CHITO-OLIGOSACCHARIDES INDUCE PAL ACTIVATION, CELL DEATH AND WATER PEROXIDE PRODUCTION IN ARABIDOPSIS CELL SUSPENSIONS

Juan Carlos Cabrera[a,b], Johan Messiaen[b], Pierre Cambier[b] and Pierre Van Cutsem[b]*

a Laboratorio de Oligosacarinas, Departamento de Fisiología y Bioquímica Vegetal, INCA, Cuba

b Unité de Recherche en Biologie Cellulaire Végétale, Facultés Universitaires Notre-Dame de la Paix, Belgium

Abstract

Chitosan oligomers are well known elicitors of biological responses in plants. Using the model plant *Arabidopsis thaliana*, we have investigated the structure-activity relationship of these elicitors. First, we prepared and characterized chitooligosaccharide mixtures with defined degrees of polymerization and degrees of acetylation. These oligosaccharides were prepared either by acid hydrolysis or enzymatically using a commercial pectinase and a selective precipitation of the products was characterized using MALDI-TOF mass spectrometry. The enzymatic method yielded shorter fragments with a higher proportion of fully deacetylated chitooligomers. On the other hand, acid hydrolysis of the starting chitosan resulted in fragments with degrees of polymerization up to sixteen and more monoacetylated residues than with the enzymatic procedure. Several oligosaccharides with increasing DA were prepared by reacetylation of well-characterized deacetylated oligomers. The effect of structural parameters and concentration of these chitooligosaccharides on defense activation in *A. thaliana* cell suspensions were studied. Depending on their DP and concentration, fully deacetylated chitooligosaccharides induced PAL activation, water peroxide synthesis and cell death in Arabidopsis 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

Since the biological activities of chitin and chitosan have often been determined using heterogeneous and/or uncharacterised 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 [1]. 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 [2]. The concentrations of oligosaccharides that are effective in plant bioassays also seem 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 [3].

In this study, we prepared two chitooligosaccharides sets of defined degrees of polymerization (DP) and several oligosaccharides with increasing degree of acetylation (DA) by reacetylation of well-characterised deacetylated oligomers. Chitin and chitosan oligosaccharides were characterized by MALDI-TOF MS and tested in suspension-cultured Arabidopsis cells for their ability to induce

phenylalanine ammonia-lyase (PAL) activity and H_2O_2 accumulation, two well-known markers of defence reactions in plants [4]. 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 *Arabidopsis 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.

Material and Methods

Preparation and characterization of chitooligosaccharides

Two set of chitooligosaccharides with different degree of polymerization were prepared as described by Cabrera et al. [5]. Briefly, for the preparation of **chitooligosaccharides with low DPs**, two grams of chitosan (Molecular weight (Mw) of 83 kDa and DA of 12%) were hydrolysed with 100 mL of concentrated HCl (37%) for 30 minutes 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 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 were prepared by enzymatic hydrolysis: Chitosan was dissolved in 0.175 M acetate buffer pH 5.5 to a final concentration of 10 g/L. This chitosan solution (90 mL) was mixed with 10 mL of commercially available Pectinex Ultra Spl (26 000 PG/mL, Novozymes A/S, Bagsvaerd, Denmark) and incubated at 37°C for 24 h. The reaction was stopped by boiling at 100°C during 15 minutes. The chitooligosaccharides with higher DPs were isolated from the chitosan hydrolyzate by selective precipitation in 90% (v/v) methanol.

Partially acetylated chitooligosaccharides were prepared by reacetylation of the high DP set of oligosaccharides: The reaction was adaptated from the method proposed by Hirano and Yamaguchi [6]. Briefly, 500 mg of chitooligosaccharides were solubilised 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, dialyzed against demonized water using a SPECTRAPOR membrane (MW cut-off of 500 Da) to eliminate the salts produced during reacetylation and freeze dried.

Mass spectrometry analysis of chitooligosaccharide mixtures: 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) 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.

Biological assays

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

Induction of PAL activity: Chitooligosaccharides to be tested were dissolved in 250 µL distilled water, filtered through a 0.22 µm membrane filter (MILLIPORE) and aseptically added to 10 mL of 3 days-old suspension-cultured cells and incubated 24 hours at 25°C under mild agitation. Five mL

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 mM mercaptoethanol. The homogenate was centrifuged at 4000 rpm for 10 minutes at 4°C. PAL (EC 4.3.1.5) activity was determined in 0.125 ml supernatant in the presence of 1.37 ml 0.1 M borate buffer (pH 8.8) supplemented with 60 mM L-Phenylalanine as described by Beaudoin-Eagan and Thorpe [7]. Protein concentration of the extracts was determined by the Bradford protein assay (BIO-RAD).

Accumulation of H_2O_2: Chitooligosaccharides to be tested were dissolved in 50 µL distilled water, filtered through a 0.22 µm membrane filter (MILLIPORE) and added to 5 mL of 3 days-old suspension-cultured cells and incubated at 25°C with shaking. Aliquots of 100 µL were removed every 4 minutes during 30 minutes, 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) according to the supplier's instructions.

Changes in **cell viability** were evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) viability assay [8]. Five mL of the cell suspension were 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 rpm during 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 95% (v/v) ethanol. The absorbance of ethanol solution was measured at 485 nm.

Results and Discution

Preparation of chitooligosaccharides of different DP and DA

In this study, we obtained and characterized two sets of chitooligosaccharides of defined DP and several chitooligosaccharides with increasing DA. Chitooligomers with low DPs were prepared by acid hydrolysis of chitosan and isolated in methanol solutions. Furthermore, 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. 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 [6].

MALDI-TOF MS analysis of chitooligomers with low DPs confirmed the presence of fully deacetylated chitooligosaccharides and *N*-acetylated oligomers carrying only one acetyl residue with DPs mainly up to 6 (Data not shown).These results were confirmed by silica TLC analysis [5].

The results of MALDI-TOF MS analysis of the chitooligosaccharides of higher DPs are presented in Table 1. Fully deacetylated chitooligomers with a DP between 5 and 9 were detected. Peaks corresponding to fragments carrying one and/or three acetyl residues were detected but in much lower quantities. The composition of the resulting reacetylated chitooligosaccharides sets are presented in the consecutive columns. These fractions had a wide distribution of acetylated residues; thus, 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. This peculiar pattern of acetylation probably reflects the complexity of the reacetylation reaction. Based on 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 *Arabidopsis thaliana*.

Table 1. MALDI-TOF MS determined composition of chitooligosaccharide mixtures obtained by reacetylation of high DP chitooligomers. Mass (Da) of detected chitooligomers is shown.

DP	No. of acetylated residues	*DA*	mmoles anhydride/g of chitooligosaccharides mixture (estimated degree of acetylation)				
			0 (0)	0.6 (40)	2.1 (65)	3.2 (75)	6.8 (85)
5	0	*0*	846.3				
	1	*20*		888.4			
	2	*40*		930.4			
	3	*60*		972.5	972.4	972.4	972.4
	4	*80*			1014.4	1014.4	1014.5
	5	*100*				1030.4	1030.4
6	0	*0*	1007.4				
	1	*17*	1049.4	1049.5			
	2	*33*		1091.5			
	3	*50*		1133.5	1133.5	1133.5	
	4	*67*		1175.5	1175.5	1175.5	1175.6
	5	*83*			1217.5	1217.5	1217.6
	6	*100*				1259.5	1259.6
7	0	*0*	1168.5				
	1	*14*	1210.5	1210.6			
	2	*24*		1252.6			
	3	*43*		1294.6	1294.6		
	4	*57*		1336.6	1336.6	1336.6	
	5	*71*			1378.6	1378.6	1378.6
	6	*86*			1420.6	1420.6	1420.7
	7	*100*					1462.7
8	0	*0*	1329.5				
	1	*12*	1371.5	1371.6			
	2	*25*		1413.6			
	3	*38*		1455.6			
	4	*50*		1497.7	1497.6	1497.6	
	5	*63*		1539.7	1539.6	1539.6	1539.7
	6	*75*			1581.6	1581.6	1581.7
	7	*88*			1623.7	1623.7	1623.7
	8	*100*					1665.7
9	0	*0*	1490.6				
	1	*11*	1532.6				
	2	*22*		1574.7			
	3	*33*		1632.7			
	4	*44*		1658.7			
	5	*56*		1700.7	1701.7	1701.7	
	6	*67*			1742.7	1742.7	1742.8
	7	*78*			1784.7	1784.7	1784.8
	8	*89*				1826.7	1826.8
	9	*100*					1842.8

Influence of DP of chitooligosaccharide on their bioactivity

Two chitosan oligomer sets with different DPs were added to 3 days-old suspension-cultured cells of Arabidopsis at a final concentration of 20 µg/mL and incubated for 24 h at 25 °C under agitation. The activity of the enzyme PAL in treated cells is presented in Figure 1. 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 [9]. Only chitosan oligomers with higher DP (DP 5-9) induced PAL activity compared to the control and low DP (DP 3-6)-treated cells (Fig. 1).

In other experiment, both chitosan oligomers sets were added to 3 days-old suspension-cultured cells of Arabidopsis at a final concentration of 300 µg/mL and the H_2O_2 accumulation was quantified (Fig. 1). Chitosan oligomers with high DPs (DP 5-9) once again induced H_2O_2 accumulation within 10 minutes 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. A rapid accumulation of active oxygen species, like 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 [10].

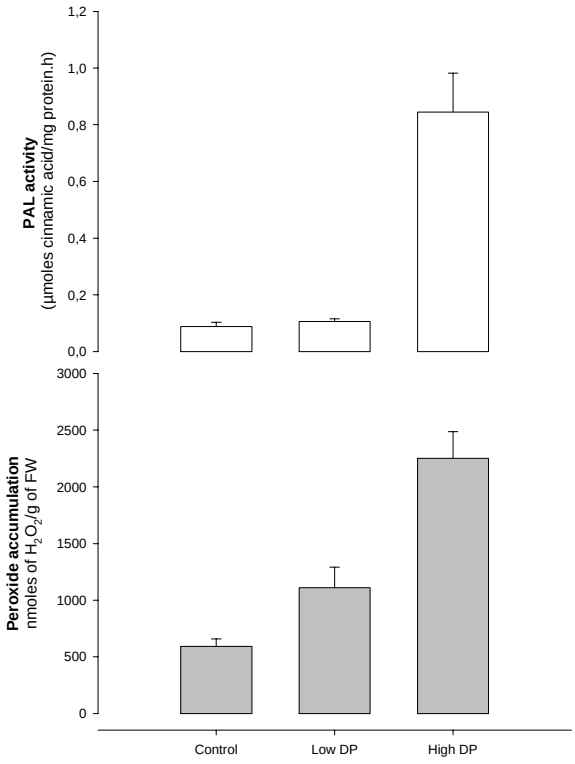


Figure 1 : Influence of the degree of polymerization of deacetylated (DA~0) short and long chitooligosaccharides on their bioactivity in Arabidopsis cell suspensions. Induction of PAL activity (chitooligosaccharides at 20 µg/mL; 24 h after application) and H_2O_2 generation (chitooligosaccharides at 300 µg/mL; 10 min after application)

Influence of DA of chitooligosaccharide on their bioactivity

When the *Arabidopsis thaliana* cell suspensions were treated with partially reacetylated chitosan oligosaccharides (20 µg/mL) of high DP (DP 5-9), the frequently observed DA dependency [1,3] of the biological response to chitooligosaccharides was not observed for the PAL activity detected. This 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. 2).
However, the effect of the degree of acetylation of the chitooligosaccharide on H_2O_2 accumulation in Arabidopsis suspension-cultured cells medium was different (Fig. 2). 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.

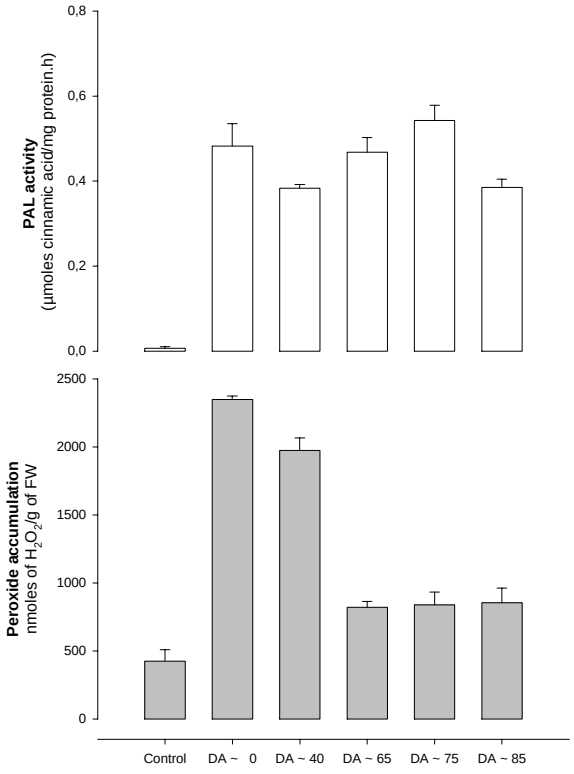
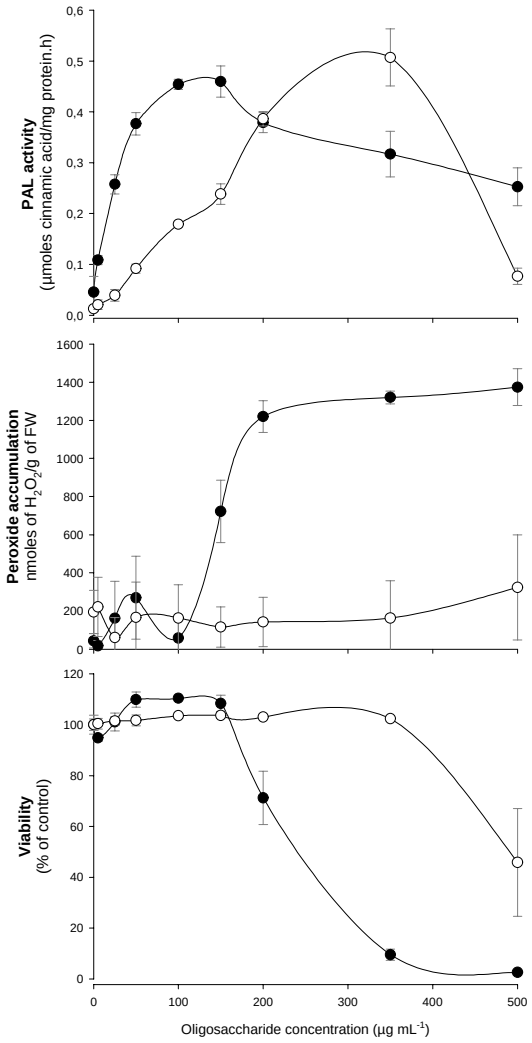


Figure 2 : Influence of the degree of acetylation of long chitooligosaccharides on their bioactivity in Arabidopsis cell suspensions. Induction of PAL activity (chitooligosaccharides at 20 µg/mL; 24 h after application) and H_2O_2 generation (chitooligosaccharides at 300 µg/mL; 10 min after application)

Influence of the concentration

A dose-response curve for fully deacetylated chitooligomers of high DP (DP 5-9) with concentrations ranging from 5 to 500 µg/mL was established (Fig. 3). 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. PAL activation reached a plateau with elicitor concentrations ranging between 100 and 150 µg/mL. When Arabidopsis cell suspensions were treated for 24 h with high concentrations (≥ 200 µg/mL) of these chitooligomers, elicited cells became colorless. This response coincided with a drastic decline in protein content of the borate extracts (Data not shown), 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. 3). These data clearly demonstrate that deacetylated chitooligosaccharide concentrations up to 100 µg/mL induce PAL in Arabidopsis cell suspensions without affecting cell viability. Above that concentration, deacetylated chitooligomers of high DP become either toxic or promote programmed cell death 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) (Data not show).

A different behavior was observed when acetylated chitooligomers (DA~65) were evaluated (Fig. 3). First, PAL activation in Arabidopsis cell suspensions reached a maximum after 24 h in the presence of 350 µg/mL of DA~65 chitooligomers. Half-maximum enzyme activation was observed at a concentration of ~150 µg/ml. Second, concentrations of these oligosaccharides up to 350 µg/mL did not induce H_2O_2 accumulation in the culture medium and did not reduce cell viability. At higher concentrations (> 350 µg/mL), 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 hours of elicitation with deacetylated (DA~0) and partially acetylated (DA~65) chitooligomers are consistent with our previous observation that the progressive acetylation of chitosan oligosaccharides results in a parallel loss of biological activity as elicitor of H_2O_2 accumulation when cells are shortly exposed to the elicitor. To further investigate if cell death induced by deacetylated chitooligosaccharides was related with 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 show). After a 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.

Figure 3 : PAL activity, H_2O_2 accumulation and cell viability in Arabidopsis cell suspension 24 hours after treatment with increasing concentrations of deacetylated chitooligomers (DA~0%) (-●-) and partially acetylated chitooligomers (DA~65%)(-○-).

Except for the DP requirements reported by others [1], 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 [3]. The concentration of high DP chitooligomers used to induce PAL activation and H_2O_2 accumulation were higher than commonly reported in rice cells or whole plants [11, 12]. Deacetylated (chitosan) high DP oligomers must be applied at a minimum concentration of 20 µg/mL (~10^{-5} M) to induce half maximum PAL activation after 24 h, and PAL activity reached a maximum in the presence of 150 µg/mL deacetylated high DP oligomers. Upon re-acetylation, the elicitor activity of the chitooligomers did not improve as frequently reported [1]. On the contrary, maximum PAL activation in the presence of chitooligomers with a DA~65 was observed at a concentration of ~300 µg/mL. 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 to re-acetylated oligomers. This result per se is interesting since 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 µg/mL, Arabidopsis cells started to produce H_2O_2 and PAL activity progressively declined (50% inhibition at 500 µg/ml). H_2O_2 accumulation was inversely proportional to the viability of the elicited cells (Fig. 3). The slow reduction of PAL

activity probably corresponded 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 cells killed by the 500 μg/ml oligochitosans treatment.
Re-acetylation 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 μg/mL) 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 5 times higher that 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 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 a H_2O_2- and PAL-independent cell death.

Acknowledgements

The authors wish to acknowledge the financial support from Région Wallonne, Belgium (Elite Internationale fellowship).

References

[1] Shibuya N, Minami E, Physiol Mol Plant P 59 (2001) 223-233.

[2] Cote F, Hahn MG, Plant Mol Biol 26 (1994) 1397-1411.

[3] Yamaguchi T, Ito Y, Shibuya, N, Trends Glycosci Glyc 12 (2000) 113–120.

[4] Somssich IE, Hahlbrock K, Trends Plant Sci 3 (1998) 86-90.

[5] Cabrera JC, Van Cutsem P, Biochem Eng J 25 (2005) 165-172.

[6] Hirano S, Yamaguchi R, Biopolymers 15 (1976) 1685-1691.

[7] Beaudoin-Eagan LD, Thorpe TA, Plant Physiol 78 (1985) 438–441.

[8] Dixon RA, In Plant cell culture: a practical approach. Dixon RA (ed.) Practical approach series, IRL Press, Oxford, (1985) 18-20

[9] Morrison TA, Buxton DR, Crop Sci 33 (1993) 1264-1268.

[10] Apel K, Hirt H, Annu Rev Plant Biol 55 (2004) 373-399.

[11] Nishizawa Y, Kawakami A, Hibi T, He DY, Shibuya N, Minami E, Plant Mol Biol 39 (1999) 907-914.

[12] Ning W, Chen F, Mao B, Li Q, Liu Z, Guo Z, He Z, Physiol Mol Plant P 64 (2004) 227-279.