Journal of Experimental Botany, Vol. 59, No. 9, pp. 2317–2324, 2008
doi:10.1093/jxb/ern096

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Early events induced by chitosan on plant cells

Bénigne-Ernest Amborabé, Janine Bonmort, Pierrette Fleurat-Lessard and Gabriel Roblin*

University of Poitiers, Laboratoire de Biochimie, Physiologie et Biologie Moléculaires Végétales, FRE CNRS 3091, 40, Avenue du Recteur Pineau, F-86022 Poitiers cedex, France

Received 16 February 2008; Revised 7 March 2008; Accepted 10 March 2008

Abstract

Chitosan (a polymer of β-1,4-glucosamine residues) is a deacetylated derivative of chitin which presents antifungal properties and acts as a potent elicitor of plant resistance against fungal pathogens. Attention was focused in this study on the chitosan-induced early events in the elicitation chain. Thus, it was shown that chitosan triggered in a dose-dependent manner rapid membrane transient depolarization of *Mimosa pudica* motor cells and, correlatively, a transient rise of pH in the incubation medium of pulvinar tissues. By using plasma membrane vesicles (PMVs), it was specified that a primary site of action of the compound is the plasma membrane H^+-ATPase as shown by its inhibitory effect on the proton pumping and the catalytic activity of the enzyme up to 250 μg ml^{-1}. As a consequence, chitosan treatment modified H^+-mediated processes, in particular it inhibited the uptake of the H^+-substrate co-transported sucrose and valine, and inhibited the light-induced H^+/K^+-mediated turgor reaction of motor cells. The present data also allowed the limit of the cytotoxicity of the compound to be established close to a concentration of 100 μg ml^{-1} at the plasma membrane level. As a consequence, chitosan could be preferably used in plant disease control as a powerful elicitor rather than a direct antifungal agent.

Key words: Chitosan, elicitor, H^+-ATPase, membrane potential.

Introduction

Chitosan (β-1,4-linked glucosamine) is a deacetylated derivative of chitin found in the composition of cell walls of many fungi (Bartnicki-Garcia, 1968). From data in previous reports, two biological roles can be ascribed to this compound. First, at defined concentrations, it presents antifungal properties as shown by its inhibitory action on the mycelial growth of a number of pathogenic fungi, including root pathogens, such as *Fusarium oxysporum* and *Pithium phanidermatum* (Leuba and Stossel, 1986; Benhamou, 1992; El Ghaouth *et al*., 1994; El Hassni *et al*., 2004), and also by its inhibitory effect on spore germination (Hadwiger and Beckman, 1980). Secondly, it acts as a potent elicitor, therefore enhancing plant resistance against pathogens (Benhamou and Thériault, 1992; El Ghaouth *et al*., 1994). It is thus an effective inducer of phytoalexin synthesis in various plant cells (Hadwiger and Beckman, 1980; Köhle *et al*., 1984), and triggers callose formation (Köhle *et al*., 1985; Conrath *et al*., 1989), lignification responses in wounded wheat leaves (Pearce and Ride, 1982), and the production of proteinase inhibitors (Walker-Simmons *et al*., 1984; Pena-Cortes *et al*., 1988). To date, only the long-term induced events have been investigated. Therefore, the aim of this work was to focus attention on the early events possibly triggered by chitosan at the cell plasma membrane level. To this end, the modification of the bioelectrical transmembrane potential on the motor cell membrane of *Mimosa pudica* was monitored. In most cases, application of an elicitor triggers a rapid depolarization of the membrane (Mathieu *et al*., 1966; Katou *et al*., 1982; Pelissier *et al*., 1986; Mayer and Ziegler, 1988; Kuchitsu *et al*., 1993; Thain *et al*. 1995). This event is the macroscopic visualization of ion migrations, namely the influx of Ca^{2+} and H^+ and the efflux of Cl^- and K^+ (Dixon *et al*., 1994; Cervone *et al*., 1997), resulting from activation of ion channels. In this regard, by measurement of pH variations in the bathing medium of pulvinar tissues and by observations of effects on plasma membrane vesicles (PMVs), it has been shown that a primary site of action of chitosan is the plasma membrane H^+-ATPase. In

* To whom correspondence should be addressed. E-mail: gabriel.roblin@univ-poitiers.fr

addition, it was observed that at relatively high concentrations, chitosan inhibited H^+-sustained nutrient uptake and K^+-driven motor cell reactions. These results are discussed in the light of the cytotoxicity of the compound.

Materials and methods

Plant materials

The sensitive plants (*M. pudica* L.) and *Cassia fasciculata* Michx. were grown in an organic compost watered daily. They were kept in climate-controlled chambers at 27.5±0.5 °C and 65±5% relative humidity. Illumination was regulated so as to give 16 h of light (photophase 06.00–22.00 h) provided by fluorescent tubes (mixing Osram fluora and Osram day-light types) with a photon flux density (400–700 nm) of 36 μmol m^{-2} s^{-1} at the plant apex. Plants of *M. pudica* and *C. fasciculata* were used when they bore 10 developed leaves.

Electrophysiological measurements

The transmembrane electrical potential was measured by the classical electrophysiological method using microelectrodes (tip diameter <1 μm, tip resistance from 5 to 30 MΩ). For details, see Moyen *et al*. (2007). Briefly, leaf was excised from the stem, and the pulvinus was fixed to the bottom of a 4 ml plexiglas chamber filled with a medium containing 1 mM NaCl, 0.1 mM KCl, and 0.1 mM $CaCl_2$, buffered at pH 5.5 with 10 mM MES/NaOH (Abe, 1981). The glass microelectrode was impaled into a motor cell of the abaxial ('extensor half') of the pulvini. Under these conditions, the resting transmembrane potential averaged at –124±19 mV from 32 assays.

Measurement of pH variations

Primary pulvini [~400 mg fresh weight (FW)] were excised from leaves, divided in transverse sections, and treated as previously described (Moyen *et al*., 2007): unbuffered incubation medium composed of 0.50 mM $CaCl_2$, 0.25 $MgCl_2$, variations of pH in the incubation medium read on a pH-meter provided with a microcombination electrode (Futura, Beckman Coulter) and linked to a potentiometric recorder. The experiments were repeated at least three times.

Preparation and use of PMVs

Purified PMVs were prepared by phase partitioning of microsomal fractions from the primary pulvini of *M. pudica* leaves according to Lemoine *et al*. (1991). Proton pumping and ATPase activity were measured as described in Noubahni *et al*. (1996) with some minor modifications. PMVs were put in the inside-out configuration by adding 0.05% brij in the assay medium (Vianello *et al*., 1982). In treated sets, chitosan was added at the given concentration 10 min before the beginning of the assay. Proton pumping activity was monitored as the decrease in the absorbance of acridine orange at 495 nm. The assay medium consisted of 50 mM KCl, 10 mM MES/TRIS (pH 6.5), 3 mM ATP, 1 mM dithiothreitol, 1 mg ml^{-1} bovine serum albumin, 20 μM acridine orange, 5 mM valinomycin, 300 mM sorbitol, and 75 μg of protein in a final volume of 1 ml. The reaction was started by addition of $MgSO_4$ to a final concentration of 3 mM. ATPase hydrolysis activity of the PMVs was measured by the phosphate released from ATP by the method of Ames (1966) in a medium containing 3 mM $MgSO_4$, 100 mM KCl, 50 mM MES/TRIS (pH 6.5), 3 mM ATP, 250 mM sucrose, and 40 μg of protein. After 5, 10, 15, and 20 min, 100 μl aliquots were sampled and mixed with the molybdate reagent which stops the reaction. In the six batches of vesicles used in this study, treatment with 0.25 mM sodium orthovanadate showed that 50–70% of the enzyme activity can be attributed to plasma membrane functioning.

PMVs either treated with chitosan as described above or non-treated (control) were fixed at room temperature (25 °C) in a mixture of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 45 min. The next steps performed at 4 °C consisted of extensive washing in the buffer followed by post-fixation in 1% (v/v) osmium tetroxide for 5 min and dehydration in an increasing ethanol series. Embedding occurred in London Resin White (LRW). Polymerization was conducted at 60 °C for 24 h. Thin sections (80 μm thick) of these chemically fixed PMVs were contrasted in uranyl acetate and lead citrate, and were observed by transmission electron microscopy using a JEM 1010 Jeol microscope operating at 80 kV.

Uptake assay of sucrose and valine

Excised primary pulvini were divided into two parts by longitudinal sections and distributed so that 10 sections constitute an experimental set, four such sets being considered for each experimental point. The tissues were pre-incubated and incubated in a medium containing 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$ buffered with 10 mM MES brought to pH 5.5 with 1 M NaOH. The pulvinar sections were rinsed in 20 ml of fresh medium for 1 h after excision and then transferred to a medium containing chitosan at the assayed concentration. After a defined incubation time, 10 sections were placed in the presence of 1 mM [^{14}C]sucrose solution (final specific activity of the medium 11 kBq ml^{-1}) and 1 mM [^{3}H]valine solution (final specific activity of the medium 9.25 kBq ml^{-1}). After incubation with the labelled substrate for 30 min, the apoplastic label was removed by three washes of 3 min each in the incubation medium. The sections were then dried, weighed, and digested in a mixture of H_2O_2, perchloric acid, Triton X-100 (2/1/1 by vol.) overnight at 55 °C. The radioactivity was then counted after addition of a scintillation medium (Ecolite, ICN) by liquid scintillation spectrometry (Packard Instr.).

Procedure for observation of dark- and light-induced turgor movements

The dark- and light- induced movements were observed on excised leaves of *C. fasciculata*. After excision, the cut petioles were dipped in distilled water for 1 h to recover. The leaves were then transferred to the assayed solution buffered at pH 5.5 with 2.5 mM MES/NaOH. The leaflet closure movements were induced by darkening the plants in the middle of the photophase and the opening movements were induced by lighting the plants in the middle of the night phase. The movements were followed by measuring the distance between the leaflets tips with a calliper square allowing the determination of angular values (Bonmort and Roblin, 1996). The dark- and light-induced movements observed on the *C. fasciculata* leaflets are similar to those triggered on *M. pudica* leaflets, but handling is easier since *C. fasciculata* is much less sensitive to mechanical shock.

Chemicals

All chemicals were purchased from Sigma-Aldrich Chimie, Saint-Quentin-fallavier, France. Chitosan from crab shells was prepared according to a method previously described by Young *et al*. (1982). A stock solution of fusicoccin (FC) at 1 mM final concentration was prepared in methanol (7% final concentration).

Results

Electrophysiological modifications induced by chitosan

The addition of chitosan to the bathing medium of *M. pudica* pulvini caused a transient depolarization of the motor cell membrane in a concentration-dependent manner at concentrations >10 μg ml^{-1} and up to 100 μg ml^{-1}. Figure 1 shows representative time courses of the bioelectrical events recorded in this range of concentrations: the depolarization occurred after a latency period (in the order of some minutes), decreasing as the applied concentration was increased, peaked at ~10–15 min, and returned to its initial resting potential after ~30–40 min even in the presence of chitosan. FC (10 μM) added at the end of each experiment triggered membrane hyperpolarization which ascertained the bioelectrical viability of the impaled cell. Quantitative results of this experiment are given in Table 1. Experiments conducted at 250 μg ml^{-1} did not give conclusive results since the motor cell reaction was so intense that the microelectrodes were expelled from the cells.

Effect of chitosan on the H^+-ATPase activity

Particular attention was paid to the proton-translocating ATPase on account of previous data showing that this enzyme is a characteristic target in the elicitation chain. Chitosan effects were therefore observed, first on the induced modification of proton fluxes in pulvinar tissues and, secondly, on the activity of the enzyme measured on PMVs.

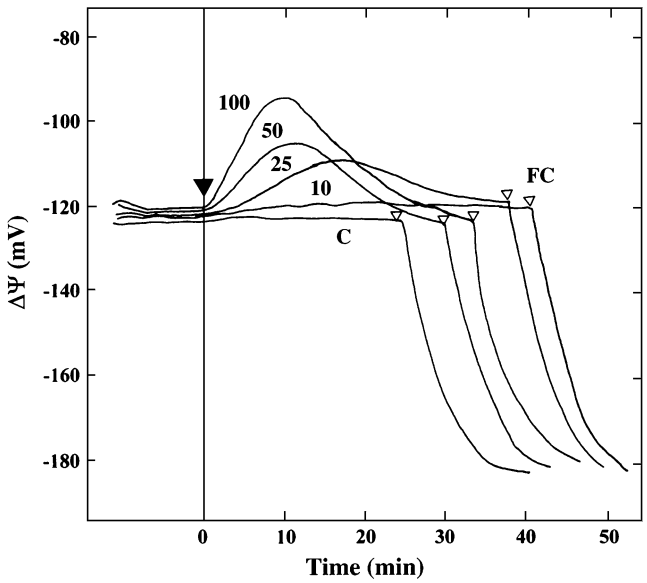

Fig. 1. Typical recordings of transmembrane potential variations in parenchyma cells of *Mimosa pudica* pulvinus induced by application of chitosan at various concentrations as indicated (in μg ml^{-1}; C: control). Chitosan was added at time 0 (black arrowhead), and 10 μM fusicoccin (FC) was added at the white arrowheads. The number of assays is indicated in Table 1.

As the result of proton pump activity, pulvinar tissues spontaneously acidified their incubation medium until an equilibrium near pH 5.0 was attained (Otsiogo-Oyabi and Roblin, 1984). Figure 2 shows representative time courses of the pH variations recorded in the bathing medium of pulvinar tissues after addition of chitosan at various concentrations (from 25 μg ml^{-1} to 250 μg ml^{-1}). After application of chitosan, a transient concentration-dependent pH rise was clearly seen at concentrations >25 μg ml^{-1}. The pH variation started after a latency period ranging from 30 s (250 μg ml^{-1}) to 3 min (25 μg ml^{-1}), peaked in the range 10 min (25 μg ml^{-1}) to 15 min (250 μg ml^{-1}), and returned to its initial value in ~2 h even in the presence of chitosan. It has been verified that this effect was not due to a modification in the buffering capacity of the compound. These data suggest that the plasma membrane H^+-ATPase may be inhibited by a chitosan treatment. In order to verify the hypothesis, proton pumping assays and determination of ATPase catalytic activity were performed following chitosan treatment of PMVs purified from primary pulvini tissues. As previously reported (Moyen *et al.*, 2007), 10 μM FC, known to activate the vanadate-sensitive ATPase, activated both processes. In contrast, data in Table 2 show that the vanadate-sensitive ATPase activity of PMVs was inhibited by chitosan in a dose-dependent manner at concentrations >10 μg ml^{-1}, a maximal inhibition of ~50% being reached at concentrations >100 μg ml^{-1}. It should be stressed that the proton pumping was more sensitive to chitosan application than the catalytic activity since it was inhibited at concentrations as low as 10 μg ml^{-1} and up to 50 μg ml^{-1}. At higher concentrations, measurement were no longer possible, indicating that PMVs were damaged. This assumption was verified by electron microscope observations. Indeed, control PMVs appeared in cross-sections as circle-shaped elements (Fig. 3A, D) whereas PMVs treated with chitosan at 50 μg ml^{-1} and 250 μg ml^{-1} were deflated, but not disrupted (Fig. 3B, C, E). This modification therefore explained the absence of proton pumping activity since the stain was no longer able to be absorbed by the vesicles in this induced configuration.

Table 1. Modifications of transmembrane potential induced by chitosan at various concentrations on the pulvinar motor cells of *Mimosa pudica*

Results correspond to means ±SD. + indicates a hyperpolarization. ND, not detectable.

Chitosan (μg ml^{-1})	Latency (min)	ΔΨ (mV)	Peaking (min)	No. of assays
10	ND	+2.4±2.4	-	10
25	3.0±0.5	+12.0±1.4	15.0±2.6	6
50	1.8±0.6	+15.6±2.0	13.0±2.0	6
100	1.6±0.6	+26.2±5.0	10.0±1.5	8

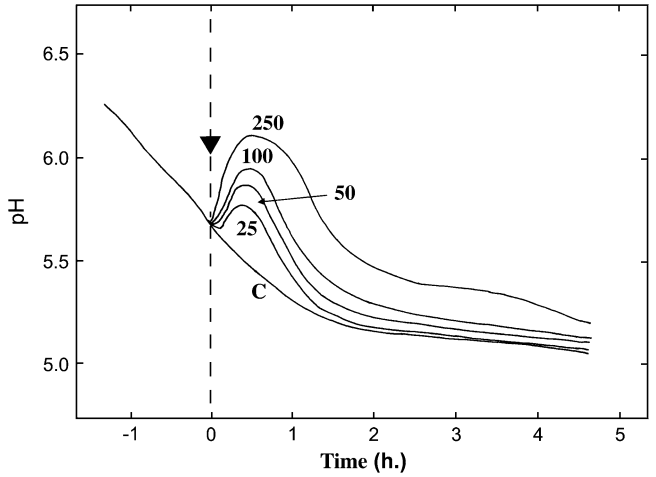

Fig. 2. Representative curves of the time course of pH variations in the bathing medium of pulvinar tissues of *Mimosa pudica* induced by treatment with chitosan at various concentrations in μg ml^{-1} as indicated. The experiments were conducted at least three times with the same general result.

Table 2. Effects of chitosan at various concentrations on proton pumping measured by OD variation and on vanadate-sensitive ATPase on PMVs purified from *Mimosa pudica* pulvini

The values are give as the mean± SD with the number of assays in parentheses and the percentage inhibition in square brackets. ND, not determined.

chitosan (μg ml^{-1})	OD variation (unit mg $prot^{-1}$ min^{-1})	Vanadate-sensitive ATPase activity (nmol Pi mg $prot^{-1}$ min^{-1})
Control	0.42±0.07 (16) –	323±33 (10) –
10	0.31±0.06 (6) [26]	314±21 (10) [3]
25	0.17±0.07 (12) [60]	305±25 (10) [6]
50	0.11±0.06 (12) [74]	255±36 (10) [21]
100	ND	185±31 (10) [41]
250	ND	166±24 (10) [49]
500	ND	156±31 (10) [52]

Subsequent effects on physiological membrane-related phenomena

Due to this inhibitory action on the plasma membrane H^+-ATPase, different processes regulated by this enzyme are expected to be disturbed by treatment with chitosan. In order to verify this expectation, two models were chosen.

First, the effect of chitosan was observed on the membrane transport of assimilates. Indeed, as a consequence of the effect observed above on the H^+-ATPase, the uptake of assimilates co-transported with H^+ is expected to be hindered. From the data in Table 3, it appears that the dose-dependent inhibitory effect of chitosan was higher and more rapid on valine uptake than on sucrose uptake.

Secondly, another type of observation was carried out on the effect of chitosan on the ion-driven osmocontractile

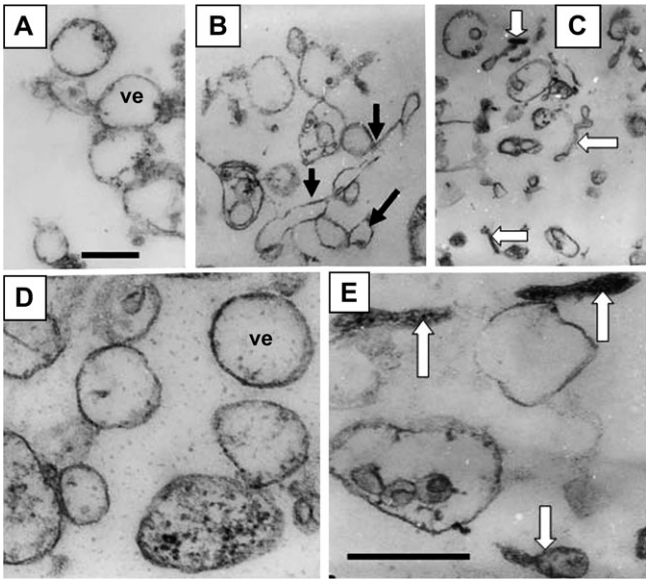

Fig. 3. Transmission electron micrographs of PMVs purified from pulvinar tissues treated by chitosan at (A, D) 0 (control), (B) 50 μg ml^{-1}, and (C, E) 200 μg ml^{-1}. Note that the circle-shaped vesicles observed in the control were distorted (black arrows) or completely deflated (white arrows) by chitosan treatment. Scale bars=0.5 μm.

Table 3. Modification of 1 mM sucrose and 1 mM valine uptake by pulvinar tissues of *Mimosa pudica,* as a function of chitosan concentration and treatment duration

Absorption of the solutes lasted 30 min. Data are means of three experiments ±SD made on 20 pulvinar halves. Numbers in parentheses indicate the percentage inhibition

	Chitosan (μg ml^{-1})			
	Control	50	100	250
Duration				
1 mM sucrose uptake (nmol mg FW^{-1})				
1 h	0.47±0.02	0.46±0.01 (0)	0.48±0.02 (0)	0.46±0.01 (0)
3 h	0.63±0.02	0.62±0.02 (0)	0.64±0.03 (0)	0.57±0.02 (8)
6 h	0.79±0.05	0.74±0.02 (5)	0.71±0.01 (10)	0.65±0.02 (18)
1 mM valine uptake (nmol mg FW^{-1})				
1 h	1.34±0.01	1.33±0.01 (0)	1.28±0.02 (4)	1.26±0.01 (6)
3 h	2.36±0.02	2.34±0.02 (0)	2.12±0.02 (10)	2.02±0.02 (14)
6 h	3.62±0.26	3.47±0.20 (4)	3.04±0.20 (16)	2.60±0.20 (28)

reactions of pulvinar cells induced by variations in light regimes (dark- and light-induced movements). Following a period of darkness during the light phase of the photoperiodic cycle, the leaflets rapidly close as a consequence of a rapid turgor loss in the pulvinar motor cells. This turgor variation is driven by an efflux of K^+ and Cl^- (Satter and Galston, 1981) and an influx of protons (Otsiogo-Oyabi and Roblin, 1984). The light-induced movements are the result of inverse ion fluxes. Applied 3 h before subjecting the plants to darkness, chitosan at high concentration (250 μg ml^{-1}) induced a partial closure of the leaflets observed after 1 h treatment. Angle

variation calculated after 30 min of darkness showed that closure was unaffected at 50 μg ml^{-1} and enhanced by 26% at 100 μg ml^{-1} and 250 μg ml^{-1} (Fig. 4A). This result suggests that the underlying K^+ efflux was activated. In contrast, the light-induced movements were strongly inhibited by the compound in a dose-dependent manner (20% at 100 μg ml^{-1}, 50% at 250 μg ml^{-1}) (Fig. 4B). These results suggest that the underlying, inwardly directed K^+ influx was strongly inhibited.

Discussion

The results of the present study show that a primary site of chitosan action is located at the plasma membrane and give additional data on the mode of action of the compound at this cellular site.

Chitosan affects physiological processes linked to the plasma membrane H^+-ATPase activity

The early perception of chitosan by plant cells was shown by the transient dose-dependent membrane depolarization (Fig. 1) and the modification of the proton fluxes displayed by the observed transient pH rise in the bathing medium of pulvinar tissues (Fig. 2). The induced transmembrane potential variation and the H^+ flux modification showed a close relationship in the latency period as well as in the peaking of the time courses in both processes, so that a causal link of H^+ entry in the cells might correspond to the depolarization of the membrane. However, the electrical potential rapidly returned to its basal level whereas the H^+ flux was more sustained, such that other compensatory ionic fluxes have to be considered to explain the discrepancy, either an efflux of cations or an influx of anions. The time course of pH variation also leads to the conclusion that H^+ fluxes were disturbed in a prolonged way and therefore may intervene in the regulation of long-term processes. Experiments on PMVs demonstrated the direct action of the chitosan on the H^+-ATPase plasma membrane, inducing the collapse of the proton motive force, subsequently impairing the energetics of the cells. As a consequence, it might be expected that the plasma membrane systems linked to the proton motive force would be impaired. Indeed, it was shown that application of chitosan led to the modification of the membrane transport of nutrients. However, note that valine uptake was more inhibited than sucrose uptake. This observation is in accordance with previous data showing that amino acid uptake was more sensitive to membrane depolarization and proton mobilization than sucrose uptake (Michonneau *et al*., 2004). The second illustrative model concerns the inhibitory effect observed on ion-sustained processes as illustrated by the modification of the H^+/K^+-regulated turgor motor cell reaction. It is noteworthy that the motor reactions driven by K^+ efflux were poorly modified, whereas reactions driven by K^+ influx were dramatically inhibited.

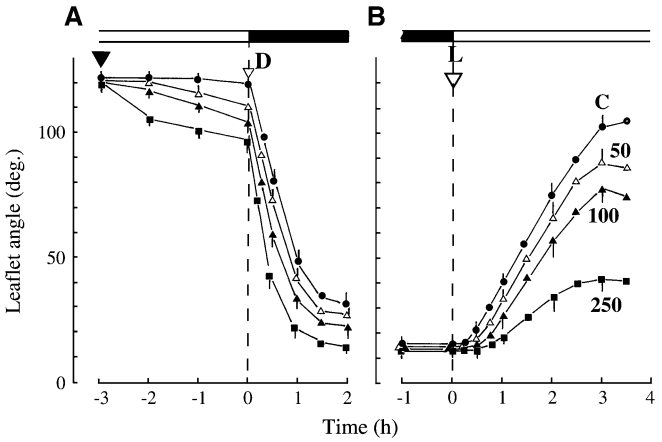

Fig. 4. Effect of chitosan at various concentrations applied for a 3 h period on the time course of the dark- (A) and light-induced (B) leaflet movements of *Cassia fasciculata*. Chitosan was applied 180 min before the onset of the dark and light period. Control (C); chitosan concentration in μg ml^{-1} as indicated. Mean ±SE; n=24.

Chitosan effect on the plasma membrane H^+-ATPase is a primary event in the elicitation chain

The above data on H^+-ATPase activity have to be considered within the scope of the involvement of chitosan in plant-induced resistance. The defence processes which are induced by specific components released from the pathogen or host cell walls during the infection are characterized by several reactions including formation of mechanical barriers, activation of extracellular proteins, and production of phytoalexins with antibiotic properties (Bell, 1981, and references therein).

Chitosan has been shown to trigger several of these reactions. Thus, it elicited callose synthesis in soybean leaves (Köhle *et al*., 1985) and induced the lignification response in wheat leaves (Pearce and Rice, 1982). It is a powerful activator of characteristic enzyme activities, i.e. chitinase and β-1,3-glucanases (Romanazzi *et al*., 2002; Amborabé *et al*., 2004), and proteinase inhibitor in tomato (Walker-Simmons *et al*., 1984). It also activates the phenylpropanoid pathway by increasing the activity of phenylalanine ammonia lyase in potato suspension culture (Dornenburg and Kow, 1997) and in cotyledons of *M. pudica* (Rossard *et al*., 2006), leading to an accumulation of many phenolic compounds (Hadwiger and Beckman, 1980; Köhle *et al*., 1984; Reddy *et al*., 1999; Rossard *et al*., 2006). It has also been shown to intervene in upstream events by triggering the oxidative burst in relation to NADPH oxidase activation (Rossard *et al*., 2006), leading to the production of H_2O_2 (Kauss and Jeblick, 1996). In addition, chitosan also activates the octadecanoid pathway as shown by an increased level of jasmonic acid in rice (Rakwal *et al*., 2002) and the

increased lipoxygenase activity in wheat (Rohland *et al.* 1997).

It should be stressed that all these reported events occurred after a time lapse of at least 30 min in the case of H_2O_2 production (Kauss and Jeblick, 1996). The present data give additional insight into the earlier events triggered by chitosan and complete the frame on the involvement of chitosan in elicitation processes. By inducing membrane depolarization, it acts in the same way as many other fungal elicitors (Mathieu *et al.*, 1966; Katou *et al.*, 1982; Pelissier *et al.*, 1986; Mayer and Ziegler, 1988; Kuchitsu *et al.*, 1993; Thain *et al.*, 1995). Similarly, H^+ influx was a characteristic reaction observed following application of various elicitors on different plant models (Dixon *et al.*, 1994; Mathieu *et al.*, 1996; Pugin *et al.*, 1997). Such modification resulted from inhibition of H^+-ATPase (Hagendoorn *et al.*, 1991; Schaller and Oecking, 1999) playing the role of a switch between the elicitor recognition and the pathogen defence signalling pathways. The direct inhibitory effect reported here on H^+-ATPase argues for this hypothesis.

Another step that should be addressed in the future concerns the characterization of a putative receptor. In a previous work, the possibility of such an interaction of chitosan with a receptor was discarded and its action was explained by an interaction of the compound with regularly spaced negative charges on the plasma membrane (Kauss *et al.*, 1989). However, the desensitization process which occurred as well in H^+ flux variation and electrophysiological events (Amborabé *et al.*, 2003) constitutes a pertinent argument for the presence of a specific receptor. This hypothesis would also help towards an understanding of the fact that the membrane potential and pH value were restored to their initial state even in the presence of the compound. In this case, receptor sites would be saturated upon arrival of the chitosan molecules, leading to disruption of the signalling reaction, only restored by a new application of the compound after a refractory period as reported previously (Amborabé *et al.*, 2003).

Chitosan induces cytotoxic effects

In some conditions, chitosan treatment resulted in the reduction and eventual loss of viability in both plant and fungal cells (Kendra and Hadwiger, 1987). This observation has prompted the evaluation of the cytotoxic effect of the compound that appeared related to the applied concentration.

In this line, two explanations can be envisaged. On the one hand, as an elicitor, chitosan may act through the hypersensitive response as shown by H_2O_2 synthesis observed in treated plant tissues (Kauss and Jeblick, 1996). However, this effect may be limited by considering the results of Lafontaine and Benhamou (1996) who noted no symptoms related to toxicity nor cytological alterations in their experimental conditions (the highest concentration used was 37.5 μg ml^{-1}). In contrast, as reported in previous studies on plant material treated with higher concentrations (200 μg ml^{-1} and up to 1000 μg ml^{-1}), chitosan destroyed cell organization (Köhle *et al.*, 1984) and induced leakage of electrolytes, UV-absorbing material, and proteins (Young *et al.*, 1982). It has been demonstrated that the leakage of this material was clearly due to the polycationic nature of chitosan which acts in a similar way to Triton X-100 (Young *et al.*, 1982), resulting in the formation of large 'pores' in the membrane, explaining, therefore, the dose-dependent deflating effect seen on treated PMVs (Fig. 3).

Antifungal effects noted on *in vitro* assays on *Botrytis cinerea* (Amborabé *et al.*, 2004) and on *Fusarium oxysporum* f. sp *albedinis* (El Hassni *et al.*, 2004) may, therefore, be the result of a general cellular cytotoxic effect not restricted to fungal cells. As a consequence, in a practical approach, the evidence is that chitosan has to be used at concentrations that do not alter plant physiology and, according to the present results on PMV ATPase activity, it did not reach the plant cell membrane at concentrations higher than 100 μg ml^{-1}. However, the actual concentration at this site is difficult to appreciate *in planta* from external treatment, since it depends on the absorption capacity, the transport into the treated leaves, and the binding capacity of the cell wall.

In conclusion, curative treatments using chitosan must be conducted with care since the increased permeability of host cells induced by the compound could facilitate infection by enabling fungal products to enter host cells. In contrast, the utilization of chitosan at concentrations that trigger elicitor action should be envisaged without restriction.

References

Abe T. 1981. Chloride ion efflux during an action potential in the main pulvinus of *Mimosa pudica*. *The Botanical Magazine (Tokyo)* **94,** 379–383.

Amborabé B-E, Aziz A, Trotel-Aziz P, Quantinet D, Dhuicq L, Vernet G. 2004. Essais d'emploi du chitosan contre *Botrytis cinerea*. *Phytoma* **571,** 26–29.

Amborabé B-E, Rossard S, Pérault JM, Roblin G. 2003. Specific perception of ergosterol by plant cells. *Comptes Rendus Biologies* **326,** 363–370.

Ames BN. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods in Enzymology* **8,** 115–118.

Bartnicki-Garcia S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Review of Microbiology* **22,** 87–108.

Bell AA. 1981. Biochemical mechanisms of disease resistance. *Annual Review of Plant Physiology* **32,** 21–81.

Benhamou N. 1992. Ultrastructural and cytochemical aspects of chitosan on *Fusarium oxysporum* f.sp. *radicis-lycopersici*, agent of tomato crown and root rot. *Phytopathology* **82,** 1185–1093.

Benhamou N, Thériault G. 1992. Treatment with chitosan enhances resistance of tomato plants to the crown and root rot pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Physiological and Molecular Plant Pathology* **41,** 33–52.

Bonmort J, Roblin G. 1996. Effect of 2,4-dichlorophenoxyacetic acid on the dark- and light-induced pulvinar movements in *Cassia fasciculata* Michx. *Plant Growth Regulation* **19,** 61–65.

Cervone F, Castoria R, Leckie F, De Lorenzo G. 1997. Perception of fungal elicitors and signal transduction. In: **Aducci P, ed.** *Signal transduction in plants*. Basel, Switzerland: Birkhaüser Verlag, 153–177.

Conrath U, Domard A, Kauss H. 1989. Chitosan-elicited synthesis of callose and of coumarin derivatives in parsley cell suspension cultures. *Plant Cell Reports* **8,** 152–154.

Dixon RA, Harrison MJ, Lamb CJ. 1994. Early events in the activation of plant defence responses. *Annual Review of Phytopathology* **32,** 479–501.

Dornenburg H, Knorr D. 1997. Evaluation of elicitor and high pressure induced enzymatic browning utilizing potato (*Solanum tuberosum*) suspension culture as a model for plant tissues. *Journal of Agriculture and Food Chemistry* **45,** 4173–4177.

El Ghaouth A, Arul J, Benhamou N, Asselin A, Bélanger RR. 1994. Effect of chitosan on cucumber plants: suppression of *Pythium aphanidermatum* and induction of defence reactions. *Phytopathology* **84,** 313–320.

El Hassni M, El Hadrami, Daayf F, Ait Barka E, El Hadrami I. 2004. Chitosan, antifungal product against *Fusarium oxysporum* f. sp. *albedinis* and elicitor of defence reactions in date palm roots. *Phytopathologia Mediterranea* **43,** 195–204.

Hadwiger LA, Beckman JM. 1980. Chitosan as a component of pea–*Fusarium solani* interactions. *Plant Physiology* **66,** 205–211.

Hagendoorn MJM, Poortinga AM, Wong Fong Sang HW, van der Plas LHW, van Walraven HS. 1991. Effects of elicitors on the plasma membrane of *Petunia hybrida* cell suspensions. *Plant Physiology* **96,** 1261–1267.

Katou K, Tomiyama K, Okamoto H. 1982. Effect of hyphal wall components of *Phytophthora infestans* on membrane potential of potato tuber cells. *Physiological Plant Pathology* **21,** 311–317.

Kauss H, Jeblick W. 1996. Influence of salicylic acid on the induction of competence for H_2O_2 elicitation. Comparison of ergosterol with other elicitors. *Plant Physiology* **111,** 755–763.

Kauss H, Jeblick W, Domard A. 1989. The degrees of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*. *Planta* **178,** 385–392.

Kendra DF, Hadwiger LA. 1987. Cell death and membrane leakage not associated with the induction of disease resistance in peas by chitosan or *Fusarium solani* f. sp *phaseoli*. *Phytopathology* **77,** 100–106.

Köhle H, Jeblick W, Poten F, Blaschek W, Kauss H. 1985. Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+}-dependent process. *Plant Physiology* **77,** 544–551.

Köhle H, Young DH, Kauss H. 1984. Physiological changes in suspension-cultured soybean cells elicited by treatment with chitosan. *Plant Science Letters* **33,** 221–230.

Kuchitsu K, Kikuyama M, Shibuya N. 1993. N-Acetylchitooligosaccharides, biotic elicitor for phytoalexin production, induce transient membrane depolarization in suspension-cultured rice cells. *Protoplasma* **174,** 79–81.

Lafontaine PJ, Benhamou N. 1996. Chitosan treatment: an emerging strategy for enhancing resistance of greenhouse tomato plants to infection by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. *Biocontrol Science and Technology* **6,** 111–124.

Lemoine R, Bourquin S, Delrot S. 1991. Active uptake of sucrose by plant plasma membrane vesicles: determination of some important physical and energetical parameters. *Physiologia Plantarum* **82,** 377–384.

Leuba JL, Stossel P. 1986. Chitosan and other polyamines: antifungal activity and interaction with biological membranes. In: **Muzzarelli R, Jeauniaux C, Gooday GW, eds.** *Chitin in nature and technology*. New York: Plenum Press, 215–222.

Mathieu Y, Lapous D, Thomine S, Laurière C, Guern J. 1966. Cytoplasmic acidification as an early phosphorylation-dependent response of tobacco cells to elicitors. *Planta* **199,** 416–424.

Mayer MG, Ziegler E. 1988. A elicitor from *Phytophtora megasperma* f. sp. *glycinea* influences the membrane potential of soybean cotyledonary cells. *Physiological and Molecular Plant Pathology* **33,** 397–407.

Michonneau P, Roblin G, Bonmort J, Fleurat-Lessard P. 2004. Valine uptake in the tap root of sugar beet: a comparative analysis with sucrose uptake. *Journal of Plant Physiology* **161,** 1299–1314.

Moyen C, Bonmort J, Roblin G. 2007. Membrane effects of 2,4-dichlorophenoxyacetic acid in motor cells of *Mimosa pudica* L. *Plant Physiology and Biochemistry* **45,** 420–426.

Noubhani AM, Sakr S, Denis MH, Delrot S. 1996. Transcriptional and post-translational control of the plant plasma membrane H^+-ATPase by mechanical treatments. *Biochimica et Biophysica Acta* **1281,** 213–219.

Otsiogo-Oyabi H, Roblin G. 1984. Effects of glycine on dark- and light-induced pulvinar movements and modification of proton fluxes in the pulvinus of *Mimosa pudica* during glycine uptake. *Planta* **161,** 404–408.

Pearce RB, Ride JP. 1982. Chitin and related compounds as elicitors of lignification response in wounded wheat leaves. *Physiological Plant Pathology* **20,** 119–123.

Pelissier B, Thibaud JB, Grignon C, Esquerré-Tugayé MT. 1986. Cell surfaces in plant–microorganisms interactions. VII. Elicitor preparations from two fungal pathogens depolarize plant membranes. *Plant Science* **46,** 103–109.

Pena-Cortes H, Sanchez-Serrano J, Rocha-Sosa M, Willmitzer L. 1988. Systemic induction of proteinase-inhibitor-II gene expression in potato plants by wounding. *Planta* **174,** 84–89.

Pugin A, Frachisse JM, Tavernier E, Bligny R, Gout E, Douce R, Guern J. 1977. Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose pathway. *The Plant Cell* **9,** 2077–2091.

Rakwal R, Tamogani S, Agrawal GK, Iwahashi H. 2002. Octadecanoid signalling component 'burst' in rice (*Oryza sativa* L.) seedlings leaves upon wounding by cut and treatment with fungal elicitor chitosan. *Biochemical and Biophysical Research Communications* **295,** 1041–1045.

Reddy MV, Arul J, Angers P, Couture L. 1999. Chitosan treatment of wheat seeds induces resistance to *Fusarium graminearum* and improves seed quality. *Journal of Agriculture and Food Chemistry* **47,** 1208–1216.

Rohland C, Balkenhohl T, Loers G, Feussner I, Grambow J. 1997. Differential induction of lipoxygenase isoforms in wheat upon treatment with rust fungus elicitor, chitin oligosaccharides, chitosan, and methyl jasmonate. *Plant Physiology* **114,** 679–685.

Romanazzi G, Nigro F, Ippolito A, Di Venere D, Salerno M. 2002. Effects of pre- and post-harvest chitosan treatments to control storage grey mold of table grapes. *Journal of Food Science* **67,** 1862–1867.

Rossard S, Luini E, Pérault JM, Bonmort J, Roblin G. 2006. Early changes in membrane permeability, production of oxidative burst and modification of PAL activity induced by ergosterol in

cotyledons of *Mimosa pudica*. *Journal of Experimental Botany* **57,** 1245–1252.

Satter RL, Galston AW. 1981. Mechanism of control of leaf movements. *Annual Review of Plant Physiology* **32,** 83–110.

Schaller A, Oecking C. 1999. Modulation of plasma membrane H^+-ATPase activity differentially activates wound and pathogen defence responses in tomato plants. *The Plant Cell* **11,** 263–272.

Thain JF, Gubb IR, Wildon DC. 1995. Depolarization of tomato leaf cells by oligogalacturonides elicitors. *Plant, Cell and Environment* **18,** 211–214.

Vianello A, Dell'Antone P, Macri F. 1982. ATP-dependent and ionophore induced proton translocation in pea stem microsomal vesicles. *Biochimica et Biophysica Acta* **689,** 89–96.

Walker-Simmons M, Jin D, West CA, Hadwiger L, Ryan CA. 1984. Comparison of proteinase inhibitor-inducing activities and phytoalexin elicitor activities of a pure fungal endopolygalacturonase, pectic fragments, and chitosans. *Plant Physiology* **76,** 833–836.

Young DY, Köhle H, Kauss H. 1982. Effect of chitosan on membrane permeability of suspension-cultured *Glycine max* and *Phaseolus vulgaris* cells. *Plant Physiology* **70,** 1449–1454.