

Xylanase treatment of plant cells induces glycosylation and fatty acylation of phytosterols

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Treatment of tobacco suspension cells (*Nicotiana tabacum* cv. KY 14) with a purified β -1,4-endoxylanase from *Trichoderma viride* [$1 \mu\text{g}$ enzyme (ml cells) $^{-1}$] caused a 13-fold increase in the levels of acylated sterol glycosides and elicited the synthesis of phytoalexins. A commercial preparation of xylanase from *Trichoderma viride* caused an identical shift in sterols. In contrast, a commercial xylanase from *Aureobasidium pullulans* had no effect on the levels of acylated sterol glycosides, but did elevate the levels of sterol esters. Treatment of the cells with Cu^{2+} or Ag^+ also evoked a severalfold increase in the levels of acylated sterol glycosides. Analysis of the various sterol lipid classes revealed that the large xylanase-induced increase in acylated sterol glycosides occurred at the expense of sterol esters, free sterols and sterol glycosides. Further analyses revealed that the most abundant phytosterol in each of the four classes of sterol lipids was β -sitosterol. Linoleic acid was the most abundant fatty acid in the sterol esters, and palmitic and linoleic acids were the most abundant fatty acids in the acylated sterol glycosides. Glucose was the only sugar moiety in the sterol glycoside and acylated sterol glycoside fractions. The results of the present study demonstrate that xylanase from *Trichoderma viride* induces a dramatic shift in the level of acylated sterol glycosides, indicating that endoxylanase was probably the active component in the cellulase enzyme preparations used in our previous study.

Key words – Elicitor, phytoalexin, phytosterol, signal transduction, sterol, xylanase.

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Introduction

We have previously reported that elicitation of tobacco suspension cells (*Nicotiana tabacum* cv. KY 14) with cellulase [Onozuka RS from *Trichoderma viride* (Karlhan Chemical Co., Santa Rosa, CA, USA)] caused a dramatic shift in the levels of phytosterols (Moreau and Preisig 1993). The culmination of this shift was a severalfold increase in the level of acylated sterol glycosides. The commercial cellulase preparation (commonly used to prepare plant protoplasts) used in the previous study is reported by the distributor to contain significant levels of xylanase activity. The same enzyme preparation was also found to contain proteins which cross-reacted with anti-

bodies prepared against a purified ethylene inducing xylanase (EIX) from *Trichoderma viride* (Fuchs et al. 1989). The present study was undertaken to investigate whether treatment of these cells with xylanases would cause the same type of sterol shift which we had previously observed with cellulase preparations.

Abbreviations – ASG, acylated sterol glycosides; cellulase RS, Onozuka cellulase RS (from *Trichoderma viride*); cellulase Tr, cellulase from *Trichoderma reesei*; EIX, ethylene inducing xylanase from *Trichoderma viride*; Pectolyase, Pectolyase Y23; SG, sterol glycosides; St, free sterols; StE, sterol esters; xylanase Ap, xylanase from *Aureobasidium pullulans*; xylanase Tv, xylanase from *Trichoderma viride*.

Materials and methods

Growth and treatment of cells

Cell suspension cultures of *Nicotiana tabacum* cv. Ky 14 were obtained from Dr Joseph Chappell, University of Kentucky, and were maintained on a modified Murishige-Skoog medium as previously described (Moreau and Preisig 1993). Cells were grown at 25°C and subcultured every 7 days. The treatments consisted of water (control) or various enzyme preparations.

Materials

Cellulase (Onozuka RS from *Trichoderma viride*) and Pectolyase (Y23 from *Aspergillus japonicus*) were obtained from Karlan Chemical Co., Santa Rosa, CA, USA. Cellulase Tr (from *Trichoderma reesei*) was obtained from Worthington Biochemical Co., Freehold, NJ, USA. Xylanases (from *Trichoderma viride* and *Aureobasidium pullulans*) and all other reagents were obtained from Sigma. Ethylene-inducing xylanase (EIX) was purified from xylan-induced *Trichoderma viride* culture filtrates as previously described (Dean and Anderson 1991).

Extraction and chromatography

Cells were separated from the culture filtrate by gentle vacuum filtration and collected on filter paper (Whatman No. 4, 20–25 µm pore size) in a Buchner funnel. After the fresh weight of the cells was quickly measured, chloroform and methanol were added, the cells were homogenized in a Polytron homogenizer (Brinkman Co., Westbury, NY, USA), and the lipids were extracted according to the method of Blich and Dyer (1959).

The culture filtrate (7–10 ml) was extracted two times with 10 ml diethyl ether. The solvents from both the cellular and media extracts were evaporated under a stream of nitrogen and the mass of each dried extract was measured. The residue (containing a mixture of lipids and phytoalexins) was redissolved in hexane/isopropanol (90:10, v/v), and the total lipid mixture was used directly for high performance liquid chromatography (HPLC) analyses. HPLC (with flame ionization detection) analyses of the sterol lipid classes were performed by using the following modifications of our previously described method (Moreau et al. 1990). Sterol esters and free sterols were analyzed on a Spherisorb 5 CN column (3 × 100 mm, Chrompack, Raritan, NJ, USA) with a flow rate of 0.5 ml min⁻¹ and a mobile phase consisting of 99.9% hexane and 0.1% isopropanol. Sterol glycosides and acylated sterol glycosides were analyzed on the same column, at the same flow rate, with a mobile phase consisting of 90% hexane and 10% isopropanol. Sesquiterpene phytoalexin analyses were performed with the same column, under isocratic conditions with hexane/isopropanol (97:3, v/v) as previously described (Moreau et al. 1992). Each experiment was performed at least three times with two independent replicates for each treatment.

Analysis of sterols, fatty acids, and sugars

Free sterols, and sterols derived from sterol esters and sterol glycosides by alkaline or enzymatic hydrolysis, respectively, were quantified by capillary gas-liquid chromatography with flame ionization detection (GLC-FID) on a 30 m × 0.25 mm id SPB-1 column from Supelco, Inc., Bellefonte, PA, USA. Sterols were identified based on their retention times relative to authentic standards with confirmation by gas-liquid chromatography-mass spectroscopy (GLC-MS) as previously described by Whitaker and Lusby (1989). Sterol esters were hydrolyzed by saponification with 1 M KOH in 80% ethanol (1 h reflux at 90°C). After they had cooled, sterols were extracted with hexane, the ethanolic phase was acidified with 6 M HCl, and free fatty acids were recovered by a second hexane extraction. The free fatty acids were then derivatized to methyl esters for GLC analysis with 14% boron trifluoride in methanol (20 min at 75°C sealed under N₂). Acylated sterol glycoside fatty acids were transesterified to methyl esters in 0.6 M methanolic KOH at room temperature (shaken for 3 h). An equal volume of water was added, the fatty-acid methyl esters (FAMES) were extracted with hexane, and the 'free' sterol glycosides were extracted with chloroform after acidification of the methanolic phase. FAMES derived from the sterol ester and acylated sterol glycoside fractions were quantified by capillary GLC-FID on a 15 m × 0.25 mm id SP-2330 column (Supelco, Inc.) as previously described (Whitaker 1992). For sterol analysis, sterol glycosides were hydrolyzed overnight with β-glucosidase (Sigma), followed by extraction with hexane/diethyl ether, 2:1 (v/v) as reported by Kesselmeier et al. (1985). For sugar analysis, sterol glycosides were hydrolyzed by refluxing for 2 h at 110°C with 2 M trifluoroacetic acid (TFA) in isopropanol/water, 3:2 (v/v). After the solutions had cooled and sterols extracted with hexane, the isopropanol/water phase containing the sugar moieties was evaporated under N₂. The sugars were derivatized to alditol acetates and analyzed by GC-MS with selective ion monitoring as described by Gross and Acosta (1991). Two prominent peaks were noted, one matching β-D-glucose. The other was tentatively identified as 1-deoxyglucose by GC-MS in the scanning mode.

Results and discussion

Treatment of tobacco suspension cells (10 ml) with 10 µg cellulase (Onozuka RS) for 4 h caused an 8-fold increase in the level of acylated sterol glycosides (ASG) (Tab. 1). In our previous paper (Moreau and Preisig 1993) we performed a time course study and noted a visible increase in ASG as early as 1 h and persisting up to 24 h. For the present study, we chose a 4-h incubation as a convenient intermediate time. Examination of the other sterol lipid classes revealed that cellulase RS also caused a small increase (24%) in the level of sterol esters and decreases in the levels of free sterols (a 45% decrease)

Tab. 1. Analyses of sterol lipids in tobacco suspension cells after various experimental treatments. Each sample flask contained 10 ml of cells. Cells were filtered and extracted after 4 h of treatment. Values are based on average molecular weights of StE=670, St=410, SG=570 and ASG=830. Values are the mean of duplicate samples from two separate experiments \pm SE.

Treatment	Sterol per culture flask (nmol)					
	StE	St	SG	ASG	Total	ASG/SG
Control	150.3 \pm 2.1	357.6 \pm 13.9	38.2 \pm 8.9	33.9 \pm 5.7	580.0 \pm 30.6	0.89
10 μ g cellulase RS	186.6 \pm 10.6	198.3 \pm 11.2	31.6 \pm 10.4	272.2 \pm 12.8	688.7 \pm 45.0	8.61
10 μ g xylanase EIX	129.1 \pm 23.4	158.0 \pm 6.1	17.9 \pm 1.9	449.3 \pm 3.4	754.3 \pm 34.8	25.10
10 μ g xylanase Tv	134.0 \pm 7.0	154.4 \pm 10.5	21.2 \pm 0.6	434.3 \pm 1.3	743.9 \pm 19.4	20.49
10 μ g xylanase Ap	273.5 \pm 17.0	327.6 \pm 9.4	28.0 \pm 2.4	33.6 \pm 3.5	662.7 \pm 32.3	1.20
10 μ g cellulase Tr	154.6 \pm 6.6	344.8 \pm 12.2	36.7 \pm 5.1	35.0 \pm 4.9	571.1 \pm 28.8	0.95
0.4 M mannitol	129.8 \pm 8.8	270.1 \pm 12.7	38.2 \pm 6.0	53.4 \pm 9.2	491.5 \pm 36.7	1.40
0.4 M mannitol + 1% cellulase RS	65.3 \pm 11.3	107.6 \pm 6.3	42.1 \pm 8.1	394.1 \pm 33.2	609.1 \pm 58.9	9.36
0.4 M mannitol + 0.1% pectolyase	138.9 \pm 11.3	283.1 \pm 7.5	40.8 \pm 3.6	72.9 \pm 2.9	535.7 \pm 25.3	1.79
0.4 M mannitol + 1% cellulase RS + 0.1% pectolyase	8.5 \pm 0.9	12.7 \pm 1.1	3.1 \pm 0.6	11.3 \pm 2.4	35.6 \pm 5.0	3.65
1 mM CuCl ₂	125.9 \pm 3.3	266.6 \pm 3.1	20.5 \pm 4.5	115.5 \pm 37.0	528.4 \pm 47.9	5.63
1 mM AgCl	65.7 \pm 6.4	149.7 \pm 6.2	8.4 \pm 1.1	250.4 \pm 37.3	474.2 \pm 51.0	2.98
10 mM CaCl ₂	136.6 \pm 11.5	354.7 \pm 4.4	28.5 \pm 1.8	31.7 \pm 3.0	551.5 \pm 20.7	1.11

and sterol glycosides (a 17% decrease). The ratio of ASG/SG was 0.89 in control cells and rose to 8.61 after the cellulase RS treatment. This compares with a 2-fold increase in ASG which was observed after 8 h in our previous study (Moreau and Preisig 1993) when we used a treatment which consisted of 10 times less cellulase (1 μ g per flask). Treatment of the cells with highly purified EIX [which exhibited only one band at 22 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); Dean and Anderson 1991] caused a 13-fold increase in the level of ASG and a decrease in the levels of each of the other three sterol lipids. The two commercially prepared xylanases (whose purity is not known) caused very different results. The first, from *Trichoderma viride*, caused a sterol shift which was almost identical to that observed with EIX. In contrast, the xylanase from *Aureobasidium pullulans* had essentially no effect on the level of ASG, but it did cause an 80% increase in the level of sterol esters and decreases in the levels of free sterols and sterol glycosides (9% and 27%, respectively). A second commercially prepared cellulase (from *Trichoderma reesei*), which has been reported to be devoid of EIX (as evidenced by the finding that it contains no proteins which cross-react with antibodies raised against EIX; Fuchs et al. 1989, Sharon et al. 1993), was tested and found to have essentially no effect on the levels of the four sterol lipid classes. Boiling each of the above ASG-inducing enzymes (cellulase RS, xylanase EIX and xylanase Tv) for 30 min eliminated their ability to cause a sterol shift (data not shown).

The commercial cellulases which were used in the present study are often used to prepare protoplasts from various plant tissues. A previous report (Kesselmeier et al. 1987) indicated that during the release of protoplasts from oat leaves, there was an increase in the levels of SG and ASG. Therefore, the next four treatments (Tab. 1)

were included to investigate the effects of conditions which have been reported for the successful preparation of protoplasts from tobacco suspension cells (Nagata et al. 1981) on the levels of the four sterol lipid classes. The simple addition of mannitol (0.4 M), which is considered isotonic for tobacco suspension cells (Nagata et al. 1981), caused a significant increase (57%) in the level of ASG, and a decrease in each of the other three sterol lipid classes. When a high concentration of cellulase (1%) was added to the mannitol, there was an additional 6-fold increase in the level of ASG (relative to the mannitol control), a 10% increase in the level of sterol glycosides, and substantial decreases in the levels of sterol esters and free sterols. When pectolyase (0.1%) was added to the mannitol, there was a 37% increase in the level of ASG (relative to the mannitol control), a slight increase in sterol glycosides and decreases in sterol esters and free sterols. In the final treatment, mannitol, cellulase and pectinase were combined, in concentrations used to prepare protoplasts, and a very large reduction in all of the sterol lipid classes was observed. With this treatment, unlike all of the others, it was noted that very few cells were trapped on the filter paper, indicating that most of the protoplasts had been released and passed through the filter paper. Because we only measured the lipids in the cells retained on the filter, the quantities of lipid in each of the sterol lipid classes were extremely low. However, the cellulase/pectolyase treatment caused the ASG/SG ratio to increase to a value of 3.61, which confirms the previous report (Kesselmeier et al. 1987) that treatment of vegetative tissue with cell-wall degrading enzymes during protoplast preparation can cause a dramatic shift in the composition of the sterol lipid classes.

Various cations have different effects on plant cells. Cu²⁺, a known stimulator of ethylene biosynthesis in tobacco leaves, elicits the production of phytoalexins in

Tab. 2. Analysis of free sterols, fatty acids and sugars in sterol lipid classes of control and xylanase-treated tobacco suspension cells. Ten ml of cells were treated for 4 h with water (Control) or 10 µg xylanase Tv (Xyl). NA, not applicable; tr, trace levels.

	St		StE		SG		ASG	
	Con	Xyl	Con	Xyl	Con	Xyl	Con	Xyl
nmol	357.6	154.4	150.3	134.0	38.2	21.2	33.9	434.3
Sterol (wt%)								
Cholesterol	1.0	1.3	1.3	1.5	0.3	0.3	1.2	0.7
Campesterol	19.2	19.9	15.5	15.1	15.2	16.9	14.7	16.8
Stigmasterol	13.1	16.7	2.2	3.5	4.3	7.6	4.2	7.9
Sitosterol	57.7	52.4	61.8	59.1	72.1	66.5	73.7	67.8
Isofucosterol	5.9	6.1	9.0	9.8	5.2	6.0	1.8	2.7
Others	3.1	3.6	10.2	11.0	2.9	2.7	4.4	4.1
Stig:Sito	0.23	0.32	0.04	0.06	0.06	0.11	0.06	0.12
Fatty acids (wt%)								
16:0	NA	NA	8.2	7.9	NA	NA	30.7	42.3
18:0	-	-	1.7	2.0	-	-	5.8	5.8
18:1	-	-	3.3	2.7	-	-	3.3	1.7
18:2	-	-	79.1	79.2	-	-	50.2	36.6
18:3	-	-	6.5	6.8	-	-	4.5	9.6
20:0	-	-	0.3	0.4	-	-	3.3	2.4
Other	-	-	0.9	1.0	-	-	2.2	1.6
Sugars (wt%)								
Glucose	NA	NA	NA	NA	tr	tr	67	86
Deoxyglu	-	-	-	-	-	-	33	14

peas and beans (Preisig et al. 1991). Although treatment with 1 mM CuCl₂ did not elicit the production of phytoalexins with these tobacco cells (data not shown), it did cause a 3-fold increase in the levels of ASG. In our previous study (Moreau and Preisig 1993), we found a nearly identical response when cells were treated with a lower concentration of CuCl₂ (0.5 mM). Treatment with 1 mM AgCl caused a 7-fold increase in the level of ASG. Ag⁺ is known to be toxic to plant tissues and has been shown to stimulate ethylene production (Mattoo et al. 1992). Ag⁺ has also been reported to be an inhibitor of ethylene action in some plant tissues and is able to enhance ethylene production in induced systems (Aharoni et al. 1979). It is not known whether the ability of Cu²⁺ and Ag⁺ to cause a sterol shift is related to their previously reported abilities to influence ethylene biosynthesis. Although Ca²⁺ has been shown to evoke many cellular responses, treatment with CaCl₂ (10 mM) did not cause any effect on the levels of sterol lipid classes.

The next series of experiments (Tab. 2) was conducted to examine the sterol, fatty acid and sugar composition of the four sterol lipid classes in control cells and in those treated with xylanase (10 µg of commercial xylanase from *T. viride* per 10 ml cells). Examination of the phytosterol composition of each of the four sterol classes in control and elicited cells revealed that in each case β-sitosterol was the most abundant phytosterol, followed by campesterol. The highest level of stigmasterol was found in the free sterol fraction, and the most significant elicitor-dependent effect was a small but consistent increase in the stigmasterol:sitosterol ratio in each of the four sterol lipid classes.

Examination of the two fatty-acid-containing sterol lipid classes revealed that linoleic acid (18:2) was the most abundant fatty acid in both. However, the ASG fraction contained considerably more palmitic acid than the sterol ester fraction. Although the fatty acids in the sterol ester fraction were unaffected by xylanase treatment, the ASG fraction showed an increase in the level of 16:0 and a decrease in the level of 18:2 upon xylanase treatment. The source of fatty acids for the increased net fatty acylation which occurred during the 13-fold increase in ASG is not known. However, we believe the fatty acids are probably derived from galactolipids and/or phospholipids (Moreau and Preisig 1993), a view supported by other studies of sterol conjugation in plant cells (Wojciechowski 1991).

The most abundant sugar in the ASG fractions in both elicited and control cells was found to be glucose. Although significant levels of deoxyglucose were found in each of the two treatments, we believe that this was an artifact generated by the particular method used for the strong acidic hydrolysis of sterol glycosides. Two procedures were used to determine whether deoxyglucose was an artifact of the hydrolysis method used in the present study (2 M TFA in isopropanol/water, 3:2 (v/v), for 2 h at 110°C). First, equal amounts of an SG sample from tomato were hydrolyzed under the relatively harsh TFA conditions and under the milder conditions of 2 M HCl in methanol for 2 h at 75°C. The strong TFA hydrolysis yielded from 15 to 30 mol % deoxyglucose, whereas HCl methanolysis gave ≤ 2 mol %. Second, tomato SG were hydrolyzed 6 h at 80°C in glacial acetic acid/0.15 M H₂SO₄, 9:1 (v/v), and the hydrolyzate passed through a

Tab. 3. Extracellular levels of phytoalexins measured 24 h after treatment with water (Control) or various enzyme preparations. Abbreviations are the same as described in Tab. 1. Values are the mean of duplicate samples from two separate experiments \pm SE.

Treatment	Phytuberol [μg (10 ml cells) $^{-1}$]	Debneyol [μg (10 ml cells) $^{-1}$]	Capsidiol [μg (10 ml cells) $^{-1}$]
Control	0	0	0
Cellulase RS			
(1 μg)	19.9 \pm 0.6	12.8 \pm 2.6	68.2 \pm 18.7
(10 μg)	15.9 \pm 2.3	28.9 \pm 0.1	178.7 \pm 9.9
Cellulase Tr			
(1 μg)	0	0	0
(10 μg)	0	0	0
Xylanase Tv			
(1 μg)	11.9 \pm 1.7	32.6 \pm 3.6	179.9 \pm 7.8
(10 μg)	40.3 \pm 4.7	14.9 \pm 3.3	81.7 \pm 10.6
Xylanase EIX			
(1 μg)	12.4 \pm 0.5	34.7 \pm 2.1	185.3 \pm 4.2
(10 μg)	47.3 \pm 3.9	14.1 \pm 1.1	77.3 \pm 6.7

Dowex 1-X8 acetate resin column, following the method of Morrison (1986). With this procedure the deoxyglucose peak was completely eliminated. We conclude from these results that the deoxyglucose obtained from the tobacco cell SG and ASG was an artifact of hydrolysis, and that glucose is the only hexose in these sterol conjugates. Glucose has usually been found to be the predominant hexose moiety in ASG and SG from plant tissues (Wojciechowski 1991).

In the final set of experiments we examined the levels of phytoalexins after treatment with each of the cellulases and xylanases (Tab. 3). Treatment with crude cellulase RS elicited high levels of debneyol and capsidiol, as previously reported (Moreau and Preisig 1993, Threlfall and Whitehead 1988, Vogeli and Chappell 1988). In contrast, treatment with the cellulase Tr, the commercial cellulase which is devoid of EIX (Fuchs et al. 1989), failed to elicit phytoalexins. Treatment with the highly purified EIX or commercial xylanase Tv also elicited very high levels of phytoalexins. Boiling each of the above phytoalexin-eliciting enzymes (cellulase RS, xylanase EIX and xylanase Tv) for 30 min eliminated their ability to elicit phytoalexins (data not shown). The results of this experiment indicate that EIX is an active elicitor of phytoalexins in these cells. These data also lead us to speculate that EIX is the active component in cellulase RS. This active component appears to be responsible for causing 1) a rapid glycosylation and fatty acylation of phytosterols and 2) elicitation of phytoalexins.

In summary, we have found that treatment of tobacco cells with a specific xylanase, certain cations (Cu^{2+} and Ag^+) and an osmoticum (mannitol) can cause a rapid shift in the proportions of the various sterol lipid classes. In addition to the previously mentioned increase in ASG and sterol glycosides reported to occur during the preparation of protoplasts from oat leaves (Kesselmeier et al. 1987), treatment of snapbean leaves with ozone has been shown to have a similar effect (Whitaker et al. 1990). It was previously reported that cellulase treatment elicited phytoalexins and inhibited the synthesis of new sterols, thus suggesting a shift of isoprene units from sterol biosynthesis to sesquiterpenoid phytoalexin biosynthesis (Chappell et al. 1989, Vogeli and Chappell 1988). Simi-

larly, Zook and Kuc (1991) found that when potato tubers were elicited with arachidonic acid there was a 10-fold increase in sesquiterpene cyclase activity and in untreated controls there was a 10-fold increase in squalene synthetase. The sterol shifts observed in the present study all occurred during a 4-h treatment, and because the level of total sterols remained quite constant during the various treatments (Tab. 1), we believe that the sterol shifts observed during the various treatments were due to a net glycosylation and fatty acylation of existing sterols, and probably did not involve de novo sterol synthesis. Although very little is known about how changes in the levels of sterol lipids may affect membranes, one study indicated that the addition of ASG (or to a lesser extent SG) to membranes caused measurable changes in thermal phase transition (Mudd and McManus 1980). A recent review has summarized some of the other environmental and chemical stimuli which can affect the proportions of the various sterol lipid classes (Wojciechowski 1991). The recent literature indicates that the sterol lipids may be the most abundant class of lipids in the plant plasma membrane (Moreau and Preisig 1993). Such rapid changes in the proportions of the various sterol lipid classes may change membrane permeability and have a profound influence on the activities and physical properties of enzymes (by either stimulating or inhibiting them) and other proteins in the plant plasma membrane.

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