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Polyamines and pectins. II. Modulation of pectic-signal transduction

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Abstract. A previous study had shown that polyamines adsorb selectively on plant cell walls according to the valence of the polyamine (Messiaen et al. 1997, Plant Physiol. 113: 387–395). In this study, the adsorption of polyamines onto isolated carrot cell walls and onto pure polygalacturonic acid was investigated in the presence of competing mono- and divalent cations (Na^{+} and Ca^{2+}). Putrescine (Put^{2+}) was unable to remove all the calcium (Ca^{2+}) from cell walls or from polygalacturonic acid. Spermidine (Spd^{3+}) and spermine (Spm^{4+}) adsorbed on all galacturonates and were able to remove Ca^{2+} completely from both the walls and the pure polygalacturonates. Therefore, Spd^{3+} and Spm^{4+}, unlike Put^{2+}, prevented polygalacturonic acid from adopting the Ca^{2+}-induced supramolecular conformation recognized by the 2F4 anti-pectin monoclonal antibody. We show that the signal transduction cascade otherwise initiated in plant cells by Ca^{2+}-bound α-1,4-oligogalacturonides was indeed blocked by both Spd^{3+} and Spm^{4+}, but not by Put^{2+}. The mobilization of cytosolic free Ca^{2+} and the cytosolic acidification usually observed after treatment with pectic fragments did not occur and the subsequent activation of phenylalanine ammonia-lyase was suppressed. It is hypothesized that the disruption by Spd^{3+} and Spm^{4+} of the Ca^{2+}-induced supramolecular conformation of pectic fragments was the cause of the inhibition of the pectic signal. We conclude that polyamines can act on plant cell physiology by modulating the transduction of the pectic signal.

Key words: *Daucus* (polyamines) – Pectin – Polyamine – Signal transduction

Abbreviations: Cad^{2+} = cadaverine; CEC = cationic exchange capacity; DA = diamine; DE^{2+} = ethanediamine; DO^{2+} = octanediamine; DP = degree of polymerization; ELISA = enzyme-linked immunosorbent assay; MoAb = monoclonal antibody; PA = polyamine; PAL = phenylalanine-ammonia lyase; PGA = polygalacturonic acid; Put^{2+} = putrescine; Spd^{3+} = spermidine; Spm^{4+} = spermine

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Introduction

Polyamines (PAs) are ubiquitous organic polycations that bind DNA and proteins and stabilize membranes, and they are probably important for the regulation of growth in plants. However, since they are frequently present in millimolar concentrations and are poorly transported, they are not considered as plant hormones.

Polyamines are involved in the control of flowering (Tiburcio et al. 1988; Wada et al. 1994; Havelange et al. 1996), fruit ripening (Rastogi and Davies 1990), embryogenesis (Bastola and Minocha 1995; Bajaj and Rajam 1996), osmotic and salt stress (Erdei et al. 1990; Tiburcio et al. 1994), cell division (Kaur-Sawhney et al. 1980) and organ elongation (Shen and Galston 1985). Diamines (DAs) and PA are also present in the plant cell wall associated with pectic polysaccharides (D'Orazi and Bagni 1987) and are believed to control the activity of pectin methylesterases (Moustacas et al. 1991; Charnay et al. 1992), lignin deposition (Angelini et al. 1993) and cell wall pH (D'Orazi and Bagni 1987). However, the molecular mechanisms underlying the effects of DAs and PAs on cell wall physiology remain unknown.

Pectin constitutes a dense molecular network that accounts for much of the mechanical properties of the cell wall. Hairy regions of pectins (rhamnogalacturonan I) coexist with smooth homopolygalacturonic domains that can dimerize according to the so-called "egg-box" model (Grant et al. 1973). By circular dichroism, the existence of this egg-box mechanism of interchain chelation of calcium has been confirmed (Gidley et al. 1980; Morris et al. 1982; Powell et al. 1982). The extent of such junction zones in the cell wall depends largely on the charge density of the polyanion,

the availability of divalent cations, and is inhibited by methylesterification and acetylesterification (Liners et al. 1992).

Pectin is also a privileged target involved in morphological processes and in host-pathogen interactions. Pectolytic enzymes release oligogalacturonides that can modulate different physiological processes within the cell wall matrix and the protoplast. At low concentrations ($< 10^{-6}$ M), α-1,4-oligogalacturonides regulate morphological responses such as the initiation of floral buds on tobacco thin-layer tissue explants (Marfà et al. 1991), fruit ripening in tomato (Brecht and Huber 1988), inhibition of root formation in tobacco (Bellincampi et al. 1995) and stem elongation in pea (Branca et al. 1988). At higher concentrations (10^{-4} M), α-1,4-oligogalacturonides trigger and modulate several defense responses such as phytoalexin synthesis in soybean (Forrest and Lyon 1990), proteinase inhibitor accumulation in tomato (Doherty and Bowles 1990), lignification in castor bean (Bruce and West 1989), hydrogen peroxide production in soybean (Legendre et al. 1993), and they activate defense-related genes in carrot (Messiaen and Van Cutsem 1993).

The biological activity of the α-1,4-oligogalacturonides depends on their length and conformation: only oligomers with a degree of polymerization (DP) higher than eight in a supramolecular Ca^{2+}-induced conformation are recognized by plant cells as signaling molecules (Messiaen and Van Cutsem 1994) that trigger morphological or defense responses. How this is achieved is far from understood. In the case of the activation of defense responses, there is strong evidence for the implication of several ions in the signal transduction cascade. Oligogalacturonides trigger a rapid and sustained calmodulin-dependent mobilization of cytosolic free Ca^{2+} and acidification of the cytosol (Mathieu et al. 1991, Messiaen et al. 1993; Messiaen and Van Cutsem 1994), concomitant with a potassium efflux, a proton influx (Mathieu et al. 1991) and hydrogen peroxide production (Legendre et al. 1993), depending on the species studied. Downstream in the signaling pathway, phosphorylation events have been reported (Farmer et al. 1991; Reymond et al. 1996; Droillard et al. 1997), but corresponding kinases have not been identified so far.

Modulation of the pectin signal also occurs before or just after its release from the cellular matrix. The signaling properties of α-1,4-oligogalacturonides depend on the nature of the pectins from which they derive and from the physico-chemical conditions existing locally in the cell wall. The presence of polygalacturonase-inhibiting protein (De Lorenzo et al. 1991), the degree of methylesterification and acetylation of pectins (Jin and West 1984), the cell wall pH (De Lorenzo et al. 1991), and the availability of some cations control the rate of degradation of pectins by pectolytic enzymes and condition the size, the amount and the half-life of the α-1,4-oligogalacturonides released in the cell wall. Since pectins can adsorb DAs and PAs, we studied the effect of cell wall DAs and PAs on the signaling properties of α-1,4-oligogalacturonides.

Binding of DAs and PAs is tightly controlled by the cell wall pH, its Ca^{2+} content and by the presence of DA- and PA-degrading enzymes (Mariani et al. 1989; Angelini et al. 1993). In a previous paper (Messiaen et al. 1997), we characterized the binding of DAs and PAs to carrot cell walls and determined the selectivity sequence for DA and PA adsorption to be spermine (Spm^{4+}) > spermidine (Spd^{3+}) ≈ Ca^{2+} > putrescine (Put^{2+}) ≈ diaminoethane (DE^{2+}) > diaminooctane (DO^{2+}). The adsorption of DAs and PAs onto cell walls was only subjected to electroselectivity and did not induce any supramolecular conformation of pectin like that induced by Ca^{2+}. We postulated that the binding of Put^{2+} was restricted to the lower-affinity binding sites of the wall pectic fraction [rhamnogalacturan I and the outer sites of dimerized polygalacturonic (PGA) chains], whereas Spd^{3+} and Spm^{4+} could displace Ca^{2+} more efficiently and have access to the high-affinity binding sites. The physiological significance of the differential binding of DAs and PAs to pectins has, so far, not been elucidated.

In this report, we have characterized the binding of DAs and PAs onto isolated carrot cell walls and onto PGA in the ionic conditions necessary for α-1,4-oligogalacturonides to adopt a Ca^{2+}-induced conformation recognized by plant cells as signaling molecules. Enzyme-linked immunosorbent assays (ELISAs) with the anti-pectin 2F4 monoclonal antibody (MoAb) were performed to analyze the effect of DAs and PAs on calcium pectate formation in solution. Knowing the proportion of each ion adsorbed onto pectins in these experimental conditions and their effect on pectin conformation, we then studied the effect of appropriate polyamine-Ca^{2+}-Na^{+}-oligogalacturonide mixtures on the activation of defense responses in carrot cells and protoplasts. We show here that Spd^{3+} and Spm^{4+}, but not Put^{2+}, inhibit both the dimerization of pectin oligomers and their biological activity as endogenous elicitors.

Materials and methods

Carrot cell suspension, protoplast preparation, dye loading and video imaging. Carrot cells suspensions were grown as described in Messiaen et al. (1997). Protoplast preparation, dye loading and measurements of cytosolic free Ca^{2+} and pH were determined as described in Messiaen and Van Cutsem (1994).

Oligogalacturonide preparation. Oligogalacturonides with a DP between 9 and 16 were obtained by acid hydrolysis of PGA (Sigma) and HPLC purification as described by Messiaen and Van Cutsem (1994).

Anti-pectin ELISA. The DAs and PAs were pre-incubated for 1 h at room temperature with PGA (0.2 mg ml^{-1}) dissolved in 150 mM NaCl and 0.5 mM $CaCl_2$ before dispensing in microtiter plates (Nunc Microwell Module F-8 immunoquality). The Ca^{2+}-induced dimerized PGA was detected using the anti-PGA monoclonal antibody 2F4 as described by Liners et al. (1992).

Cell stimulation and assay of phenylalanine ammonia-lyase (PAL). The DAs and PAs were incubated for 1 h at room temperature with the α-1,4-oligogalacturonides (DP 9–16) in the presence of 150 mM

NaCl and 0.5 mM $CaCl_2$. Carrot cells and protoplasts were treated for 6 h at room temperature with the α-1,4-oligogalacturonide-DA or PA mixtures. The α-1,4-oligogalacturonides and DAs/PAs were used at final concentrations of 0.2 mg ml^{-1} and 2 mM, respectively. Phenylalanine ammonia-lyase activities and protein contents were assayed as described in Messiaen and Van Cutsem (1994).

Polyamine adsorption onto carrot cell walls. Daucus carota cell walls (1 mg) were soaked in 10 ml Put^{2+}, Spd^{3+} or Spm^{4+} solutions in the presence of 0.5 mM $CaCl_2$ and/or 150 mM NaCl, and the solutions were renewed five times (once every hour). Cell walls were then pelleted by centrifugation at 13 000 *g* for 15 min at room temperature and the supernatant discarded. The cell walls were dried for 48 h at 50 °C and weighed using an electrobalance (model 708501; Sartorius, Van der Heyden, Brussels, Belgium). The total cationic exchange capacity (CEC) of the cell walls was determined after equilibration in a 0.5 mM $CaCl_2$ solution.

Polyamine adsorption on PGA. A 1.5-mL aliquot of PGA (Sigma) solution (1 mg ml^{-1}) was dialyzed (Spectra/por Membrane, MWCO 3500; Spectrum Medical Industries, Los Angeles, Calif., USA) against 50 ml of DA or PA (Put^{2+}, Spd^{3+} or Spm^{4+} respectively) solution in 0.5 mM $CaCl_2$ and/or 150 mM NaCl. The dialyzing solutions were renewed five times (once every hour) to reach equilibrium.

Determination of DAs, PAs and Ca^{2+}. The amounts of DA (or PA) and Ca^{2+} adsorbed onto carrot cell walls or onto PGA were determined by HPLC and atomic absorption spectrophotometry (Messiaen et al. 1997). No replications of the data points of Figs. 1 and 2 have been performed because testing many samples once over a range of experimental conditions (i.e. different PA concentrations) provides more convincing results than repeating the experiments at only a limited number of conditions.

Results

Binding of DAs and PAs to carrot cell walls. In the absence of competing cations, the binding of Put^{2+} to the cell walls increased steeply to reach a plateau at 70% of the total wall CEC (0.8 eq kg^{-1}) while some Ca^{2+} was carried over from the culture medium, even at a 5 mM Put^{2+} concentration (Fig. 1A). In the presence of

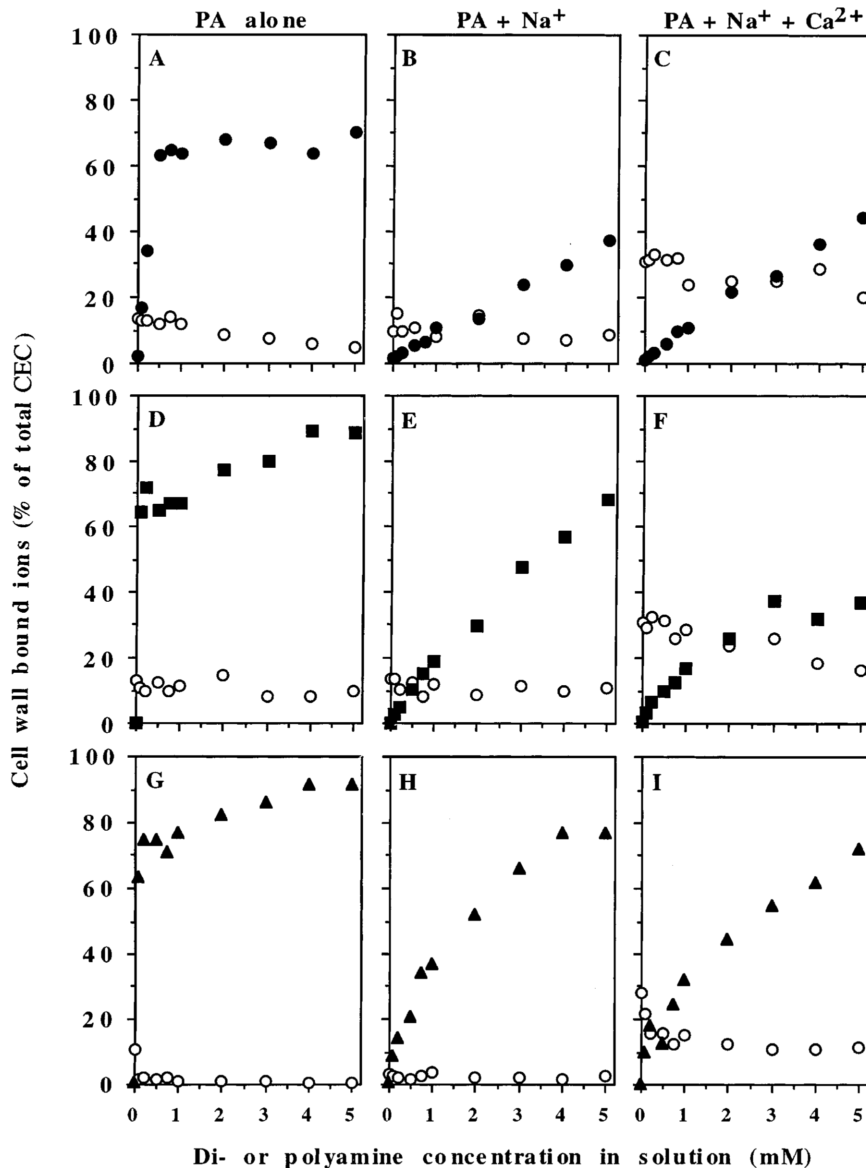

Fig. 1A–I. Proportion of Ca^{2+} (○) and DA-PA (●, ■ and ▲) adsorbed onto carrot cell walls in the absence of competing ions (**A,D,G**), in the presence of 150 mM NaCl (**B,E,H**), and in the presence of 150 mM NaCl + 0.5 mM $CaCl_2$ (**C,F,I**). Carrot cells were treated with Put^{2+} (**A–C**; ●), with Spd^{3+} (**D–F**; ■) and with Spm^{4+} (**G–I**, ▲)

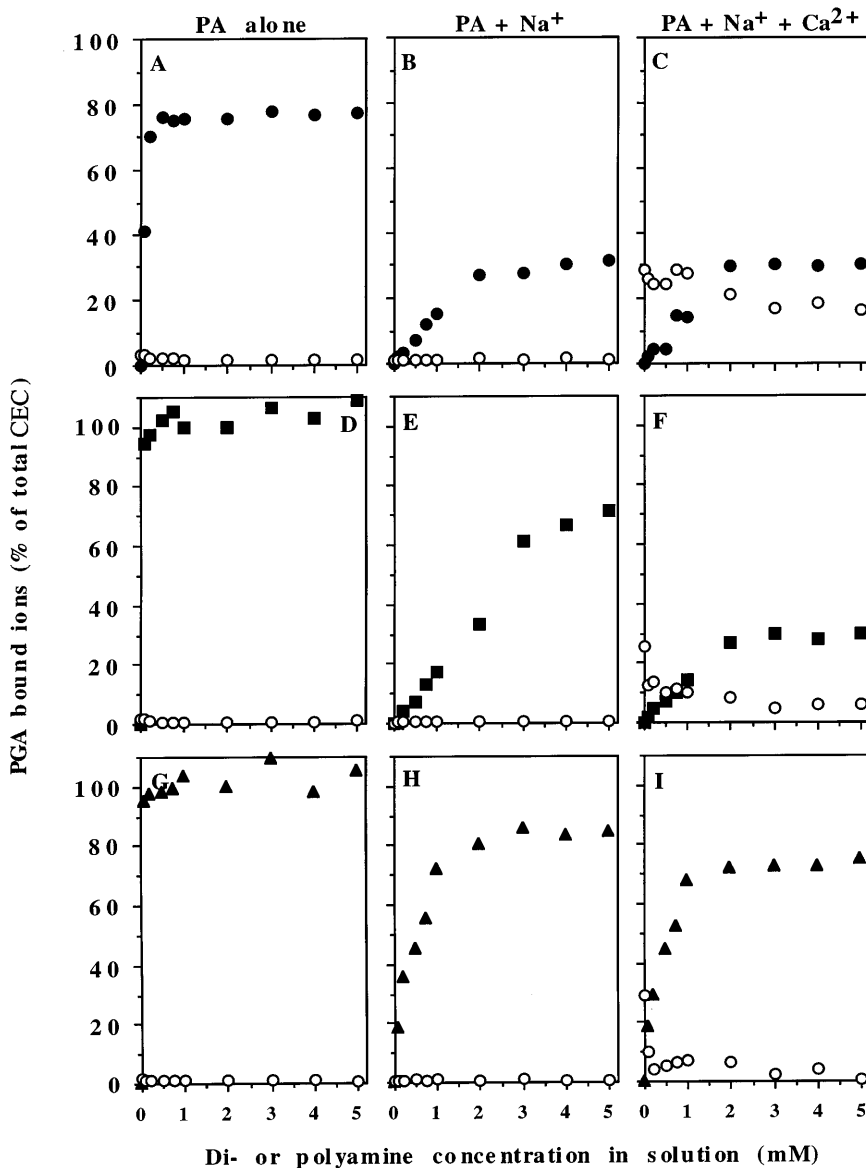

Fig. 2A–I. Proportion of Ca^{2+} (○) and DA-PA (●, ■ and ▲) adsorbed on to PGA in the absence of competing ions (**A**,**D**,**G**), in the presence of 150 mM NaCl (**B**,**E**,**H**) and in the presence of 150 mM NaCl + 0.5 mM $CaCl_2$ (**C**,**F**,**I**). The PGA samples were treated with Put^{2+} (**A–C**; ●), with Spd^{3+} (**D–F**; ■) and with Spm^{4+} (**G–I**; ▲)

150 mM NaCl (Fig. 1B), Put^{2+} binding was severely reduced to less than 40% of the CEC. When both NaCl (150 mM) and $CaCl_2$ (0.5 mM) were present (Fig. 1C), the wall Put^{2+} did not undergo any further reduction, but even slightly increased, showing that Put^{2+} and Ca^{2+} did not compete for the same binding sites. Only higher Put^{2+} concentrations were able to remove some Ca^{2+} from the cell walls.

In the absence of competing cations, Spd^{3+} and Spm^{4+} binding reached about 90% of the wall CEC (Fig. 1D,G), the remaining carboxylates being most probably protonated. Sodium chloride (150 mM) reduced the Spd^{3+} and Spm^{4+} binding by 25% and 15%, respectively (Fig. 1E,H), and when $CaCl_2$ (0.5 mM) was present in addition to NaCl, the binding of Spd^{3+} and Spm^{4+} was further reduced down to 40% and 70% of the CEC, respectively (Fig. 1F,I). The sum of Ca^{2+} and Spd^{3+} in Fig. 1F is surprisingly lower than in Fig. 1E where Ca^{2+} is absent from the equilibrium solution. This phenomenon is opposite to that observed with Put^{2+}: in the presence of 0.5 mM $CaCl_2$ in solution, the total amount (in equivalents) of Put^{2+} and Ca^{2+} in the walls (Fig. 1C) was higher than in the absence of Ca^{2+} (Fig. 1B).

In Fig. 1C,F and I, the Ca^{2+} content (30% of the CEC in the absence of DA and PA) decreased more steeply and reached lower levels with increasing valence of the organic counterion, especially Spm^{4+}.

Binding of DAs and PAs to PGA in the presence of sodium and Ca^{2+}. The binding of Put^{2+} (Fig. 2A) to PGA in the absence of competing cations reached up to 80% of the theoretical CEC of PGA (5.2 eq kg^{-1}). Both in the absence (Fig. 2B) and presence (Fig. 2C) of 0.5 mM $CaCl_2$, 150 mM NaCl caused the binding of Put^{2+} to PGA to reach a plateau at 30% of the CEC for 2 mM Put^{2+}, Na^+ being the counterion for the remaining 70% of the CEC. This showed once more that Put^{2+} and Ca^{2+} did not compete for the same binding sites.

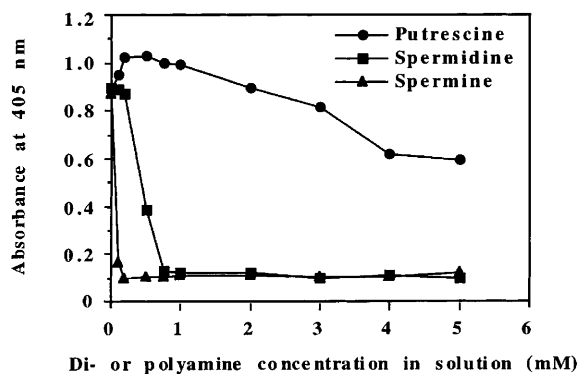

Fig. 3. Effect of PAs on the recognition of PGA by the 2F4 anti-PGA MoAb in the presence of 150 mM NaCl + 0.5 mM $CaCl_2$. Polygalacturonic acid was pre-incubated in the presence of different concentrations of Put^{2+} (●), Spd^{3+} (■) and Spm^{4+} (▲). The absorbance measurements (n = 5 for each datum point) had a standard deviation <0.1 absorbance unit

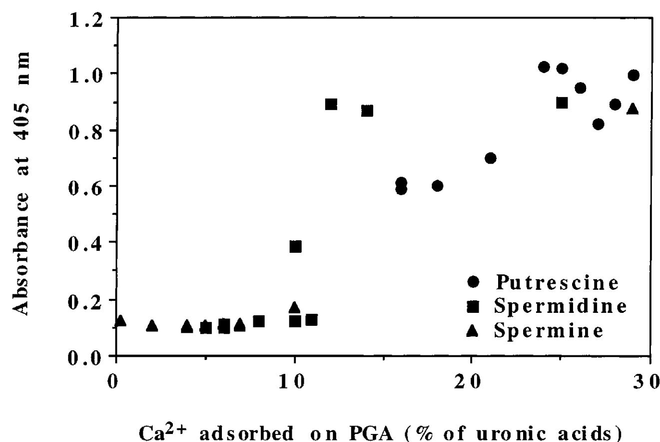

Fig. 4. Recognition of PGA by the 2F4 MoAb in the presence of Put^{2+} (●), Spd^{3+} (■) and Spm^{4+} (▲) as a function of the residual Ca^{2+} adsorbed on PGA (expressed as a percentage of uronic acids)

Both Spd^{3+} and Spm^{4+} adsorption reached 100% of the CEC of PGA when present as the sole counterions (Fig. 2D,G). In the presence of 150 mM NaCl, they occupied at most 70% and 85% of the binding sites, respectively (Fig. 2E,H). When 0.5 mM $CaCl_2$ was present in solution in addition to Na^+, the binding of Spd^{3+} and Spm^{4+} was reduced to 30% and 70% of the binding sites, respectively (Fig. 2F,I).

Effect of DAs and PAs on the Ca^{2+}-induced dimerization of PGA. In the absence of Ca^{2+}, none of the DAs and PAs tested was able to induce a pectic conformation recognizable by the 2F4 MoAbs (not shown). When 0.5 mM Ca^{2+} was present in the ELISA solutions, the PAs were able to differentially modulate the binding of the antibodies to the pectins.

For Put^{2+} concentrations lower than 1 mM, the recognition of calcium pectate by the 2F4 MoAb was slightly improved in comparison with the control without Put^{2+} (Fig. 3). On increasing the Put^{2+} concentration, the absorbance of the test slightly decreased. On addition of Spd^{3+} or Spm^{4+}, the recognition of pectates by the 2F4 antibody steeply decreased. Spermine was more effective than Spd^{3+} in preventing the formation of calcium pectates: only 0.1 mM Spm^{4+} was necessary in the equilibrium solution to inhibit the 2F4 response by 50%, instead of 0.5 mM Spd^{3+} (Fig. 3). The 2F4 inhibition plateau was reached when either 10% of the PGA uronates were associated with Spd^{3+}, or 18% were associated with Spm^{4+} as deduced from Fig. 2F and 2I, respectively.

Polyamines clearly removed Ca^{2+} and thereby hindered pectin dimerization. A very small amount (0.1 mM) of Spm^{4+} in the bathing solution caused a decrease in the Ca^{2+} associated with PGA from 29% in the control down to 10%, and correlatively reduced to background level the recognition of Ca^{2+}-dimerized PGA by the antibodies. When the data of Fig. 3 are plotted, not as a function of the external DA or PA equilibrium concentrations, but as a function of the pectin-bound Ca^{2+} (Fig. 4), Ca^{2+} adsorption clearly appears to be the key factor for antibody binding to pectin with a threshold at about 10% of the CEC of PGA. Above that Ca^{2+} content, a conformational change of PGA takes place to form the egg boxes recognized by the 2F4 antibodies. Below that threshold Ca^{2+} content, no epitope is maintained in solution. This confirms results reported in Messiaen et al. (1997) which indicate that PA adsorption is not cooperative and only results from electrostatic interactions, which means that no egg-box-type conformation of pectin is induced.

The behavior of Put^{2+} was further compared with the effect of shorter and longer DAs on recognition of soluble PGA by the 2F4 MoAb (Fig. 5). Only Put^{2+} (butanediamine) increased antibody binding to PGA at diamine concentrations ≤1 mM. All the other diamines

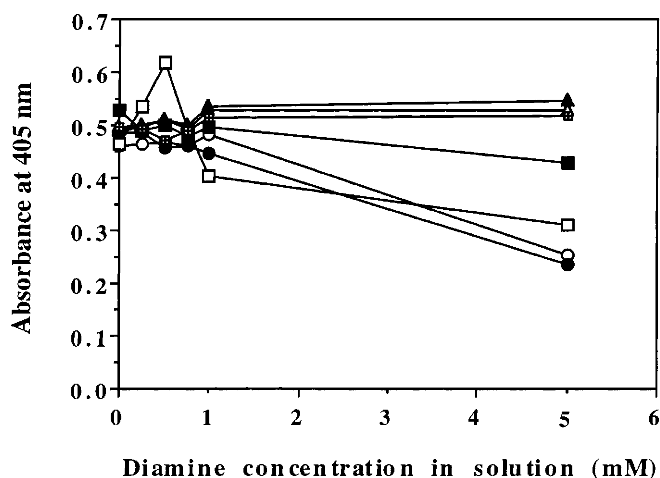

Fig. 5. Effect of DA concentrations on the recognition of PGA by the 2F4 anti-PGA MoAb in the presence of 150 mM NaCl + 0.5 mM $CaCl_2$. Polygalacturonic acid was pre-incubated in the presence of different concentrations of diaminoethane (○), diaminopropane (●), diaminobutane (□), diaminopentane (■), diaminohexane (△), diaminoheptane (▲) and diaminooctane (⊟). The absorbance measurements (n = 5 for each datum point) had a standard deviation <0.1 absorbance unit

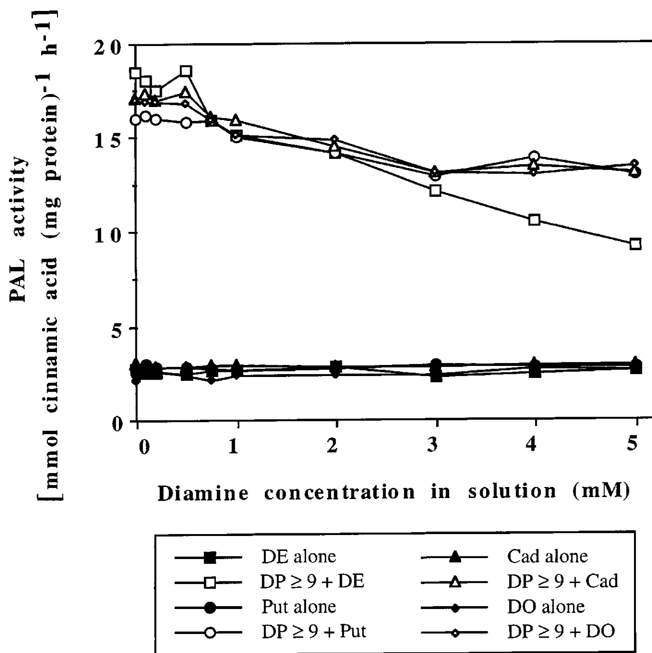

Fig. 6. Effect of DA concentrations on the activation of PAL in carrot cells treated with 0.2 mg ml^{-1} α-1,4-oligogalacturonides (DP 9–16) in the presence of 150 mM NaCl and 0.5 mM $CaCl_2$. The PAL measurements (n = 5 for each datum point) had a standard deviation <2 mmol cinnamic acid (mg protein)$^{-1}$ h^{-1}

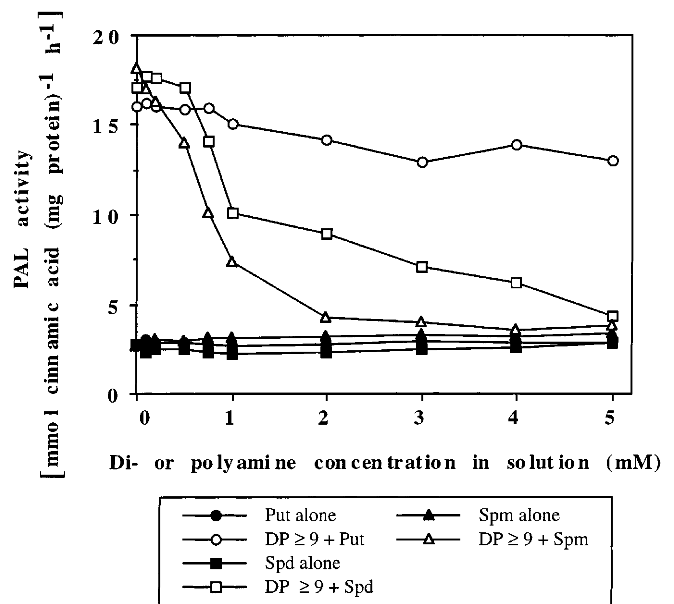

Fig. 7. Effect of DA and PA concentrations on the activation of PAL in carrot cells treated with 0.2 mg ml^{-1} α-1,4-oligogalacturonides (DP 9–16) in the presence of 150 mM NaCl and 0.5 mM $CaCl_2$. The PAL measurements (n = 5 for each datum point) had a standard deviation <2 mmol cinnamic acid (mg protein)$^{-1}$ h^{-1}

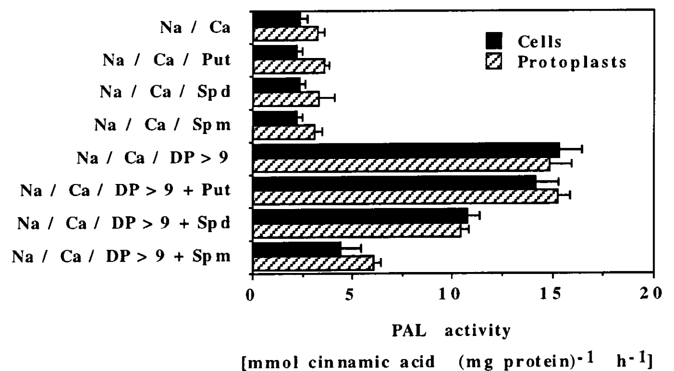

Fig. 8. Effect of DAs and PAs on the activation of PAL in carrot cells and protoplasts treated with 0.2 mg ml^{-1} α-1,4-oligogalacturonides (DP 9–16) in the presence of 150 mM NaCl and 0.5 mM $CaCl_2$. Putrescine, Spd^{3+} and Spm^{4+} were added at a final concentration of 2 mM. The error bars represent the standard deviation (n = 5 for each datum point)

either did not interfere with the required pectin conformation (hexane-, heptane- and octane-diamines) or decreased its formation and thus its recognition by the antibodies (ethane-, propane-, pentane-diamines).

From these results, we could devise PA (or DA)-Na^+-Ca^{2+} mixtures appropriate for modulating the signaling properties of α-1,4-oligogalacturonides in carrot cells and protoplasts.

Effect of DAs and PAs on activation of PAL by α-1,4-oligogalacturonides. The activation of PAL was not affected by DAs or PAs alone (Figs. 6,7). In the presence of α-1,4-oligogalacturonides, increasing DA concentrations moderately inhibited PAL activation (Fig. 6), whereas Spd^{3+} and Spm^{4+} were far more effective in suppressing PAL activation (Fig. 7). A 50% inhibition of the PAL response was observed for a 0.75 mM Spm^{4+} or a 2 mM Spd^{3+} concentration. An almost complete PAL inhibition was reached at a 5 mM concentration of both PAs (Fig. 7). Similar results were obtained with carrot protoplasts (Fig. 8), showing that the removal of the cell wall did not change the sensitivity of the protoplasts to the addition of α-1,4-oligogalacturonides.

Modulation by DAs and PAs of cytosolic responses induced by α-1,4-oligogalacturonides. Carrot protoplasts were treated with α-1,4-oligogalacturonides in the presence of 2 mM DE^{2+}, Put^{2+}, cadaverine (Cad^{2+}), DO^{2+}, Spd^{3+} or Spm^{4+}, and the cytosolic free Ca^{2+} and pH were monitored for 30 min using indo-1 and SNARF-1 fluorescent dyes, respectively. In the absence of pectic fragments, the DAs and PAs tested had no effect on the cytosolic free Ca^{2+} and pH (Figs. 9,10). Putrescine combined to pectic fragments induced a stronger Ca^{2+} mobilization than pectic fragments alone. However, DE^{2+}, Spd^{3+}, Spm^{4+} induced a nearly 50% inhibition of the Ca^{2+} response, whereas Cad^{2+} and DO^{2+} were less effective in inhibiting the mobilization of cytosolic free Ca^{2+} (Figs. 9,10). Similar observations could be made in terms of cytosolic pH: DE^{2+}, Spd^{3+} and Spm^{4+} partially inhibited cytosolic acidification, while all the other DAs had no significant inhibitory effect on the pH drop (Figs. 9,10).

An additional negative control of protoplast elicitation was performed by preincubating the pectic

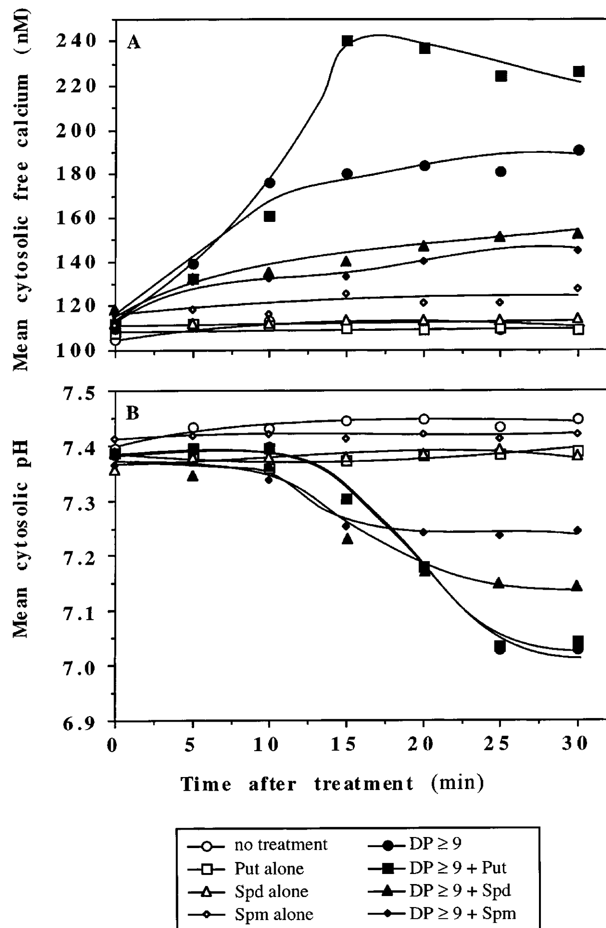

Fig. 9A,B. Effect of DAs on cytosolic free Ca^{2+} (**A**) and cytosolic pH (**B**) in carrot protoplasts treated with 0.2 mg ml^{-1} α-1,4-oligogalacturonides (DP 9–16) in the presence of 150 mM NaCl and 0.5 mM $CaCl_2$. Diamines were added at a final concentration of 2 mM. Each point corresponds to the mean cytosolic free Ca^{2+} or mean cytosolic pH calculated by integrating pixel values over the whole surface of 10 protoplasts. All cytosolic free Ca^{2+} and pH data points had a standard deviation <15 nM and <0.1 pH unit, respectively

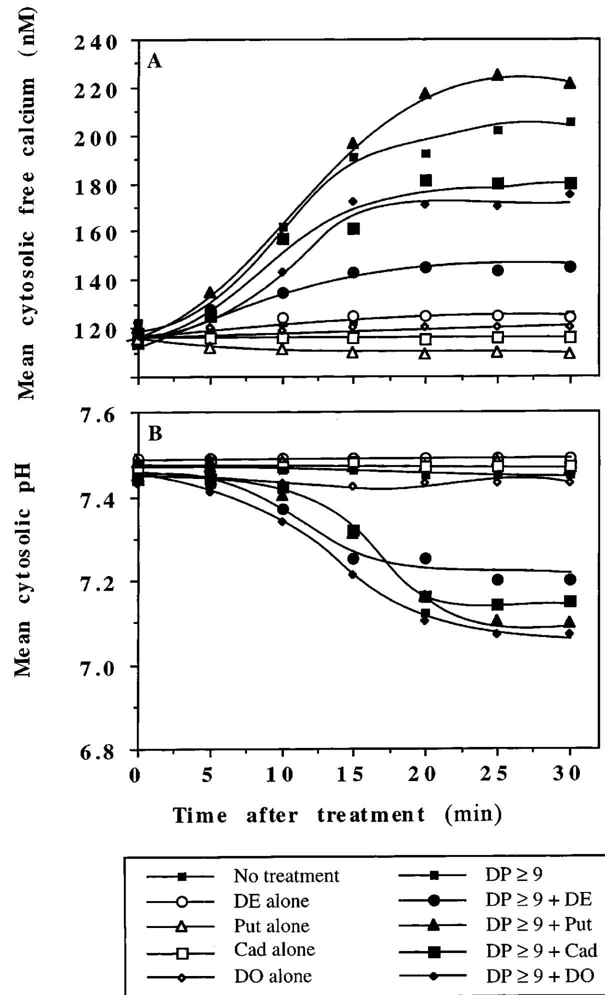

Fig. 10A,B. Effect of PAs on cytosolic free Ca^{2+} (**A**) and cytosolic pH (**B**) in carrot protoplasts treated with 0.2 mg ml^{-1} α-1,4-oligogalacturonides (DP 9–16) in the presence of 150 mM NaCl and 0.5 mM $CaCl_2$. Polyamines were added at a final concentration of 2 mM. Each point corresponds to the mean cytosolic free Ca^{2+} or mean cytosolic pH calculated by integrating pixel values over the whole surface of 10 protoplasts. All cytosolic free Ca^{2+} and pH data points had a standard deviation <15 nM and <0.1 pH unit, respectively

fragments with the 2F4 MoAb prior to treatment. The antibody largely prevented subsequent cytosolic Ca^{2+} increase and pH drop (Fig. 11).

Discussion

Diamines and PAs are organic polycations that adsorb differentially on plant cell wall uronates (Messiaen et al. 1997). Knowing that oligouronates are elicitors in plants, we investigated a possible modulatory effect of DAs and PAs on pectic elicitation. Since the previously published physico-chemical results were obtained using nonphysiological concentrations of PAs, or in the absence of Ca^{2+} and monovalent cations, we performed PA adsorption isotherms on both isolated cell walls and pure PGA, under the conditions required for elicitation. From the equilibrium concentrations of cations and PAs adsorbed on polyuronates, we could then interpret ELISA tests designed to quantify the amounts of pectic chains that had adopted the elicitating conformation. We could then choose the PA concentrations to be used in signal transduction experiments.

Adsorption isotherms of DAs and PAs. The difference in binding capacity of cell walls and PGA for Put^{2+}, Spd^{3+} and Spm^{4+} reflects both the heterogeneity of the binding sites and structural differences between PAs. Figure 2B,C shows unequivocally that the plateau reached by Put^{2+} is similar in the presence or absence of Ca^{2+}. These results confirm previous conclusions (Messiaen et al. 1997): Put^{2+} cannot access higher-affinity sites that instead of Put^{2+}, either protonate (Fig. 2A) or bind Na^{+} or Ca^{2+} when present (Fig. 2B,C).

On the contrary, Spd^{3+} and Spm^{4+} in pure solutions electrostatically compensated all PGA uronates

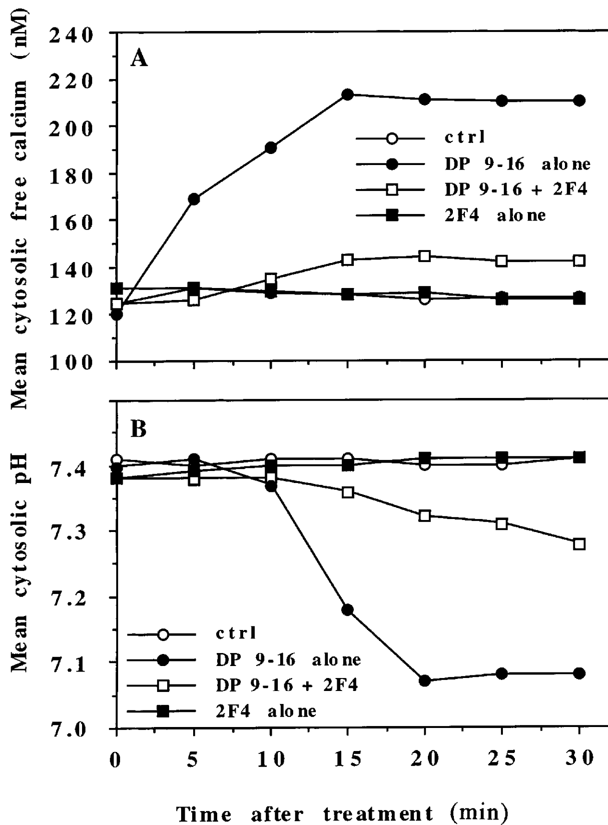

Fig. 11A,B. Effect of an excess of 2F4 monoclonal antibody on the mobilization of cytosolic free Ca^{2+} (**A**) and on cytosolic acidification (**B**) in carrot protoplasts treated with 0.2 mg ml^{-1} α-1,4-oligogalacturonides (DP 9–16) in the presence of 150 mM NaCl and 0.5 mM $CaCl_2$. Each point corresponds to the mean cytosolic free Ca^{2+} and mean cytosolic pH calculated by integrating pixel values over the whole surface of 10 protoplasts. All cytosolic free Ca^{2+} and pH data points had a standard deviation < 15 nM and < 0.1 pH unit, respectively

(Fig. 2D,G). In the presence of Na^+, the PAs reached lower plateaus, depending on their valences. The most peculiar observation came from the comparison of Fig. 2E and 2F: the presence of only as little as 0.5 mM Ca^{2+} in the equilibrium solution lowered the Spd^{3+} plateau by more than 50%. The only plausible explanation for that Spd^{3+} drop is that the binding of even small amounts of Ca^{2+} induced a drastic reorganization of the spatial arrangement of polygalacturonates which became sterically much less accessible to spermidine. We know (Messiaen et al. 1997) that DA and PA adsorption only involves electroselectivity. Unlike Ca^{2+}, DAs and PAs are thus unable to induce a cooperative dimerization of pectins. At first sight, the difference between these two isotherms (Fig. 2E,F) could simply be attributed to the presence of Ca^{2+} causing the transition of PGA from isolated chains to dimerized pectins with lower affinity for Spd^{3+}.

However, this is probably an oversimplification and the explanation must be more subtle. The egg-box formation only happens when more than 10% of the uronates bind Ca^{2+} (Fig. 4) but the drop in Spd^{3+} adsorption occurs at external Spd^{3+} concentrations higher than 2 mM. At those high external PA concentrations, the Ca^{2+} content of PGA decreased continuously, but even these small amounts of Ca^{2+}, insufficient to allow egg-box formation, were enough to induce a state of pectin different from isolated or dimerized chains but much less favourable for Spd^{3+} adsorption.

Conformation of PGA in the presence of PAs. The inhibition of the PGA recognition by the 2F4 MoAb in the ELISA test can be correlated with the adsorption of DAs and PAs onto PGA in the presence of 150 mM NaCl and 0.5 mM $CaCl_2$ (Fig. 2C,F,I). The stronger the affinity of DAs or PAs for PGA, the stronger the inhibition of the Ca^{2+}-induced dimerization of PGA in solution. For example, Put^{2+} was unable to remove much Ca^{2+} from PGA (Fig. 2C) and PGA was still recognized by the 2F4 MoAb (Fig. 3). The cations Spd^{3+} and Spm^{4+} readily lowered Ca^{2+} to less than 10% of the CEC (Fig. 4), and the subsequent binding of the 2F4 MoAb was prevented (Fig. 3). We verified that the absence of PGA recognition was not due to DAs or PAs binding to the antibodies. Briefly, 2F4 samples were incubated with each DA or PA (2 mM) for 1 h at room temperature and dialyzed overnight against the Tris-Na-Ca buffer of Liners et al. (1992). The dialyzed 2F4-DA or -PA mixtures were then used as primary antibodies in an ELISA test. No difference could be found between 2F4 controls and DA- or PA-preincubated antibodies. If PAs strongly interfered with the antibody, one would expect a reduction of the antibody binding to the pectin, even after dialysis. This was not the case (data not shown).

The inhibition of the 2F4 MoAb by DAs (Fig. 5) was size-dependent: the shorter the DA, the stronger the inhibition. Diamines longer than cadaverine (diaminopentane) did not inhibit PGA recognition by the antibodies. This is closely correlated with the ability of the DA to adsorb onto wall pectins, and to compete with Ca^{2+} on the cation-exchange sites (Messiaen et al. 1997). The case of Put^{2+} at low concentration is interesting since it was the only DA to increase the antibody recognition. Putrescine, being first adsorbed on low-affinity sites, i.e. on isolated chains and outer charges of dimerized pectins, and having little access to higher-affinity sites, i.e. to the inner faces of the egg boxes, probably stabilized the conformation that better bound the antibodies.

Elicitation by oligouronates in the presence of PAs. In carrot, α-1,4-oligogalacturonides with a DP ≥ 9 in a Ca^{2+}-induced conformation trigger a rapid and sustained increase in cytosolic free Ca^{2+} and a drop in cytosolic pH. These responses are essential signals leading to the activation of defense-related genes such as PAL (Messiaen and Van Cutsem 1994).

If we look at the PAL-elicitating activity of pectic fragments incubated with DAs and PAs (Fig. 6), it turns out that the results conform to the general trend observed in the ELISA tests in Fig. 3, i.e. a rapid disappearence of dimerized oligopectates, and thus of the elicitors, when Spd^{3+} and Spm^{4+} are present.

However, the decrease in the elicitating capacity of the fragments occurs at higher PA concentrations, probably for three reasons. First, cells were treated with oligomers in the PAL assay, since the PGA we used in the ELISA tests has no elicitating capacity in this bioassay. Conversely, oligomers cannot be used in an ELISA test for steric reasons. We expect oligomers and polymers to have different affinities for the same cations and thus to behave slightly differently with regard to their supramolecular conformation.

Second, the PAL activation tests of Figs. 6 and 7 were performed on living cell suspensions and protoplasts, and no monitoring of the actual Ca^{2+} and DA or PA concentrations at the surface of the plasmalemma could be achieved. Calcium ions could be exchanged from the cell walls or pumped out of the protoplasts; polyamines could be secreted or metabolized. Neither did we attempt to keep constant the polyamine concentrations in the culture medium after the start of the 24-h experiments.

Third, pectins were present in cell walls and probably secreted around the protoplasts during the course of the experiments. We do not know whether pectolytic enzymes could have produced additional fragments.

However, despite all these limitations, the conclusions are clear: Spm^{4+}, which is the most potent inhibitor of the supramolecular conformation of pectins, is also the PA that downregulates most strongly the PAL-elicitating capacity of the oligouronates. The mechanism of action is simple: the PA has no effect by itself, but it prevents the pectic oligomers from adopting the egg-box conformation recognized by putative receptors. The efficiency of this mechanism depends on the valence of the PAs, with $Spm^{4+} > Spd^{3+} > Put^{2+}$. This is in agreement with our previous observations reporting the same selectivity sequence of wall pectins for DAs and PAs (Messiaen et al. 1997). The suppression of cell elicitation by altering or masking the conformation of the pectic elicitor could be achieved not only by Spd^{3+} and Spm^{4+}, but also by the addition of EGTA (data not shown) or by preincubating the pectic fragments with the 2F4 MoAb itself (Fig. 11).

Physiological implications. This work shows that PA binding to pectic oligomers modulates the pectic signal these oligomers generate in plant cells. The divalent Put^{2+} stabilizes the egg-box conformation of the pectic fragments. Conversely, Spm^{3+} and Spd^{4+} hinder the dimerization of the pectins by removing Ca^{2+} from the egg boxes, preventing the triggering of the signaling cascade. The ratio of DAs to higher PAs has been shown to be directly correlated with elongation rate (Shen and Galston 1985).

This mechanism of pectic-signal modulation requires the presence of a minimum amount of PAs in the cell walls. What is meant by "a minimum amount" is still a matter of debate. The absolute wall concentration of PAs is probably not relevant to the problem: what matters are the ratios of PAs to pectins and PAs to cations close to the plasma membrane, as well as the $Put^{2+}/(Spd^{3+} + Spm^{4+})$ ratio.

Acidic pectins able to form egg boxes in the presence of Ca^{2+} are normally not found close to the plasmalemma of healthy cells (data not shown) but are confined to cell corners. In pathological conditions, invading bacteria and fungi secrete large amounts of pectolytic enzymes. De-esterified pectic fragments are then released in the cell wall and are free to diffuse to the plasmalemma. *Erwinia carotovora*, for example, releases free oligogalacturonides from carrot cells at concentrations higher than 0.1 mM (data not shown) which was shown to be the minimum concentration required for elicitor activity of pectic fragments. This happens concomitantly with an increase in the apoplastic free Ca^{2+} content (Messiaen and Van Cutsem 1994).

Recent publications give estimates of PA concentrations in different plant systems. Bajaj and Rajam (1996) studied PA involvement in the morphogenesis of long-term callus cultures of rice. The total PA content rises from 1.2 mmol kg^{-1} fresh weight in two-week-old callus to 24 mmol kg^{-1} fresh weight in 20-month-old callus that has severely reduced morphogenesis. The young callus has a Put^{2+}/Spd^{3+} ratio of 2.79 which, 20 months later, increases to 7.07. The morphogenetic capacity of the aged rice callus culture can be greatly improved by blocking PA accumulation and lowering the Put^{2+}/Spd^{3+} ratio with a 5 mM Spd^{3+} treatment. Ye et al. (1997) studied the correlation between elevated levels of Put^{2+} and oxidant-stress resistance. They observed free Put^{2+}, Spd^{3+} and Spd^{4+} levels up to 40, 76 and 330 mmol kg^{-1} fresh weight in a drought-resistant cultivar, and systematically lower Put^{2+} levels in a sensitive one.

Although these whole-cell concentrations are far higher than the 2 mM PAs used in this study, the in-vivo distribution of these PAs between the walls and the protoplasts is not known, even if PAs in mature plant tissues are reported to be primarily associated with the cell wall (Galston and Kaur-Sawhney 1995). However, in the case of an infection by a fungal or bacterial pathogen, we expect the PAs to be released in the apoplasm after plasmalemma breakdown. The wall degradation by an endopolygalacturonase itself releases bound PAs in the apoplastic fluid in addition to Ca^{2+} (Mariani et al. 1989). The in-vivo amounts of free PAs in the wall should then be largely sufficient to bind solubilized α-1,4-oligogalacturonides and modulate their biological properties. In some systems, such as pathogen attack, PAs could then play a specific role by modulating the transduction of the pectic signal.

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