na buffer containing 0.1% Tween 20 before addition of 50 μL of HRP-labeled sheep antimouse (SAM) immunoglobulin (1:5000 in 50 mM acetate buffer containing Ca/Na solution) for 60 min at 37°C. After a second washing cycle, the binding of the antibodies was revealed by incubation for 20 min in dark at room temperature with 100 μL of enhanced K-blue TMB substrate (Neogen Corporation, Lexington, KY).

Bioassay

Suspension-cultured cells derived from leaves of *A. thaliana* strain L-MM1 ecotype *Landsberg erecta* were grown in Murashige and Skoog medium (4.43 g L⁻¹) with sucrose (30 g L⁻¹), 0.5 mg L⁻¹ naphthalene acetic acid, 0.05 mg L⁻¹ 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 fresh medium every 7 days.

Cell viability assay

Seven-day-old cells were treated with OGA, COS and their combination were expressed as percentages of the concentrations detected in control solutions without added chitosan oligosaccharides.

Alkalization and K⁺ efflux assay

Seven-day-old cells were filtrated on Miracloth (Calbiochem, Merck, Darmstadt, Germany), washed and equilibrated in a K⁻ free medium containing 10 mM sucrose, 0.5 mM Ca²⁺, 50 mM Na⁺ and 0.5 mM 2-(N-morpholino)-ethanesulfonic acid 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.

OGA, COS and their combination were dissolved and pre-equilibrated for 1 h in the Ca/Na bioassay solution. Five-milliliter aliquots of washed cell (100 mg fresh weight mL⁻¹) were placed in glass vials, and the incubation medium was changed for equal volumes of assayed solutions using a Pasteur pipette and agitated on a rotatory shaker at 150 rpm. The extracellular pH and K⁺ concentrations were determined in aliquots of the incubation medium obtained by rapid filtration of the cells through Miracloth. pH was monitored with a Hamilton Biotrace pH electrode, and K⁺ concentrations were determined in 1 N HCl using an atomic absorption spectrophotometer (PU 9200X, Pye-Unicam, Cambridge, UK).

Statistical analysis

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

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**Conflict of interest statement**

None declared.

**Abbreviations**

BSA, bovine serum albumin; CD, circular dichroism; COS, chitosan oligomers; DA, degree of acetylation; DP, degree of polymerization; ELISA, enzyme-linked immunosorbent assay; GaMlg, goat antimouse immunoglobulin; HRP-SAM, horseradish peroxidase-labeled sheep antimouse; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MoAbs, monoclonal antibodies; OGA, oligogalacturononides; PEI, polyethyleneimine; PGA, polygalacturonic acid; TTC, 2,3,5-triphenyltetrazolium chloride; WAK, wall-associated kinases.

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

**References**


