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Size, acetylation and concentration of chitooligosaccharide elicitors determine the switch from defence involving PAL activation to cell death and water peroxide production in Arabidopsis cell suspensions

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Chitosan oligomers are known elicitors of plant defence mechanisms. In this work, chitooligosaccharides of different degrees of polymerization and degrees of acetylation were prepared and characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The effect of the degree of polymerization (DP), degree of acetylation and concentration of these chitooligosaccharides on defence activation in *Arabidopsis thaliana* suspension-cultured cells was studied. Our study results show that fully deacetylated chitooligosaccharides (chitosan oligomers) induce, depending on their DP and concentration, phenylalanine ammonia-lyase (PAL) activation, H_2O_2 synthesis and cell death in *A. thaliana* cell suspensions. The progressive reacetylation of the chitosan oligomer elicitors progressively impaired their ability to enhance H_2O_2 accumulation and cell death, but did not affect the activation of PAL.

Introduction

Oligosaccharide fragments derived from chitin, a linear polysaccharide composed of 4-linked β-*N*-acetylglucosaminyl residues and common component of fungal cell walls, and chitosan, its de-*N*-acetylated derivative, have been shown to elicit defence responses in various plants. *N*-acetylchitooligosaccharides bind to a specific receptor (Bradley Day et al. 2001, Okada et al. 2002) and induce membrane depolarization (Kuchitsu et al. 1993, Kikuyama et al. 1997), ion fluxes (Kuchitsu et al. 1997), the production of reactive oxygen species (ROS) (Kuchitsu et al. 1995) and phytoalexin synthesis (Ren and West 1992, Yamada et al. 1993) in suspension-cultured rice cells. They also elicit lignification in wheat (Barber et al. 1989), ion flux and protein phosphorylation in cultured tomato cells (Felix et al. 1993), chitinase activity in melon (Roby et al. 1987), phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activity in soybean leaves (Khan et al. 2003) and glucanase gene transcription in cultured barley cells (Kaku et al. 1997).

Chitosan-derived oligosaccharides elicit defence responses in different plants, mostly dicots such as the accumulation of phytoalexins in pea pods (Hadwiger and Beckman 1980), in suspension-cultured soybean cells (Kohle et al. 1984), parsley cells (Conrath et al. 1989), the synthesis of ROS (Lee et al. 1999), the accumulation of defence-related proteinase inhibitors in tomato and potato leaves (Walker-Simmons and Ryan 1984, Peña-Cortes et al. 1988), the synthesis of callose

Abbreviations – DA, degree of acetylation; DP, degree of polymerization; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MW, molecular weight; NAA, naphthalene acetic acid; PAL, phenylalanine ammonia-lyase.

in suspension-cultured parsley (Conrath et al. 1989), tomato (Grosskopf et al. 1991) and *Catharanthus roseus* cells (Keen 1975). Whether chitosans interact with a specific membrane receptor is not known, but the early activation of a MAP kinase cascade seems to be a common signalling pathway used by both deacetylated (Vasconsuelo et al. 2003) and acetylated (Wan et al. 2004) elicitors.

More recently, microarray and proteomic analyses of chitin elicitation were characterized either in liquid-cultured *Arabidopsis thaliana* cells or in seedlings. These studies confirmed the activation of several previously described defence genes and unravelled some genes of unknown function also affected by chitooligomers (Ramonell et al. 2002, Zhang et al. 2002, Akimoto-Tomiyama et al. 2003, Wan et al. 2004). Moreover, Zhang et al. (2002) showed that the early activation of three genes, encoding a MAP kinase, a zinc-finger protein and a lectin-like protein, by chitin oligomers of DP 2–8 needs a phosphorylation event within 20 min after the addition of the elicitor. Using signal transduction mutants of the jasmonate (JA), the ethylene and the salicylic acid (SA) pathways, they showed that chitin triggers a signalling cascade that is independent of JA, SA and ethylene.

As the biological activities of chitin and chitosan have often been determined using heterogeneous and/or uncharacterized oligosaccharide or polymer mixtures, the size and structure requirements for oligochitins and chitosan oligomers to have a biological activity are difficult to ascertain. Additionally, the optimum size and structure of these oligosaccharides for elicitor activity are different depending on the experimental systems (Shibuya and Minami 2001). The oligosaccharides generally must have a DP>4 to induce a biological response, but beyond that requirement, it is not possible to generalize about structural features essential for their biological activity (Cote and Hahn 1994). The concentrations of oligosaccharides that are effective in plant bioassays seem also to be different for both elicitors and dependent on the plant model used. The concentrations of chitosan-derived oligosaccharides required to trigger defence responses are usually much higher than those necessary for chitin oligosaccharides to elicit similar defence responses (Yamaguchi et al. 2000).

In this study, we prepared two chitooligosaccharides sets of defined degree of polymerization (DP) and several oligosaccharides with increasing DA by reacetylation of well-characterized deacetylated oligomers. Chitin and chitosan oligosaccharides were characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and tested in suspension-cultured Arabidopsis cells for their ability to induce PAL activity and H_2O_2 accumulation, two well-known markers of defence reactions in plants (Somssich and Hahlbrock 1998). We showed that fully deacetylated chitooligosaccharides (chitosan oligomers) induce, depending on their DP and concentration, PAL activation, H_2O_2 synthesis and cell death in *A. thaliana* cell suspensions. The progressive reacetylation of the chitosan oligomer elicitors progressively impaired their ability to enhance H_2O_2 accumulation and cell death, but did not affect the activation of PAL.

Materials and methods

Chitin and chitosan preparation

Chitin from Cuban lobster was supplied by Mario Muñoz Pharmaceutical Laboratories (La Habana, Cuba) and used to prepare chitosan under heterogeneous conditions following the methodology described by Cabrera et al. (2000). The molecular weight (MW) of the chitosan obtained was found to be 82.9 kDa by viscosimetry as deduced from an intrinsic viscosity (η) of 416 ml g^{-1} and its DA was 12% as determined by titration.

Preparation of elicitors

Chitooligosaccharides with low DPs

Chitosan (2 g) was dissolved in 100 ml of dilute acetic acid and dried under vacuum until the content became a gelatinous paste. Hundred millilitre of concentrated HCl (37%) was added, and the suspension was heated for 30 min at 72°C under stirring. The hydrolysis reaction was stopped by immersion in an ice bath. Most of the solvent and HCl was evaporated under vacuum. The residue was then resuspended in water and the solution evaporated, those last two operations being repeated twice. The residue was finally dissolved in water, and the solution was brought to pH 6.5 by addition of concentrated 10 *M* NaOH. The neutralized chitosan hydrolysates were precipitated with a final methanol concentration of 90% (v/v). The supernatant containing chitooligosaccharides with low DPs was concentrated under reduced pressure.

Chitooligosaccharides with high DPs

Chitosan was dissolved by overnight shaking at room temperature in 0.175 *M* acetate buffer pH 5.5 to a final concentration of 10 g l^{-1}. This chitosan solution (90 ml) was mixed with 10 ml of commercially available

Pectinex Ultra SPL (26 000 PG ml^{-1}, Novozymes A/S, Bagsvaerd, Denmark) and incubated at 37°C for 24 h. The reaction was stopped by boiling at 100°C during 15 min. The chitooligosaccharides with higher DPs were isolated from the chitosan hydrolysate by selective precipitation in 90% (v/v) methanol. The resulting pellet was washed several times with 90% (v/v) methanol, re-dissolved in water, exhaustively dialysed against deionized water using a SPECTRAPOR membrane (MW cut-off of 500 Da) and freeze dried.

Reacetylation of high DP chitooligosaccharides

The reacetylation reaction was adaptated from the method proposed by Hirano and Yamaguchi (1976). Briefly, 500 mg of chitooligosaccharides was solubilized in 40 ml of 2% acetic acid and diluted with 8 ml of methanol. Reacetylation was performed by the drop by drop addition of various quantities of acetic anhydride under fast stirring at room temperature. The solution was further stirred for 2 h, neutralized with 1 *M* NaOH, dialysed against deionized water using a SPECTRAPOR membrane (MW cut-off of 500 Da) in order to eliminate the salts produced during reacetylation and freeze dried.

Mass spectrometry analysis of chitooligosaccharide mixtures

Approximately 0.5 µl of the sample solution was mixed on the spectrometer target with 2 µl of a solution of 2,5-dihydroxybenzoic acid as matrix (15 mg ml^{-1}) prepared in 30% aqueous ethanol. Mass spectra were recorded on a Bruker Ultraflex mass spectrometer (Bruker Daltonik, Bremen, Germany) in the positive ion mode using a nitrogen laser (337 nm, 3 ns pulse width, 3 Hz). All spectra were measured in the reflector mode using external calibration.

Cell culture

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}) and 0.5 µg ml $^{-1}$ of NAA and 0.05 µg ml^{-1} of kinetin, pH 5.7. Cultures were maintained under a 16/8-h light/dark photoperiod, at 25°C, on a rotary shaker at 100 r.p.m.. Cells were diluted 10-fold in fresh medium every 7 days.

Bioassays for elicitor activity

PAL activity

Chitooligosaccharides to be tested were dissolved in 250 µl of distilled water, filtered through a 0.22-µm membrane filter (Millipore, Bedford, MA) and aseptically added to 10 ml of 3-day-old suspension-cultured cells and incubated 24 h at 25°C under mild agitation. Five millilitre of the reaction mixture was centrifuged for 5 min at 100 *g* and 4°C to collect the cells. Cells were homogenized at 4°C in 1 ml of 0.1 *M* borate buffer (pH 8.8) containing 2 m*M* mercaptoethanol. The homogenate was centrifuged at 4000 *g* for 10 min at 4°C. PAL (EC 4.3.1.5) activity was determined in 0.125 ml of supernatant in the presence of 1.37 ml of 0.1 *M* borate buffer (pH 8.8) supplemented with 60 m*M* L-phenylalanine as described by Beaudoin-Eagan and Thorpe (1985). Protein concentration of the extracts was determined by the Bradford protein assay (Bio-Rad Laboratories Gmbh, Munchen, Germany).

Cell viability

Cell viability was determined using 2,3,5-triphenyltetrazolium chloride (TTC) viability assay (Dixon 1985). Five millilitre of the cell suspension was centrifuged for 5 min at 100 *g* and 4°C to collect the cells. Cells were incubated with 2 ml of 0.05 *M* KH_2PO_4 at pH 8.0 containing 0.6% TTC for 15 min in the dark at 20°C. This mixture was centrifuged at 4000 *g* for 1 min, and the water-insoluble red formazan accumulated by living cells was extracted by heating at 60°C for 15 min with 3 ml of 95% (v/v) ethanol. The absorbance of ethanol solution was measured at 485 nm.

H_2O_2 measurement

Chitooligosaccharides to be tested were dissolved in 50 µl of distilled water, filtered through a 0.22-µm membrane filter (Millipore) and added to 5 ml of 3-day-old suspension-cultured cells and incubated at 25°C with shaking. Aliquots of 100 µl were removed every 4 min during 30 min, quick spin centrifuged and the H_2O_2 concentration was measured in the supernatant using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Paisley, UK) according to the supplier's instructions. Each experiment was performed at least three times. The coefficient of variation of H_2O_2 concentrations was generally comprised between 6 and 10% and was not indicated on data point for the sake of clarity of the figures.

Results

Preparation of chitooligosaccharides of different DP and DA

As the degree of polymerization of chitooligosaccharides is a determining structural factor for their bioactivity (Hahn 1996), two sets of these oligosaccharides with defined DPs were prepared by different ways. Chitooligomers with low DPs were prepared by acid hydrolysis of chitosan and isolated in methanol solutions. MALDI-TOF MS analysis confirmed the presence of fully deacetylated chitooligosaccharides and *N*-acetylated oligomers carrying only one acetyl residue with DPs mainly up to 6 (Fig. 1A). Peaks corresponding to hepta- and octa-oligomers were detected at much lower intensities. These results were confirmed by silica TLC analysis (Cabrera and Van Cutsem 2005).

Chitooligosaccharides with higher DPs were prepared by enzymatic hydrolysis in the presence of Pectinex Ultra Spl and further isolated by selective precipitation in 90% methanol. The MALDI-TOF MS analysis of the chitooligosaccharides prepared is presented in Fig. 1B. In this unambiguous mass spectrum, fully deacetylated chitooligomers with a DP between 5 and 9 were detected as sodium and/or potassium adduct ions. Peaks corresponding to fragments carrying one and/or three acetyl residues were detected in much lower quantities. We did not determine the amount of each oligomer product, because no linear correlation between the relative ion intensities in the spectrum and their absolute amounts has yet been established. However, it is widely accepted that the relative ion intensity corresponding to one oligomer reflects the relative quantity of this oligomer in the mixture (Akiyama et al. 1995, Li et al. 2004). As reported previously, selective precipitation of hydrolysis products in the presence of 90% methanol reduced the proportion of low DP oligomers (Cabrera and Van Cutsem 2005).

In order to obtain chitooligosaccharides with a similar DP distribution but with an increasing degree of acetylation, the high DP chitooligosaccharides were submitted to reacetylation using different proportions of acetic anhydride in the presence of methanol as scavenger groups in order to minimize *O*-acetylation, which decreases the efficiency and reproducibility of the reacetylation process (Hirano and Yamaguchi 1976). Reacetylated chitooligosaccharides detected by MALDI-TOF MS analysis as a function of the molar ratio of added acetic anhydride are shown in Table 1 and compared to the original chitooligosaccharides. An estimated DA of each prepared mixture was assigned as a

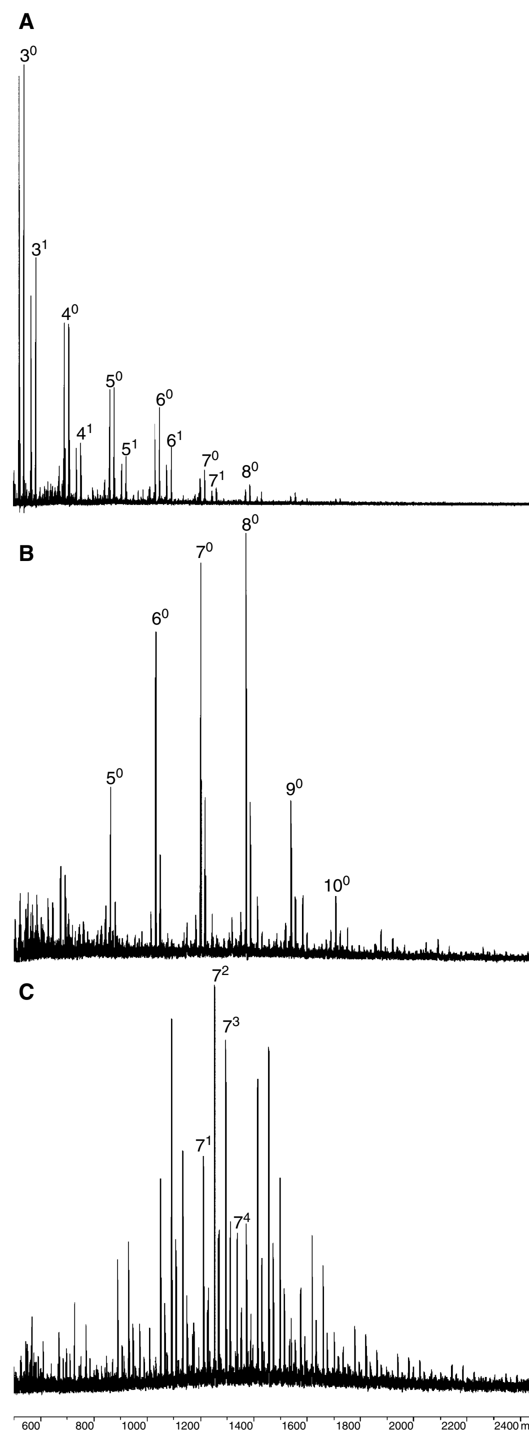

Fig. 1. MALDI-TOF MS analysis of the chitooligosaccharides. (A) Chitooligomers of low DPs obtained by acid hydrolysis, (B) chitooligomers of high DPs obtained by enzymatic degradation of chitosan with Pectinex Ultra SPL, (C) reacetylated chitooligomers of high DPs with DA approximately 40. Identified peaks are labelled as DP^{Ac}, where DP indicates degree of polymerization and Ac, the number of acetyl groups. DA, degree of acetylation; DP, degree of polymerization; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Table 1. MALDI-TOF MS determined composition of chitooligosaccharide mixtures obtained by reacetylation of high DP chitooligomers. Detected chitooligomers are shown in grey. DA, degree of acetylation; DP, degree of polymerization; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

			mmoles anhydride g^{-1} of chitooligosaccharides mixture (estimated degree of acetylation)				
DP	No. of acetylated residues	DA	0 (0)	0.6 (40)	2.1 (65)	3.2 (75)	6.8 (85)
	0	0	■				
	1	20		■			
5	2	40		■			
	3	60		■	■	■	■
	4	80			■	■	■
	5	100				■	■
	0	0	■				
	1	17					
	2	33		■			
6	3	50		■	■	■	
	4	67		■	■	■	■
	5	83			■	■	■
	6	100				■	■
	0	0	■				
	1	14		■			
	2	24		■			
7	3	43		■	■		
	4	57		■	■	■	
	5	71			■	■	■
	6	86			■	■	■
	7	100					■
	0	0	■				
	1	12					
	2	25		■			
	3	38		■			
8	4	50			■	■	
	5	63			■	■	■
	6	75			■	■	■
	7	88			■	■	■
	8	100					■
	0	0	■				
	1	11					
	2	22		■			
	3	33		■			
9	4	44		■			
	5	56		■	■	■	
	6	67			■	■	■
	7	78			■	■	■
	8	89				■	■
	9	100					■

function of the structure of the chitooligosaccharides detected in each spectrum. Different chitooligosaccharide sets with increasing DA were obtained depending on the amount of acetic anhydride added in the reacetylation reaction. The resulting reacetylated chitooligosaccharides had a wide distribution of acetylated residues. In other words, chitooligosaccharides with similar DP but with a different DA and presumably in different proportions were detected in the end mixture. For example, in the MALDI-TOF spectrum of the mixture with estimated DA of 40%, peaks of *N*-acetylated heptamers carrying between one and four *N*-acetyl residues were detected (Fig. 1C). This peculiar pattern of acetylation probably reflects the complexity of the reacetylation reaction. On the basis of the spectroscopic data, we also concluded that the reacetylation reaction

did not alter the degree of polymerization of the source chitooligosaccharides. We were thus able to prepare chitooligomers with different patterns of acetylation and equal degrees of polymerization. These now characterized chitooligosaccharides were used to study the influence of the DA on their biological activity as elicitors of PAL activation and H_2O_2 accumulation in the model plant *A. thaliana*.

Chitooligosaccharides induce PAL activity

PAL is a key enzyme in the phenylpropanoid pathway producing precursors of secondary metabolites, including lignin, flavonoid pigments and phytoalexins, some of which play key roles in a range of plant–pathogen interactions (Morrison and Buxton 1993). As previous experiments indicated that PAL activity in elicitor-treated Arabidopsis suspension-cultured cells peaked after at least 24 h of elicitation (Thonar personal communication), we measured the effect of fully deacetylated chitooligosaccharides (chitosan oligomers) on PAL activity after 24 h. Two chitosan oligomer sets with different DPs were added to 3-day-old suspension-cultured cells of Arabidopsis at a final concentration of 20 μg ml^{-1} and incubated for 24 h at 25°C under agitation. Only chitosan oligomers with higher DP (DP 5–9) induced PAL activity compared with the control and low DP (DP 3–6)-treated cells (Fig. 2A).

When the *A. thaliana* cell suspensions were treated with partially reacetylated chitosan oligosaccharides (20 μg ml^{-1}) of high DP (DP 5–9), the frequently observed DA dependency (Yamaguchi et al. 2000, Shibuya and Minami 2001) of the biological response to chitooligosaccharides was not observed in our experimental system. The PAL activity response was very similar to that observed in the presence of deacetylated chitosan oligomers and did not vary significantly when the DA was increased to 85% (Fig. 2B).

A dose–response curve for PAL activation by fully deacetylated chitooligomers of high DP (DP 5–9) with concentrations ranging from 5 to 500 μg ml^{-1} was established (Fig. 2C). PAL activation in suspension-cultured cells of Arabidopsis was strongly dependent on the amounts of elicitor added. Half-maximum enzyme activation was observed at a chitooligosaccharide concentration of about 20 μg ml^{-1}. PAL activation reached a plateau in Arabidopsis cells treated with elicitor concentrations ranging from 100 to 150 μg ml^{-1}. At higher concentrations of chitosan oligomers ($\geq$150 μg ml^{-1}), PAL activity and protein content of the borate extracts declined. PAL activation in the presence of chitosan oligosaccharides at a concentration between 100 and 150 μg ml^{-1} coincided with a browning of the elicited cells (not shown), a cellular response already reported in other plant systems and resulting from the accumulation of phenolic compounds (Hahlbrock and Scheel 1989).

Chitooligosaccharides induce H_2O_2 accumulation

A rapid accumulation of active oxygen species, such as H_2O_2, is a signal of a hypersensitive response in plants. This response, known as the oxidative burst, has been implicated in plant disease resistance (Apel and Hirt 2004). The generation of H_2O_2 in Arabidopsis suspension-cultured cells treated with increasing doses of deacetylated chitooligosaccharides with high DP (DP 5–9) up to a final concentration of 500 μg ml^{-1} was evaluated (Fig. 3A). The cells did not significantly accumulate H_2O_2 in response to elicitor concentrations lower than 300 μg ml^{-1}. Above that concentration, elicitor-induced H_2O_2 accumulation occurred within 4–8 min after treatment and within 10 min leveled off at an intermediate value. Higher elicitor concentrations induced H_2O_2 synthesis at an almost similar rate. The maximum H_2O_2 level reached was equivalent, but 400 and 500 μg ml^{-1} concentration of oligosaccharides allowed a sustained H_2O_2 accumulation for up to 30 min.

In a next step, two chitosan oligomer sets with different DPs were added to 3-day-old suspension-cultured cells of Arabidopsis at a final concentration of 300 μg ml^{-1} and the kinetics of H_2O_2 accumulation were recorded (Fig. 3B). Chitosan oligomers with high DPs (DP 5–9) once again induced H_2O_2 accumulation within 4 min after treatment. Similar to the PAL response, Arabidopsis cells treated with low DP (DP 3–6) chitosan oligomers and control cultures did not clearly enhance their H_2O_2 production.

The effect of the degree of acetylation of the chitooligosaccharide on H_2O_2 accumulation in Arabidopsis suspension-cultured cell medium was further evaluated (Fig. 3C). The lowest acetylated chitooligosaccharides exhibited the highest H_2O_2 production-inducing activity, although the DA = 40 chitooligomers were slightly less efficient. Chitooligosaccharides with a higher DA (DA > 40) did clearly not promote any H_2O_2 accumulation. These data suggest that the progressive acetylation of the chitosan oligosaccharides results in a parallel loss of their biological activity as elicitors of H_2O_2 synthesis in Arabidopsis.

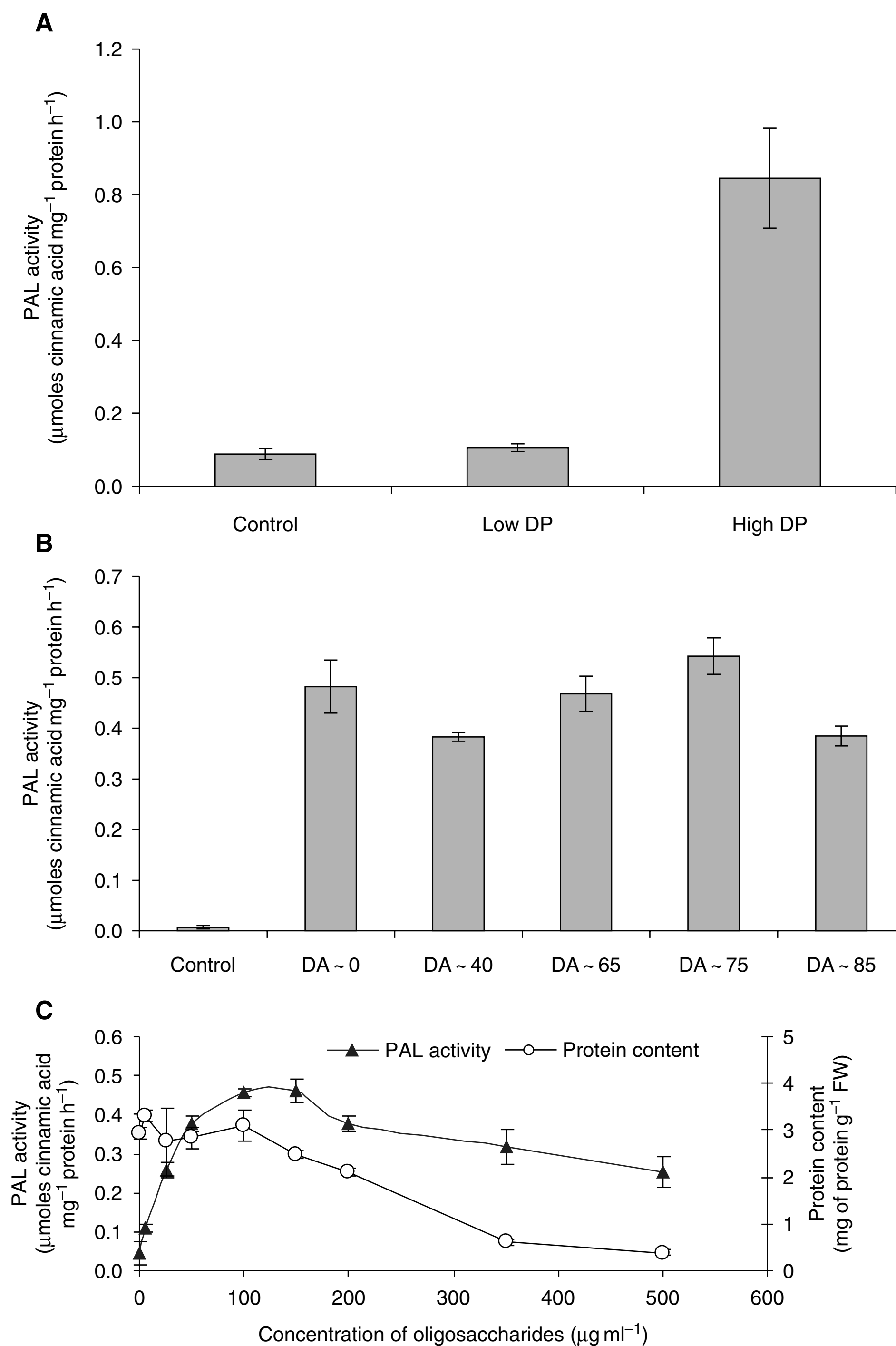

Fig. 2. Induction of PAL activity in Arabidopsis cell suspensions. Influence of the degree of polymerization (DP) of deacetylated (DA approximately 0) short and long chitooligosaccharides (20 μg ml^{-1}; 24 h after application) on their activity (A); influence of the degree of acetylation (DA approximately 0% up to DA approximately 85%) of high DP chitooligosaccharides at 20 μg ml^{-1} on their activity (B); dose–response curve of PAL activity and protein content in Arabidopsis cell suspensions 24 h after application of a chitooligosaccharide preparation of high DP and DA approximately 0% (C). Data are mean ± SD of triplicate samples from one representative of two independent experiments. PAL, phenylalanine ammonia-lyase.

Effect of chitooligosaccharides on cell viability

When Arabidopsis cell suspensions were treated for 24 h with high concentrations (≥200 μg ml^{-1}) of fully deacetylated chitooligomers of high DP (DP 5–9), elicited cells became colourless. This response coincided with a drastic decline in protein content of the borate extracts (Fig. 2C), an increase of H_2O_2 accumulation in the culture medium and with cell death as revealed by the 2,3,5-triphenyltetrazolium chloride viability assay (Fig. 4). These data clearly demonstrate that deacetylated chitooligosaccharide concentrations up to 100 μg ml^{-1} induce PAL in Arabidopsis cell suspensions without affecting cell viability. Above that

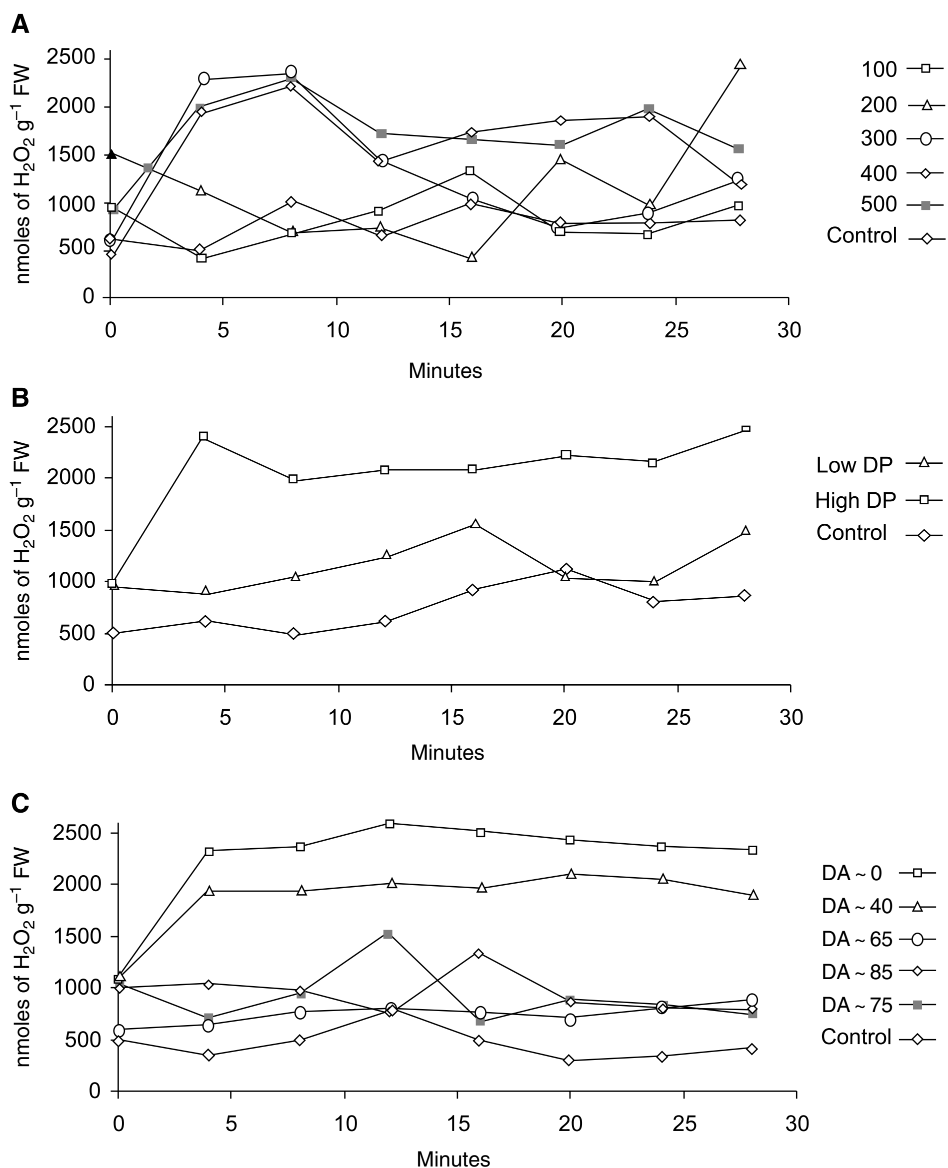

Fig. 3. Time–response curves of H_2O_2 generation in Arabidopsis cell suspension cultures by different concentrations (μg ml^{-1}) of chitooligosaccharides of high DP and DA approximately 0% (A); by chitooligosaccharides of different degrees of polymerization at 300 μg ml^{-1} (B) and by chitooligosaccharides of high DP and different degrees of acetylation (between DA approximately 0% and DA approximately 85%) at 300 μg ml^{-1} (C). All data shown in Fig. 3 are from one representative experiment of at least three independent replicates. DA, degree of acetylation; DP, degree of polymerization.

concentration, deacetylated chitooligomers of high DP either become toxic or promote programmed cell death (PCD) and loss of membrane permeability. Deacetylated chitooligomers of low DP (DP 3–6) did not affect Arabidopsis cell viability even at higher concentration (500 μg ml^{-1}) (data not shown).

A different behaviour was observed when largely acetylated chitooligomers (DA approximately 65) were evaluated (Fig. 4). First, PAL activation in Arabidopsis cell suspensions reached a maximum after 24 h in the presence of 350 μg ml^{-1} of chitooligomers (DA approximately 65). Half-maximum enzyme activation was observed at a concentration of approximately 150 μg ml^{-1}. Second, concentrations of these oligosaccharides up to 350 μg ml^{-1} did not induce H_2O_2 accumulation in the culture medium and did not reduce cell viability. At higher concentrations (>350 μg ml^{-1}), cell viability and PAL activity decreased, and a relatively small H_2O_2 accumulation was observed.

The differences in H_2O_2 accumulation in the culture medium of Arabidopsis cells after 24 h of elicitation with deacetylated (DA approximately 0) and partially acetylated (DA approximately 65) chitooligomers are consistent with our previous observation that the progressive acetylation of chitosan oligosaccharides results in a parallel loss of biological activity as

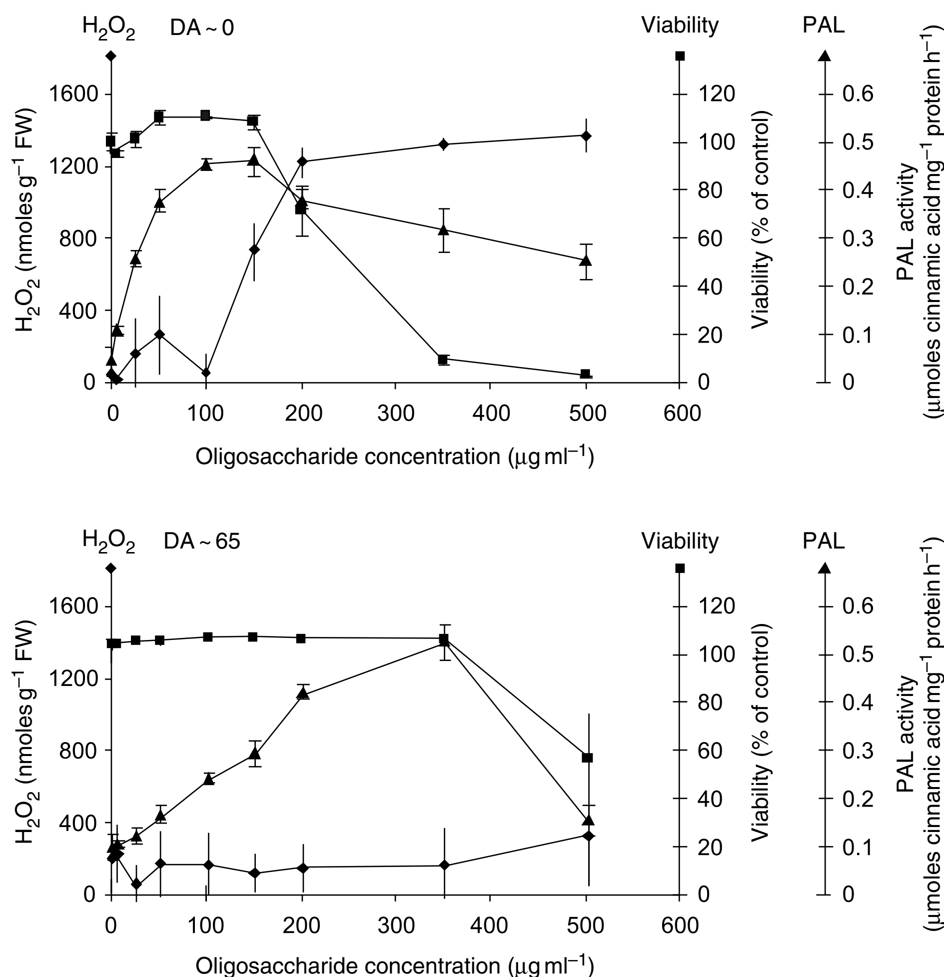

Fig. 4. Cell viability, H_2O_2 accumulation and PAL activity in Arabidopsis cell suspension 24 h after treatment with increasing concentrations of deacetylated chitooligomers (DA approximately 0%) and partially acetylated chitooligomers (DA approximately 65%). DA, degree of acetylation; PAL, phenylalanine ammonia-lyase.

elicitor of H_2O_2 accumulation when cells are shortly exposed to the elicitor. To further investigate whether cell death induced by deacetylated chitooligosaccharides was related to their ability to enhance H_2O_2 accumulation, we treated Arabidopsis cell suspensions with increasing concentrations of H_2O_2 and measured cell viability (data not shown). After 24-h treatment, exogenously added H_2O_2 did not affect Arabidopsis cell viability. This result suggests that H_2O_2 accumulation in the culture medium of elicited Arabidopsis cells is not directly involved in triggering cell death in the presence of deacetylated chitooligosaccharides.

Discussion

In this study, we obtained two sets of chitooligosaccharides of defined DP and several chitooligosaccharides with increasing DA characterized by MALDI-TOF MS. We examined the effect of the DP, DA and concentration of those chitooligosaccharides on PAL activation and H_2O_2 accumulation in *A. thaliana* suspension-cultured cells. These two widely used markers of defence responses have already been shown to be efficient markers of elicitation response in other plant systems treated with chitin oligomers (Khan et al. 2003).

Except for the DP requirements reported by others (Shibuya and Minami 2001), the defence responses of our Arabidopsis cell suspension were different in some aspects from those observed in other elicitation plant models treated with purified or polymeric chitosan or chitin (Yamaguchi et al. 2000). The concentration of high DP chitooligomers used to induce PAL activation and H_2O_2 accumulation was higher than that commonly reported in rice cells or whole plants (Nishizawa et al. 1999, Ning et al. 2004). Deacetylated (chitosan) high DP oligomers must be applied at a minimum concentration of 20 µg ml^{-1} (approximately 10^{-5} *M*) to induce

half-maximum PAL activation after 24 h, and PAL activity reached a maximum in the presence of 150 $\mu g\ ml^{-1}$ of deacetylated high DP oligomers. Upon reacetylation, the elicitor activity of the chitooligomers did not improve as frequently reported (Shibuya and Minami 2001). On the contrary, maximum PAL activation in the presence of chitooligomers with a DA approximately 65 was observed at a concentration of approximately 300 $\mu g\ ml^{-1}$. Increasing the number of acetyl residues on the chitooligosaccharide elicitor resulted in a shift of the dose–response curve towards higher concentrations. In our Arabidopsis cell suspension, chitosan oligomers appeared thus to be more efficient in triggering PAL activity compared with reacetylated oligomers. This result per se is interesting, as all plant systems do not necessarily respond to chitosan oligomers.

The lower concentrations of chitooligomers able to induce PAL were ineffective in triggering H_2O_2 accumulation whatever the duration of the incubation. When deacetylated chitooligomers were applied at concentrations higher than 300 $\mu g\ ml^{-1}$, Arabidopsis cells started to produce H_2O_2 and PAL activity progressively declined (50% inhibition at 500 $\mu g\ ml^{-1}$). H_2O_2 accumulation was inversely proportional to the viability of the elicited cells (Fig. 4). The slow reduction of PAL activity corresponds probably to a loss of PAL enzymes in the supernatant by cell leakage due to membrane damage in the presence of high, maybe toxic, elicitor concentrations. This is consistent with a decrease of the protein content of the elicited cells, also compatible with the persistence of a significant PAL activity of dead cells killed by the 500 $\mu g\ ml^{-1}$ of oligochitosan treatment.

Reacetylation of the chitooligomers interestingly resulted in an inhibition of H_2O_2 accumulation for both short and long exposures to the acetylated elicitor. Above a DA of 65%, elicited cells did not accumulate H_2O_2 and did not die, even in the presence of high elicitor concentrations. Only very high concentrations ($>$500 $\mu g\ ml^{-1}$) were able to reduce both PAL activity (80% inhibition) and cell viability (50% inhibition), without significantly increasing H_2O_2 concentration after 24 h. From these observations, it was tempting to attribute cell death to H_2O_2 accumulation in the culture medium of cells treated with deacetylated chitooligosaccharides. However, exogenously added H_2O_2 did not induce any significant drop of cell viability. Even if we do not know exactly the H_2O_2 concentration perceived by the cells in that experiment, we could show that H_2O_2 concentrations five times higher than those produced by the cells were unable to kill these cells. This clearly suggests that H_2O_2 accumulation in the culture medium of Arabidopsis cells is not directly responsible for cell death. In this new context of elicitation, the presence of acetyl residues on the chitooligosaccharide elicitor allowed PAL activation but prevented cell death. On the other hand, the removal of the acetyl groups resulted in a more complex and concentration-dependent elicitation response: the cell response switched from H_2O_2-independent PAL activation at low elicitor concentrations ($<$150 $\mu g\ ml^{-1}$) to H_2O_2 accumulation and H_2O_2- and PAL-independent cell death at high elicitor concentrations ($>$150 $\mu g\ ml^{-1}$). Whether chitosan oligomers killed cells by activating an H_2O_2-independent PCD or simply by necrosis is still unknown in our system. Unlike *N*-acetyl-chitooligosaccharides for which a receptor has been identified (Okada et al. 2002), the way chitosan oligomers are perceived by plant cells is still unknown and often considered as a non-specific interaction of a polycation with negatively charged plasma membrane phospholipids that mimics and activates a common MAP kinase-dependent defence response (Shibuya and Minami 2001).

Similar complex elicitation responses were observed in suspension-cultured soybean cells treated with polymeric chitosan (Zuppini et al. 2003). Low concentrations of polymeric chitosan (50 $\mu g\ ml^{-1}$) induce the mobilization of cytosolic calcium, H_2O_2 accumulation in the culture medium, the activation of chalcone synthase and evoke a caspase-like-dependent PCD. High concentrations (200 $\mu g\ ml^{-1}$) of the same elicitor however were unable to induce cytosolic calcium mobilization and H_2O_2 accumulation, and cell death became caspase-like-independent. In rice, both elicitors (deacetylated and acetylated chitooligosaccharides) are able to induce H_2O_2 accumulation. In cell suspensions, polymeric chitosans trigger H_2O_2-dependent activation of chitinase, glucanase, PR1 and PAL (Lin et al. 2005). In rice whole plants, *N*-acetyl-oligosaccharides elicit the synthesis of H_2O_2, PCD and the activation of several defence genes (Ning et al. 2004).

Although the central role of H_2O_2, and ROS in general, in several physiological responses has been strongly documented for both biotic and abiotic stresses (Neill et al. 2002, Apel and Hirt 2004, Laloi et al. 2004, Mittler et al. 2004), its implication in PCD is still under debate. In some plant models, H_2O_2 induces PCD (Desikan et al. 1998, Houot et al. 2001, Ren et al. 2002, Gechev and Hille 2005) and in others it appears not to be required (Glazener et al. 1996, Dorey et al. 1999, Sasabe et al. 2000, Ichinose et al. 2001). Moreover, in H_2O_2-dependent PCD, PCD is only observed when the H_2O_2 concentration reaches a threshold value as a result of a complex sequence of events including H_2O_2 perception, suppression of H_2O_2 scavengers (de Pinto et al. 2002), activation of a

MAPK signalling cascade (Ren et al. 2002) and the co-ordinated regulation of at least 150 genes shown to be involved in controlling H_2O_2 homeostasis (Mittler et al. 2004). In our Arabidopsis elicitation model, PAL activation, H_2O_2 accumulation and cell death appeared to be three independent responses that resemble the response of tobacco cells treated with elicitin (Sasabe et al. 2000) in which apoptotic cell death, oxidative burst and PAL gene expression only share an early common calcium second messenger and then separate into three distinct signalling pathways.

The diversity of elicitation responses observed in different plant models in the presence of chitooligosaccharides probably reflects the complexity of the perception of these elicitors by plant cells. The extracellular matrix of cells grown in liquid medium is different from that found in tissues of whole plants in terms of porosity, individual polysaccharide composition and enzymatic activities. The presence of the polyanionic pectins in the cell wall probably affects differentially the diffusion of polycationic chitosans and *N*-acetyl-chitooligosaccharides towards the plasma membrane. Similarly, the availability of chitinases and other chitin hydrolytic or binding activities in the plant cell wall could drastically reduce the elicitor concentration effectively perceived by the protoplast. The level of expression of oligochitin receptors (Bradley Day et al. 2001, Okada et al. 2002), or of other oligochitin-binding proteins (Li and Claeson 2003, Peumans et al. 2003), could be developmentally regulated and also explain the discrepancies between different plant models (cell suspensions, seedlings and whole plants) and plant species. For example, Okada et al. (2002) showed that carrot, wheat, barley, rice and tobacco BY-2N cells responded differently to *N*-acetyl-chitooligosaccharides, although their plasma membranes all contained a high-affinity binding site for *N*-acetyl-chitooligosaccharides.

In conclusion, this report shows that the degree of acetylation of the chitooligosaccharide elicitors in Arabidopsis cell suspension is an important structural requirement controlling differentially the onset of two independent defence responses (PAL and/or H_2O_2 accumulation) in a concentration-dependent manner. High DA also protects elicited cells against an H_2O_2- and PAL-independent cell death.

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