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In tobacco BY-2 cells xyloglucan oligosaccharides alter the expression of genes involved in cell wall metabolism, signalling, stress responses, cell division and transcriptional control.

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Abstract

Xyloglucan oligosaccharides (XGOs) are breakdown products of xyloglucans, the most abundant hemicelluloses of the primary cell walls of non-Poalean species. Treatment of cell cultures or whole plants with XGOs results in accelerated cell elongation and cell division, changes in primary root growth, and a stimulation of defence responses. They may therefore act as signalling molecules regulating plant growth and development. Previous work suggests an interaction with auxins and effects on cell wall loosening, however their mode of action is not fully understood. The effect of an XGO extract from tamarind (*Tamarindus indica*) on global gene expression was therefore investigated in tobacco BY-2 cells using microarrays. Over 500 genes were differentially regulated with similar numbers and functional classes of genes up and down-regulated, indicating a complex interaction with the cellular machinery. Up-regulation of a putative xyloglucan endotransglycosylase/hydrolase-related (*XTH*) gene supports the mechanism of XGO action through cell wall loosening. Differential expression of defence-related genes supports a role for XGOs as elicitors. Changes in the expression of genes related to mitotic control and differentiation also support previous work showing that XGOs are mitotic inducers. XGOs also affected expression of several receptor-like kinase genes and transcription factors. Hence, XGOs have significant effects on expression of genes related to cell wall metabolism, signalling, stress responses, cell division and transcriptional control.

(216 words)

Key words

BY-2 cells, cell cycle, cell walls, microarray analysis, *Nicotiana tabacum*, xyloglucan oligosaccharides.

Introduction

The cellulose/hemicellulose network of the primary cell wall provides structural support as well as physically regulating wall expansion [10,19]. Xyloglucans are the most abundant hemicelluloses of the primary cell walls of non-Poalean species and may have a functional role in hydrogen bonding to, and tethering of, the cellulose microfibrils to each other [46].

Changes in xyloglucan structure have important effects on plant defences. For example, the Arabidopsis mutant, *mur*3, is compromised in xyloglucan galactosyltransferase activity [45], resulting in abnormal xyloglucan structure. This mutant has elevated levels of salicylic acid, exhibits constitutive activation of defence-related genes and is resistant to the pathogen *Hyaloperonospora parasitica* [74].

Xyloglucan oligosaccharides (XGO) are derived from breakdown of xyloglucans and can be defined as oligomers of 1,4-linked β-D-Glc*p* residues. Both chain length and the substitutions in the glucan backbone define different classes of XGO and their nomenclature is through combinations of F, X, G and L, each demarcating modifications of specific oligosaccharides [29, 61]. For example, the archetypal seed xyloglucan from *Tamarindus indica* L. comprises XXXG, XXLG, XLXG, and XLLG oligosaccharides in the molar ratio 1.4:3:1:5.4 [79]. *In vivo,* XGOs are generated by the action of xyloglucan endo-transglycosylase/hydrolase (XTH) [26] on xyloglucans, and are then modified by the action of α-fucosidase, α-xylosidase, β-galactosidase and β-glucosidase [28]. *XYLOGLUCAN ENDO-TRANSGLYCOSYLASE/HYDROLASE* (*XTH*) genes encode proteins with two different catalytic activities. These have very different effects on xyloglucan structure: xyloglucan endo-transglycosylase (XET) (xyloglucan:xyloglucosyl transferase; EC 2.4.1.207) catalyzes non-hydrolytic cleavage and ligation of xyloglucan chains, while xyloglucan endo-hydrolase (XEH) activity (xyloglucan-specific endo-*β*-1,4-glucanase; EC 3.2.151) results in xyloglucan chain shortening. Although *XTH* has also been referred to as *XYLOGLUCAN ENDO-*

TRANSGLUCOSYLASE/HYDROLASE [61], this is not strictly correct as the activity involves the transfer of a whole glycan chain and not just one glucosyl residue [26]. These enzymes are encoded by complex gene families consisting of differentially regulated members that are likely to be important in fine-tuning the *in vivo* composition of the XGOs [35, 26]. *In vitro*, specific oligosaccharides can be produced from xyloglucan by partial digestion with cellulase [β (1-4)–D-glucanase].

A number of different types of oligosaccharides can be elicitors that activate plant defence responses [53]. They are recognized by different cell surface receptors, resulting in a stimulation of direct metabolic pathways and an increase in systemic acquired resistance (SAR) [3, 66]. These include fungal- derived oligosaccharides such as those from β glucan, chitin and chitosan, but also oligogalacturonides derived from pectic cell wall fragments. Less is known about the effects of XGOs, although there are reports of them affecting the hypersensitive response induced by tobacco necrosis virus [67, 69]. XGOs also promoted phytoalexin accumulation in soybean cotyledons [60] and increased ethylene production in tomato fruit, perhaps as part of a hypersensitive response to biotic stress [16]. They have also been commercially patented as plant defence boosters [42]. However, at least in *Arabidopsis* cultured cells, their bioactivity in eliciting early defence responses (medium alkalization, ion effluxes and peroxide accumulation) appears to be less than that of other oligosaccharides derived from plant cell walls (oligogalacturonides) and fungal cell walls (chitosan oligogalacturonides) (Cabrera lab, unpublished results). Oligosaccharins also affect responses to abiotic stress. In winter wheat the oligosaccharin XGAG accumulates during cold acclimation and exogenous treatments with this oligosaccharin increased freezing tolerance [81, 82].

Bioactive oligosaccharides, termed oligosaccharins, also have effects on growth and development that are not obviously related to disease resistance. XGOs play a role in the regulation of plant growth [73, 80], an effect that depended on the presence of a terminal L-fucose [52]. However, XGOs derived from tamarind (*Tamarindus indica* L.) seeds that do not have a terminal L-fucose also have positive effects on plant growth [1, 2] causing an increase in primary root elongation in *Arabidopsis thaliana* but a deceleration of the rate of lateral root formation [31]. Part of these growth effects may be attributed to a shorter cell cycle: treatment of tobacco BY-2 cells with tamarind seed XGOs resulted in a shortening of G1 whilst mitotic cell size remained constant [31]. Indeed, XGOs could well be novel, naturally occurring signaling molecules [30].

The mode of action of XGOs in modulating plant growth is poorly understood. At low concentrations (10^{-8}-10^{-9} M), XGOs may antagonize auxin signalling [50] and inhibit pea stem segment growth, whereas at higher concentrations (10^{-4} M) they had cell wall loosening effects similar to those elicited by auxin [50]. In azuki bean (*Vigna angularis*) epicotyls, cell wall loosening was associated with a modulation of xyloglucan endotransglycosylase/hydrolase (XTH) towards its xyloglucan degrading activity [39], increasing cell wall extensibility.

Treatment of cultured tobacco cells with 0.1-1 mM XXXG resulted in a decrease in cell size, accompanied by a rounding of the cells, but acceleration of cell growth and shortening in cell doubling time resulting in an increase in cell number during the logarithmic phase of culture growth [38]. These effects were attributed to a reduction in the molecular weight of the endogenous xyloglucan, resulting in cell wall loosening. Use of fluorescently labelled XXXG demonstrated that the exogenous XGO was incorporated into the cell wall xyloglucan and was associated with cell expansion [38]. Transgenic expression of genes encoding xyloglucan degradative enzymes such as *Aspergillus aculeatus* xyloglucanase in poplar [58], *Arabidopsis* cellulase in poplar [63] or poplar cellulase in *Arabidopsis* [59], are consistent with the effects of exogenous XGO treatments, confirming an association between xyloglucan breakdown and increased cell expansion.

To our knowledge, changes in gene expression following XGO treatment have not been investigated before now. To gain insight into the mechanism of XGO action at the molecular level, we exposed the tobacco (*Nicotiana tabacum* L.) BY-2 cell line to a natural mixture of XGOs derived from tamarind (*Tamarindus indica* L.) seeds, followed by microarray analysis. Global gene expression was significantly altered by XGO treatment with changes in the expression of genes related to defence, abiotic stress, signalling and cell wall metabolism. The up-regulation of a putative xyloglucan endotransglycosylase-related (*XTH*) gene suggests a dual mechanism of XGO action on cell wall loosening. Changes in the expression of genes related to cell cycle control and differentiation further support a role for XGOs as mitotic inducers.

Materials and methods

Xyloglucan Oligosaccharides (XGO)

XGOs were extracted from tamarind (*Tamarindus indica* L.) seeds and purified as described previously [17, 31]. *Trichoderma viride* cellulase (SIGMA) was used to digest the xyloglucan (XG) polysaccharide, and the XG oligosaccharides produced were isolated by ultrafiltration (Amicon centrifugal filter devices MWcut off 5000 Da) and dialysis (Spectra/Por MWcut off 500 Da). Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) spectrometry [49] was used to determine XGO composition. The mass spectrum showed the presence of XGO ions with m/z of 791, 953, 1085, 1247 and 1409 corresponding to $(M+Na)^+$ adduct ions of XXG, XXGG, XXXG, XXLG/XLXG (XGO isomers are not distinguished by MALDI-TOF analysis), and XLLG [**Online Resource** Figure 1]. The mixture was predominantly XLLG and XXLG, and a lower proportion of XXXG XXGG and XXG, as classified by Fry *et al.* [29] [**Online Resource** Table 1]. The relative proportion of xyloglucan oligosaccharides obtained by MALDI and HAEC-PAD analysis (data not show) were similar. The profiles and relative proportions of xyloglucan oligosaccharides were in good agreement with those reported previously for this plant species [79, 7].

Culture of Tobacco BY-2 Cells and Experimental Treatments

The tobacco (*Nicotiana tabacum* L.) BY-2 cell line was cultured on BY-2 medium [43] and subcultured at 7 d intervals as described previously [27]. To assess the effect of XGOs on BY-2 fresh weight, 10 mL of a cell suspension was transferred to 95 mL of fresh medium supplemented with XGOs at 0.1, 10 or 100 mg L^{-1} (0.8, 8 or 80 μM), or fresh medium as a control. Cell mass (fresh weight) was determined after 7 days of culture by centrifugation and weighing the pellet of three independent cultures. For determination of mitotic index and cell area, 20 μL of cells was removed from the culture and mixed immediately with 1 μL Hoechst stain (Bisbenzimide Sigma, 100 μg mL^{-1} in 2 % (v/v) Triton X-100) and analysed with an Olympus BH2 fluorescence microscope (UV $\lambda = 420$ nm). The mitotic index (the sum of prophase, metaphase, anaphase, and telophase mitotic figures as a percentage of all cells) was measured daily for a minimum of 300 cells per slide on random transects across the coverslip on one slide from each of three independent cultures per sampling time per treatment. Interphase and mitotic cell areas were measured using SigmaScan® (Jandel Scientific, San Rafael, CA, USA). All the measurements were performed daily throughout the 7 d culture period.

RNA extraction

For RNA extraction, BY-2 cells were sampled 1 h following subculture into BY2 medium (day 0) and then on day 2 (log phase) grown with or without 0.1 mg L^{-1} of XGO. Cells were collected by centrifugation, frozen in liquid nitrogen, and stored at -80 °C until required. Total RNA was extracted using the Ambion® RNAqueous-Micro Kit (Ambion, Austin, USA), according to the manufacturer's instructions. RNA was extracted from replicate cultures separately for use in the microarray and real-time PCR analysis thus providing biological replicates for the experiment.

Real time PCR analysis

Total RNAs were isolated as described above and then treated with DNase I (Ambion, Austin, USA). They were then converted to cDNAs using a First Strand Synthesis Kit for RT-PCR, RETROscript™ (Ambion, Austin, USA), according to the manufacturer's instructions. Quantitative RT-PCR was performed with the use of ABsolute™ QPCR SYBR® Green Mix which is optimised for SYBR® Green I assays (Thermo Fisher Scientific Inc., ABgene®, UK). Gene specific primers designed and used to analyze transcript abundance are shown in [**Online Resource** Table 2]. All the primers were designed using the programme Primer3: available online (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) [62].

Real-time amplification was carried out in a 20 µL total volume containing 300-400 nM of each primer and 10 µL SYBR Green Mix (ABsolute™ QPCR Thermo Fisher Scientific Inc., ABgene®, UK or PowerSYBR Green PCR Master Mix Applied Biosystems). Thermal cycling conditions were set at 15 min at 95 °C, followed by 45 cycles consisting of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C in a Real-Time PCR Detection System Rotor-Gene 6000 (Corbett Life Science, QIAGEN) or 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a StepOne™ Real-Time PCR System (Applied Biosystems). The mean of triplicate reactions was used to estimate transcript copy number. To utilize the comparative Ct method of relative quantitation of gene expression, validation experiments were performed on all target gene primers [primer pairs listed in **Online Resource** Table 2). To test primer specificity, melting curve analysis (from 60 °C to 95 °C with an increasing heat rate of 0.5 °C s^{-1}) was performed following amplification. Relative quantification of gene expression was carried out using 2^{-DDCT} or comparative Ct method [44]. Expression levels were normalized using the elongation factor 1-alpha mRNA [18] [**Online Resource** Table 2].

Microarray Analysis

For microarray analysis, total RNA was isolated as described above from two biological replicates on day 2 of culture (log phase) for both treatments, 0.1 mg L^{-1} of XGO and control (no XGO). Array analysis was performed at the Nottingham Arabidopsis Stock Centre (The University of Nottingham, UK) using the Affymetrix service for Tobacco Transcriptomics. Samples from the two independent biological replicates for each treatment were subjected to hybridization with the Probe Array Type ATCTOBa520488 of Tobacco Expression Atlas (TobEA), containing 43768 genes [24]. Unigenes were previously annotated using BLASTX based on the best hit (e-value $<1 \times 10^{-10}$) against a database of protein sequences from *Arabidopsis thaliana* (Arabidopsis Information Resource (TAIR) (http://arabidopsis.org/index.jsp)) and also using the program Blast2GO [15] against a database of non-redundant proteins from Genbank [24].

Statistical analyses

Growth data were evaluated statistically using t-tests (GraphPad Software, Inc.) available online (http://www.graphpad.com/quickcalcs/ttest1.cfm). All microarray data were processed by the NASC's Affymetrix Service using the MAS5 algorithm [33]. Statistical tests were carried out using the program GeneSpring GX ver 11.0 (Agilent, Technologies, Inc. 2009, Santa Clara, CA, USA) with Benjamini and Hochberg false discovery rate multiple testing correction MTC [5]. Array data are expressed as FCA Absolute (fold change) with associated p value.

Gene Ontology (GO) analysis (GeneSpring GX 11.0.1) was carried out using a custom Perl script based on GO annotation from the TAIR 8 release (as of May 2012). A Contingency χ^2 test and t-tests were performed using Minitab15 (Minitab Inc., PA, USA).

Results

Exogenous XGOs stimulated growth and mitotic activity in tobacco BY-2 cell cultures

XGO treatment altered fresh weight of 7-day old cell cultures; the most significant increase was obtained with a 0.1 mg L^{-1} XGO treatment (Fig. 1A). This concentration was therefore selected for all further experiments. BY-2 cells treated with 0.1 mg L^{-1} XGO showed a peak in the mitotic index on day 2 of culture whereas in untreated control cultures the mitotic index peaked on day 3 (Fig. 1B). Indeed on day 2 the mitotic index in the XGO treated cells was significantly higher than in the control cells confirming the known promotion of cell proliferation by these XGOs [31].

Mitotic cell size data conformed to an inverse temporal pattern compared with mitotic indices, regardless of treatment. It was large on day 1, smaller on days 2-4 and large once more on day 6 (Fig. 1C). When treated with 0.1 mg L^{-1} XGO the size of mitotic cells on day 2 of culture was significantly smaller compared to controls, although on all other days cell size was not significantly changed compared to untreated controls.

The effect of 0.1 mg L^{-1} XGO treatment on the expression of *CDKB1;2*, as a marker for mitotic activity, was investigated (Fig. 1D). The pattern of *CDKB1;2* expression was similar in 0.1 mg L^{-1} XGO treated and control cells; expression in both cultures peaked between day 1 and day 2, partly coinciding with the peak in mitotic index.

Global gene expression in BY-2 cells is modified by exogenous XGO treatment

Having confirmed a positive effect on cell proliferation elicited by the 0.1 mg L^{-1} XGO treatment, an Affymetrix array representing 43,768 genes was screened to identify changes in global gene expression associated with XGO treatment. The second day of cell culture was selected as the point when this treatment elicited the greatest difference in mitotic index and cell area. Changes in gene expression on day 2 of culture with and without the treatment with 0.1 mg L^{-1} XGO were therefore compared. A total of 591 genes were differentially expressed (more than a 2-fold change relative to the reference, with a *P*-value of less than 0.05) [25] [**Online Resource** Table 3]. Principal Component Analysis (PCA) revealed that the XGO treatment replicates were tightly clustered, and were well separated from the untreated controls indicating a clear difference in overall transcriptional profile (Fig. 2).

Of the 591 differentially expressed genes the number whose expression was up-regulated (334 genes) was higher than those down-regulated (257 genes). Putative functions, processes or responses could only be defined for 146 of these genes, due to incomplete annotation of the tobacco genome to date. Of these, 89 were up-regulated and 63 down regulated [**Online Resource** Table 3]. Based on gene ontology (GO) annotations and homology to genes of known function in The Arabidopsis Information Resource (TAIR) (http://arabidopsis.org/index.jsp), a putative protein function could be ascribed to 140 of these genes (Table 1), dividing them into 28 different functional groups as listed in Table 1.

The largest group was related to proteolysis, of which substantially more were up- rather than down-regulated. Cytoskeletal and transferase-related genes were more highly represented amongst genes that were down-regulated whereas chromatin remodelling, proteolysis-related, oxidoreductases and transporters were more highly represented amongst the up-regulated genes. However the overall pattern of differentially expressed genes between the different classes did not differ significantly between those that were up-or down-regulated (analysed by a contingency χ^2 test).

Not all the genes could be confidently categorised in relation to a biological or cellular function. However of particular functional significance in relation to the mechanism of action of XGOs, were the three genes related to cell wall metabolism, a group of 33 genes with functions related to signal transduction and stress responsiveness, and four genes related to cell division ([**Online Resource** Table 3]; Table 2 and Table 3). The 10 genes related to chromatin remodelling and transcriptional control (Table 1) are also of interest in relation to the effects of XGOs on other down-stream processes.

The expression of selected genes, showing significant changes in expression on the microarrays, was further tested by real time RT-PCR. These were selected to represent functional groups of specific interest in relation to the role of XGOs: cell wall remodelling, signal transduction and auxin responses, and defence responses. For this experiment, expression with and without XGO treatment was compared both after 2 d to confirm the array result and also after only 1 h treatment to establish whether the XGOs elicited any very rapid transcriptional responses (Fig 3). The individual results are described below.

Cell wall metabolism

Three of the differentially expressed genes have putative functions in cell-wall architecture. Expression of genes encoding a putative XTH-related protein and a cell wall invertase were both up-regulated by 3.4- and 2.9-fold respectively, while a gene encoding a putative (1-4)-beta-mannan endohydrolase was down-regulated by 2.9-fold. The closest match to the *XTH*-like gene (CV020867) in *Arabidopsis thaliana* was to *XTH9* (AT4G03210), encoding an enzyme involved in loosening and rearrangement of the cell wall and maximally expressed in vegetative and floral shoot apices [34]. The tobacco Expressed Sequence Tag (EST) used to design oligos for the microarray was 64% homologous to the Arabidopsis *XTH9* at the amino acid level and includes the XTH conserved active site motif [61]. Real time RT-PCR confirmed the up-regulation of the tobacco *XTH*-like gene in the XGO treated cultures compared to controls on day 2 of culture (Fig 3A). Furthermore it also revealed a very rapid up-regulation of the *XTH*-like gene expression within 1 h of XGO addition. Expression of this gene fell in both control and XGO treated cells from day 0 to day 2 of culture.

Signal transduction and responses

Nineteen genes putatively related to signal transduction or signal responses showed differential expression (Table 2), ten were up-regulated whereas nine were down-regulated. Up-regulated genes included those with putative functions in calcium mediated signalling, and responses to auxin and jasmonic acid (JA). Down-regulated genes included those with putative functions in development and phototropism, and signal transduction of brassinosteroids.

Ten genes with homology to kinases were differentially expressed; four were up-regulated, while the other six were down-regulated. Two of the kinase genes showed closest homology to phosphofructokinase B type family (PFKB-type), putatively involved in metabolic functions [56] and one is related to cytoskeletal functions. The remaining genes showed homology to receptors such as RLKs, serine/threonine protein kinase family and leucine rich repeat family, all of which may have roles in signalling [64].

A gene with homology to an *Arabidopsis* GTP-binding family protein (AT5G54840) was down-regulated on the arrays (3.4-fold). In *Arabidopsis* this gene (*AtSGP1*) is expressed in the quiescent centre of the root apical meristem, columella of the root cap, guard cells and stele, and may play an important role in signalling of cell fate/ cell differentiation [4]. Real time PCR confirmed the down-regulation of expression with XGO treatment (Fig. 3B) both after the 2 d time period tested in the arrays and also less dramatically, but still significantly after just 1h of XGO treatment. In contrast, expression of this gene increased significantly in the two days of culture in the untreated control cells.

Auxin-induced genes not specifically related to signalling also included a gene with homology to *Medicago truncatula NODULIN21* (*MtN21*) (up-regulated by 2.4-fold), and genes encoding proteins with functions in carbohydrate metabolism, e.g., β-galactosidase (up-regulated by 2.6-fold), which are regulated by auxin in other plant species [11]. Expression of *DW001943*, a gene showing 79% homology to an *Arabidopsis* auxin-responsive gene (AT2G04850, [55]) at the amino acid level was also up-regulated in the arrays (3.1-fold). This was confirmed by real-time PCR where the expression of this gene on day 2 of culture was significantly higher when grown in the presence of XGO than without XGO (Fig. 3C). Furthermore, expression of this gene was strongly induced following the 1 h exposure to XGO on day 0 suggesting a very rapid response, but then fell during continuous exposure to XGO over the 2 d culture period. Conversely in control cells cultured without XGO, expression rose between day 0 and day 2 of culture.

Stress responsive genes

Several of the differentially expressed genes also have putative functions in stress responses, both biotic (seven genes) and abiotic (11 genes) (Table 3). Differentially expressed genes related to elevated biotic stress included a chitinase-like gene with closest homology to

AT3G12500, a gene involved in the ethylene/ JA mediated signalling pathway during systemic acquired resistance [75]. Two tobacco targets were homologous to this gene, one of which was up- and the other-down regulated. Expression of a gene with homology to *Arabidopsis JAZ8* (CQ809070; jasmonate-zim-domain protein 8, AT1G30135), was also up-regulated (by 2.7-fold). Although the overall homology to the *Arabidopsis* gene is low, the tobacco EST contains the TIFY sequence which is required as part of the ZIM domain for protein-protein interactions between JAZ family proteins [13]. In *Arabidopsis*, JAZ proteins act as repressors of JA signalling and mediate various jasmonate-regulated processes, including defence [12]. Up-regulation of the tobacco *JAZ8*-like gene with XGO treatment compared to untreated control cells on day 2 of culture was verified by real time RT-PCR. In control cells expression was undetectable at either time point, but was rapidly induced by the 1 h XGO exposure on day 0. Expression levels then fell in continuous exposure to XGO after 2 d of culture [**Online Resource** Fig 2].

A gene with closest homology to *Arabidopsis LOL1* (AT1G32540) was down-regulated 3.4-fold. The homology between the tobacco EST (EB428982) and *LOL1* covers one of the three *LOL1* zinc finger domains [23]. *LOL1* encodes a DNA binding protein which promotes cell death and is involved in the hypersensitive response. Reduced *LOL1* expression was reflected by the real-time PCR results (Fig. 3D). Remarkably, expression of this gene was highly induced by the 1 h XGO treatment on day 0 indicating a rapid response to the XGO treatment but fell between day 0 and day 2 of culture in both control and XGO treated cells.

Genes relating to iron deficiency, heat, including two heat shock proteins (HSPs), cold and hypoxia were all up-regulated (Table 3). However, genes related to dehydration, cold, DNA repair and wounding, were all down-regulated.

Cell cycle related genes

Six genes on the array showing altered expression with XGO treatment have putative functions in cell cycle control. Three were up- and three were down-regulated. One of the up-regulated genes shows homology to *Arabidopsis TSK* (*TONSOKU*, AT3G18730) (3.7-fold), which encodes a protein necessary for cell cycle progression at G2/M phase [71]. Also there was a 4.7-fold up regulation of a microtubule motor gene (encoding a kinesin-like protein), and a 2-fold up regulation of a gene with homology to *Arabidopsis GAMMA-H2AX* (gamma histone variant *H2AX*, AT1G54690. Interestingly, the gene encoding the kinesin-like protein is preferentially expressed in mitotic BY-2 cells and appears to function mainly in cell division [47]; γ-H2AX in *Arabidopsis* plays a role in meiotic processes [9].

All three of the down-regulated genes with putative functions in cell division showed homology to kinesin-like proteins. One of these (BP130115, down-regulated by 2.5-fold) was most highly expressed in the log phase of BY-2 cells [48] and may be involved in cytokinesis. The other two: EB448475 and BP527174 show closest homology to an *Arabidopsis* kinesin motor protein (AT5G65460) involved in cytokinesis [77] and actin mediated chloroplast movement [70].

Chromatin remodelling, histone associated and transcriptional control

Three up-regulated genes had putative functions related to histone modification and chromatin remodelling. These included a histone deacetylase (BP529582) (2.1-fold) and a gene with homology to a meiosis specific histone protein (EB449808) (*H2AX*), up-regulated by 2-fold already discussed above. In addition, a tobacco gene (U01961) with homology to *Arabidopsis HAC1* (AT1G79000), was also up-regulated by 4.5-fold. *HAC1* is a H3/H4 histone acetyltransferase involved in the regulation of flowering time [21].

Seven transcription factors were differentially expressed (Table 4). Four with homology to *MADS5, MYB68, DUO1* and *ZAT6* were up-regulated, whereas three with homology to a

C3HC4-type RING finger, *BHLH093* and *NAC* domain transcription factors were down-regulated. Two of the up-regulated transcription factors show homology to *Arabidopsis* genes involved with root development: *MYB68* is maximally expressed in roots [54] and *ZAT6* helps regulation of phosphate homeostasis during root development [22]. Less is known about the *Arabidopsis* homologues to the down-regulated transcription factors, although *BHLH093* may have a role in stomatal development [57].

Discussion

XGOs stimulate growth

Changes in fresh weight and the higher and anticipated mitotic index peak with XGO treatment of tobacco BY-2 cells confirm previous reports [31, 38] that XGOs stimulate cell division both as individual compounds and as the natural extract containing a mixture of XGOs used here. The fall in mitotic cell size in both control and XGO treated cells during the peak of mitotic index, regaining original size by the end of the culture period, is in line with previous observations in our lab [65]. The null effect of XGO on cell size is also in agreement with the finding that XGO treatment of BY-2 cells shortens G1 whilst mitotic cell size remained constant [31]. Kaida *et al.* [38] found a reduction in cell size associated with XGO treatment of a different tobacco cell culture system (XD-6 derived from *Nicotiana tabacum* L. var. Xanthi). This is in agreement with the significant reduction in cell area at day 2 of culture in the XGO treated cells found here.

The coincidence between timing of the increase in the mitotic index and peak in *CDKB1;2* expression are consistent with a previous report of *CDKB1* RNA expression during the complete BY-2 cell growth cycle [68], where this gene was highly expressed within the exponential growth phase and then declined substantially as cells exited the cell cycle and entered stationary phase. *CDKB1* transcripts and protein accumulate during S, G2, and M phases and their associated kinase activity peaks during mitosis [36].

Microarray analysis reveals changes in the expression of genes with putative functions in cell wall metabolism, the cell cycle, auxin and stress responses

The clear differentiation between expression profiles of XGO treated and untreated BY-2 cells shown by PCA and the similar proportions of up- or down-regulated genes indicate that the cellular effects seen with XGO treatment involve complex changes in gene expression.

In a previous microarray analysis characterizing gene expression during normal growth of BY-2 cells, Matsuoka et al [48] found that log phase cells predominantly expressed DNA/chromosome duplication gene homologues. In addition, many genes for basic transcription and translation machineries, as well as proteasomal genes, were up-regulated at this growth phase. Our findings are consistent with these previous results. However, we show here that when challenged with XGOs differentially expressed genes include those related to cell wall metabolism, the cell cycle, auxin responses as well as stress responses: both biotic and abiotic.

A putative XTH-related gene was up-regulated by XGO treatment

The up-regulation of a gene with close homology to an *XTH* by XGO treatment is consistent with an increase in xyloglucan endotransglycosylase activity in response to XGOs in *Azuki* bean hypocotyls [39], which correlated with increased cell wall extensibility and xyloglucan breakdown. Kaku et al. [39] suggested that the XGOs may stimulate endotransglycosylation by acting as acceptor substrates. Data presented here show increased transcription of an *XTH*-like gene in response to XGOs. The very rapid transcriptional up-regulation of the *XTH*-like expression following only 1 h of XGO treatment shown here suggests a direct effect of the XGOs on transcription, stimulating increased enzyme production in addition to effects on enzyme activity [39]. The fall in transcript levels with continuous XGO treatment is likely due to a feedback system ensuring homeostasis of cell wall turnover.

XGO treatment results in changes in the expression of genes related to cell division and differentiation

Part of the positive growth effects seen in previous studies [1, 2, 31] in response to XGO treatment can be attributed to increased competence of cells to enter mitosis shown here and in Kaida et al [38], although they did not report on changes in the mitotic index peak or effects on gene expression. Of significance in this context is the up-regulation of *TSK* (*TONSOKU*) reported here, which is required during the cell cycle. *tsk* mutants are delayed in G2/M progression [71] which may be caused by activation of the G2/M checkpoint, or defects in mitosis. TSK localizes to the ends of spindle microtubules during mitosis, and defects in TSK cause disruption of the cell division plane [72]. Thus TSK is probably required for correct organisation of the spindle structure.

Up-regulation of a gene encoding a kinesin-like protein, *TBK1*, is consistent with its preferential expression in mitotic BY-2 cells [47] suggesting a role during cell division. Thus the treatment with XGOs may also be affecting cell division through up-regulation of genes that are required for mitosis. Moreover, down-regulation of the *AtSGP1*-like gene, involved in cell fate/ cell differentiation signalling in *Arabidopsis* [4], is consistent with an effect of XGOs in promoting mitosis and repressing differentiation.

XGO's treatment affects the expression of genes related to signalling by and responses to plant growth regulators

Since exogenous XGOs elicit clear cellular effects, directly or indirectly, it follows that this signal must be perceived and transduced within the cell. The finding that four genes with homology to serine/threonine (Ser/Thr) kinases and four with homology to leucine rich repeat (LRR) proteins were differentially expressed in response to the XGO treatment is thus consistent with a signalling role for XGOs.

A class of Ser/Thr protein kinases that are tightly bound to the cell wall, named wall-associated kinases (WAKs), are candidate receptors for oligogalacturonides (OGs) released from the plant cell wall. Notably WAKs bind these oligosaccharides *in vitro* [6, 8, 20]. Possibly other members of the WAK family also bind XGOs. Thus future work to characterise the Ser/Thr receptor-like genes that are differentially expressed in response to exogenous XGOs will be an important step towards understanding the mode of action of these oligosaccharides in plants.

Another class of receptors that could be mediating the signal transduction of xyloglucans comprises leucine-rich repeat transmembrane protein kinases (LRRs) as they are involved in response to several plant growth regulators; e.g., brassinosteroids [41], ethylene [78] and gibberellins [76].

Given the early reports suggesting an interaction between XGOs and auxins [51, 52] we noted here the differential expression of several auxin responsive genes which supports this interaction. The complexity of the interaction between XGOs and auxin [51, 52] is reflected in the transcript levels of an auxin-responsive gene (DW001943), which was very rapidly up-regulated following just 1 h of treatment with XGOs but then fell during the following 2 d.

The up-regulation of this gene in control cultures, mirroring the rise in the mitotic index, is consistent with the expression of another auxin-responsive gene (*arcA*) in cultured BY-2 cells [37] whose expression fell in parallel with a fall in the mitotic index.

XGOs as elicitors of plant defences and responses to stress

One notable finding from the microarray analysis was the differential expression of several stress responsive genes, which supports earlier reports that XGOs may have a role in acting as elicitors of plant defence [67, 69]. The differential expression of chitinase genes supports previous reports of the effects of xyloglucan fragments prepared from tamarind seeds and pea stems. Increased activity of peroxidase, beta-1,3-glucanase and chitinase occurred in the extracellular fluid of cucumber cotyledons which relates to the hypersensitive response of cucumber to Tobacco Necrosis Virus (TNV) [67]. The rapid up-regulation of two defence-response related genes, *JAZ8* and *LOL1*- like genes in response to the XGOs, followed by a decline over the 2 d culture period is similar to the wounding response of *JAZ8* in *Arabidopsis* which is rapidly induced by wounding [13] with maximal levels after 1 h thereafter falling off.

Also of interest were the class of differentially expressed genes that have putative roles in abiotic stress responses, including genes that respond to all the stress related plant growth regulators. To our knowledge, although other oligosaccharins have been associated with responses to abiotic stress [81, 82], a link between XGO treatment and abiotic stress had not previously been made. Effects of XGOs on abiotic stress responses require further work.

Conclusions

We show for the first time, that XGO treatment of tobacco BY-2 cells with a natural admixture of XGOs, thus representing more closely XGOs in vivo, elicits substantial changes in gene expression. These changes cover several important biological processes which are probably related to XGO function in whole plants. Of particular significance is the finding that XTH activity may be promoted through transcriptional activation. Up-regulation of genes promoting mitosis, and down regulation of genes promoting differentiation with XGO treatment explains the increase in mitotic cells. Our data also support reports of positive effects of XGOs as elicitors, and further suggest that XGOs may be involved in abiotic stress responses.

Supplementary information (online resources)

Supplementary Fig 1 MALDI-TOF mass spectra of xyloglucan oligosaccharides (NB XGO isomers are not distinguished by MALDI-TOF analysis).

Supplementary Fig 2 Comparative expression (by real-time PCR) of *JAZ8*-like gene in BY2 cells treated with 0.1 mg L^{-1} XGO at day 0 (X-0) and day 2 (X-2) of culture (mean ± S.E., n= 3, different letters indicate statistically different means $P < 0.05$).

Supplementary Table 1: XGO composition in cellulase hydrolysates of *Tamarindus indica* L. xyloglucan as determined my MALDI-TOF mass spectrometry.

Supplementary Table 2: Primers used for real time PCR analysis. The gene identification corresponds to the sequence annotated in the GeneBank database of NCBI (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and used as template for primer design. F: forward and R: reverse primers.

Supplementary Table 3: Results of microarray analysis: probes that were up or down regulated by ≥2-fold and putative functions where data are available.

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FIGURE LEGENDS

Fig. 1 Effects of XGO treatment in the tobacco BY2 cell line on: (A) growth (fresh weight of cell culture after 7 d culture, mean ± S.E. n ≥ 3), (B) mitotic index (% frequency of cells in division; mean ± S.E., n= 3), (C) mitotic cell area (μm^2, mean ± S.E. n= 15-20) and (D) expression of CDKB1;2 ± XGO by real-time PCR; (±S.E., n=2) over 3 days of culture * =$P <$ 0.05, *** = $P < 0.001$ compared to 0 mgL^{-1} XGO on each day).

Fig. 2 Principal Components Analysis (PCA) of the microarray data (two replicates each of XGO treated and control). All genes are plotted with respect to first and second principal components. Samples occupying similar position in PC space share similar gene expression trends.

Fig. 3 Expression pattern (by real-time PCR) of (A) *XTH*-like gene (CV020867), (B) a GTP binding protein, (C) a putative auxin-responsive gene (DW001943), (D) a *LOL1*-like gene (EB428982) in BY2 untreated cells on day 0 (C-0) and day 2 (C-2) of culture and in cells treated with 0.1 mg L^{-1} XGO (X-0 and X-2) (mean ± S.E., n= 3, different letters indicate statistically different means $P < 0.05$).

TABLES

Table 1: Functional groups of genes whose expression was up- or down-regulated (>2-fold) in response to XGO treatment

Predicted protein function	**up**	**down**	**total**	**%**
ATPase	0	2	2	1.5
biosynthesis	2	2	4	3.0
calcium binding	1	1	2	1.5
carbohydrate binding	2	3	5	3.7
cell wall metabolism	2	1	3	2.2
chaperonin/HSPs	3	0	3	2.2
chitinase	1	1	2	1.5
chromatin /histone associated	4	0	4	3.0
cyt P450	2	0	2	1.5
cytoskeleton	1	5	6	4.5
Glutathione-S-transferase	1	0	1	0.7
GTPase	1	1	2	1.5
hydrolase	4	2	6	4.5
kinase	4	5	9	6.7
lipid binding	2	2	4	3.0
metabolism	1	3	4	3.0
nucleic acid binding	6	6	12	9.0
oxidoreductase	5	1	6	4.5
phosphatase	0	2	2	1.5
photosynthesis	4	5	9	6.7
proteolysis	12	5	17	12.7
protein binding	4	6	10	7.5
receptor	2	0	2	1.5
ribosomal	1	1	2	1.5
secondary metabolism	3	0	3	2.2
transcription factor	3	3	6	4.5
transferase	0	5	5	3.7
transporter	6	0	6	4.5
unknown	6	0	6	4.5

Table 2: Differentially expressed genes related to signal transduction and responses, whose expression was up- or down-regulated >2-fold in response to XGO treatment.

Probe Set ID	FCA absolute (Fold change on array)	Closest Arabidopsis homologue AGI code	Description
Up-regulated			
DW001943_at	3.1	AT2G04850	auxin-responsive protein-related
BP134562_at	2.2	AT5G38210	serine/threonine protein kinase family protein
BP526893_at	3.4	AT5G43020	leucine-rich repeat transmembrane protein kinase, putative
C4219_at	2.5	AT4G28950	*ROP9 (RHO-RELATED PROTEIN FROM PLANTS 9*)
BP526893_at	3.4	AT5G43020	leucine-rich repeat transmembrane protein kinase, putative
BP192587_at	2.5	AT4G24480	serine/threonine protein kinase, putative
MT203B_at	2.7	AT1G30135	*JAZ8/TIFY5A (JASMONATE-ZIM-DOMAIN PROTEIN 8)*
BP529083_at	2.7	AT3G63060	circadian clock coupling factor *ZGT*
BP136882_at	2.9	AT3G01400	armadillo/beta-catenin repeat family protein
EB442205_at	3.8	AT2G26190	calmodulin-binding family protein
Down regulated			
BP526151_at	2.1	AT2G16250	leucine-rich repeat transmembrane protein kinase, putative
BP128689_at	2.2	AT1G15750	*TPL/WSIP1 (WUS-INTERACTING PROTEIN 1);* protein binding
BP129606_at	3.1	AT1G24650	leucine-rich repeat family protein / protein kinase family protein
C2467_at	2.3	AT2G30520	*RPT2 (ROOT PHOTOTROPISM 2);* protein binding
C9904_at	2.4	AT5G60900	*RLK1 (RECEPTOR-LIKE PROTEIN KINASE 1)*
C2929_at	3.4	AT5G54840	GTP-binding family protein
BP131989_at	2.6	AT4G31160	transducin family protein / WD-40 repeat family protein
BP130215_at	2.6	AT3G13670	serine/threonine protein kinase family protein
C4477_at	4.8	AT1G32130	involved in brassinosteroid-regulated gene expression.

Table 3: Differentially expressed genes related to stress responses whose expression was up- or down-regulated >2-fold in response to XGO treatment.

Probe Set ID	FCA absolute (Fold change on array)	Closest Arabidopsis homologue AGI code	Description	Response to/ function
Up-regulated				
DQ131889_x_at	2.1	AT4G31970	*CYP82C2* (cytochrome P450)	hypoxia
C9400_at	2.8	AT4G31940	*CYP82C4* (cytochrome P450)	low Fe
C6549_at	3.5	AT3G12500	*ATHCHIB (BASIC CHITINASE);* chitinase	biotic stress
C2859_s_at	2.6	AT1G78380	*ATGSTU19 (GLUTATHIONE TRANSFERASE 8)*	drought, oxidative stress
C5973_at	3.4	AT1G53540	17.6 kDa class I small heat shock protein (*HSP17.6C-CI*)	heat shock protein
BP132586_at	2.2	AT2G26890	*GRV2 (KATAMARI2*); binding / heat shock protein binding	heat shock protein
C2748_at	3.3	AT1G72860	disease resistance protein (TIR-NBS-LRR class), putative	biotic stress
MT203B_at	2.7	AT1G30135	*JAZ8/TIFY5A (JASMONATE-ZIM-DOMAIN PROTEIN 8)*	biotic stress
CV016057_at	2.6	AT2G15970	*COR413-PM1* (cold regulated 413 plasma membrane 1)	cold
EB447067_s_at	3.6	AT1G65870	disease resistance-responsive family protein	biotic stress
CN498873_s_at	6.1		induced by the bacterial effector protein AvrPto	biotic stress
Down-regulated				
EB425750_at	3.2	AT1G70670	caleosin-related family protein	drought, ABA
C8646_at	2.9	AT3G12500	*ATHCHIB (BASIC CHITINASE);* chitinase	biotic stress
EB428982_at	3.4	AT1G32540	*LOL1 (LSD ONE LIKE 1)*	biotic stress
BP528192_at	3.7	AT1G80210	*BRCA1/BRCA2-CONTAINING COMPLEX 36 HOMOLOG A*	DNA repair
BP192659_at	3.2	AT5G53000	*TAP46 (2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 KD)*	cold
DW001183_at	2.1	AT1G55480	*ZKT*, phosphorylated at Thr and Ser residues after wounding	wounding
BP132027_at	2.5	AT4G18030	SAM methyl transferase family protein	dehydration

Table 4: Differentially expressed genes with homology to transcription factors whose expression was up- or down-regulated >2-fold in response to XGO treatment.

Probe Set ID	FCA absolute (Fold change on array)	Closest Arabidopsis homologue AGI code	Description	Response to/ function
Up-regulated				
AF068724_at	2.9	AT1G69120	MADS box protein *MADS5*	MADS domain transcription factor
BP526619_at	2.2	AT5G65790	*MYB 68* (myb domain protein 68)	response to gibberellin stimulus, response to salicylic acid stimulus
EB643472_x_at	2.7	AT3G60460	*DUO1 MYB* transcription factor	required for male gamete formation
BP528590_at	3.5	AT5G04340	*C2H2 (ZINC FINGER OF ARABIDOPSIS THALIANA 6; ZAT6)*	Root development and phosphate homeostasis
Down-regulated				
C249_at	2.1	AT3G14320	zinc finger (*C3HC4*-type RING finger) family protein	Highly expressed in seed
EB678910_at	3.1	AT5G65640	*BHLH093 (BETA HLH PROTEIN 93)*	Maximal expression in floral apex and hypocotyl, role in stomatal development
EB683185_at	2.1	AT2G24430	*ANAC038/ANAC039*	NAC domain, seed-specific expression

In tobacco BY-2 cells xyloglucan oligosaccharides alter the expression of genes involved in cell wall metabolism, signalling, stress responses, cell division and transcriptional control.

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Abstract

Xyloglucan oligosaccharides (XGOs) are breakdown products of xyloglucans, the most abundant hemicelluloses of the primary cell walls of non-Poalean species. Treatment of cell cultures or whole plants with XGOs results in accelerated cell elongation and cell division, changes in primary root growth, and a stimulation of defence responses. They may therefore act as signalling molecules regulating plant growth and development. Previous work suggests an interaction with auxins and effects on cell wall loosening, however their mode of action is not fully understood. The effect of an XGO extract from tamarind (*Tamarindus indica*) on global gene expression was therefore investigated in tobacco BY-2 cells using microarrays. Over 500 genes were differentially regulated with similar numbers and functional classes of genes up and down-regulated, indicating a complex interaction with the cellular machinery. Up-regulation of a putative xyloglucan endotransglycosylase/hydrolase-related (*XTH*) gene supports the mechanism of XGO action through cell wall loosening. Differential expression of defence-related genes supports a role for XGOs as elicitors. Changes in the expression of genes related to mitotic control and differentiation also support previous work showing that XGOs are mitotic inducers. XGOs also affected expression of several receptor-like kinase genes and transcription factors. Hence, XGOs have significant effects on expression of genes related to cell wall metabolism, signalling, stress responses, cell division and transcriptional control.

(216 words)

Key words

BY-2 cells, cell cycle, cell walls, microarray analysis, *Nicotiana tabacum*, xyloglucan oligosaccharides.

Introduction

The cellulose/hemicellulose network of the primary cell wall provides structural support as well as physically regulating wall expansion [10,19]. Xyloglucans are the most abundant hemicelluloses of the primary cell walls of non-Poalean species and may have a functional role in hydrogen bonding to, and tethering of, the cellulose microfibrils to each other [46].

Changes in xyloglucan structure have important effects on plant defences. For example, the Arabidopsis mutant, *mur*3, is compromised in xyloglucan galactosyltransferase activity [45], resulting in abnormal xyloglucan structure. This mutant has elevated levels of salicylic acid, exhibits constitutive activation of defence-related genes and is resistant to the pathogen *Hyaloperonospora parasitica* [74].

Xyloglucan oligosaccharides (XGO) are derived from breakdown of xyloglucans and can be defined as oligomers of 1,4-linked β-D-Glc*p* residues. Both chain length and the substitutions in the glucan backbone define different classes of XGO and their nomenclature is through combinations of F, X, G and L, each demarcating modifications of specific oligosaccharides [29, 61]. For example, the archetypal seed xyloglucan from *Tamarindus indica* L. comprises XXXG, XXLG, XLXG, and XLLG oligosaccharides in the molar ratio 1.4:3:1:5.4 [79]. *In vivo,* XGOs are generated by the action of xyloglucan endo-transglycosylase/hydrolase (XTH) [26] on xyloglucans, and are then modified by the action of α-fucosidase, α-xylosidase, β-galactosidase and β-glucosidase [28]. *XYLOGLUCAN ENDO-TRANSGLYCOSYLASE/HYDROLASE* (*XTH*) genes encode proteins with two different catalytic activities. These have very different effects on xyloglucan structure: xyloglucan endo-transglycosylase (XET) (xyloglucan:xyloglucosyl transferase; EC 2.4.1.207) catalyzes non-hydrolytic cleavage and ligation of xyloglucan chains, while xyloglucan endo-hydrolase (XEH) activity (xyloglucan-specific endo-*β*-1,4-glucanase; EC 3.2.151) results in xyloglucan chain shortening. Although *XTH* has also been referred to as *XYLOGLUCAN ENDO-*

TRANSGLUCOSYLASE/HYDROLASE [61], this is not strictly correct as the activity involves the transfer of a whole glycan chain and not just one glucosyl residue [26]. These enzymes are encoded by complex gene families consisting of differentially regulated members that are likely to be important in fine-tuning the *in vivo* composition of the XGOs [35, 26]. *In vitro*, specific oligosaccharides can be produced from xyloglucan by partial digestion with cellulase [β (1-4)–D-glucanase].

A number of different types of oligosaccharides can be elicitors that activate plant defence responses [53]. They are recognized by different cell surface receptors, resulting in a stimulation of direct metabolic pathways and an increase in systemic acquired resistance (SAR) [3, 66]. These include fungal- derived oligosaccharides such as those from β glucan, chitin and chitosan, but also oligogalacturonides derived from pectic cell wall fragments. Less is known about the effects of XGOs, although there are reports of them affecting the hypersensitive response induced by tobacco necrosis virus [67, 69]. XGOs also promoted phytoalexin accumulation in soybean cotyledons [60] and increased ethylene production in tomato fruit, perhaps as part of a hypersensitive response to biotic stress [16]. They have also been commercially patented as plant defence boosters [42]. However, at least in *Arabidopsis* cultured cells, their bioactivity in eliciting early defence responses (medium alkalization, ion effluxes and peroxide accumulation) appears to be less than that of other oligosaccharides derived from plant cell walls (oligogalacturonides) and fungal cell walls (chitosan oligogalacturonides) (Cabrera lab, unpublished results). Oligosaccharins also affect responses to abiotic stress. In winter wheat the oligosaccharin XGAG accumulates during cold acclimation and exogenous treatments with this oligosaccharin increased freezing tolerance [81, 82].

Bioactive oligosaccharides, termed oligosaccharins, also have effects on growth and development that are not obviously related to disease resistance. XGOs play a role in the regulation of plant growth [73, 80], an effect that depended on the presence of a terminal L-fucose [52]. However, XGOs derived from tamarind (*Tamarindus indica* L.) seeds that do not have a terminal L-fucose also have positive effects on plant growth [1, 2] causing an increase in primary root elongation in *Arabidopsis thaliana* but a deceleration of the rate of lateral root formation [31]. Part of these growth effects may be attributed to a shorter cell cycle: treatment of tobacco BY-2 cells with tamarind seed XGOs resulted in a shortening of G1 whilst mitotic cell size remained constant [31]. Indeed, XGOs could well be novel, naturally occurring signaling molecules [30].

The mode of action of XGOs in modulating plant growth is poorly understood. At low concentrations (10^{-8}-10^{-9} M), XGOs may antagonize auxin signalling [50] and inhibit pea stem segment growth, whereas at higher concentrations (10^{-4} M) they had cell wall loosening effects similar to those elicited by auxin [50]. In azuki bean (*Vigna angularis*) epicotyls, cell wall loosening was associated with a modulation of xyloglucan endotransglycosylase/hydrolase (XTH) towards its xyloglucan degrading activity [39], increasing cell wall extensibility.

Treatment of cultured tobacco cells with 0.1-1 mM XXXG resulted in a decrease in cell size, accompanied by a rounding of the cells, but acceleration of cell growth and shortening in cell doubling time resulting in an increase in cell number during the logarithmic phase of culture growth [38]. These effects were attributed to a reduction in the molecular weight of the endogenous xyloglucan, resulting in cell wall loosening. Use of fluorescently labelled XXXG demonstrated that the exogenous XGO was incorporated into the cell wall xyloglucan and was associated with cell expansion [38]. Transgenic expression of genes encoding xyloglucan degradative enzymes such as *Aspergillus aculeatus* xyloglucanase in poplar [58], *Arabidopsis* cellulase in poplar [63] or poplar cellulase in *Arabidopsis* [59], are consistent with the effects of exogenous XGO treatments, confirming an association between xyloglucan breakdown and increased cell expansion.

To our knowledge, changes in gene expression following XGO treatment have not been investigated before now. To gain insight into the mechanism of XGO action at the molecular level, we exposed the tobacco (*Nicotiana tabacum* L.) BY-2 cell line to a natural mixture of XGOs derived from tamarind (*Tamarindus indica* L.) seeds, followed by microarray analysis. Global gene expression was significantly altered by XGO treatment with changes in the expression of genes related to defence, abiotic stress, signalling and cell wall metabolism. The up-regulation of a putative xyloglucan endotransglycosylase-related (*XTH*) gene suggests a dual mechanism of XGO action on cell wall loosening. Changes in the expression of genes related to cell cycle control and differentiation further support a role for XGOs as mitotic inducers.

Materials and methods

Xyloglucan Oligosaccharides (XGO)

XGOs were extracted from tamarind (*Tamarindus indica* L.) seeds and purified as described previously [17, 31]. *Trichoderma viride* cellulase (SIGMA) was used to digest the xyloglucan (XG) polysaccharide, and the XG oligosaccharides produced were isolated by ultrafiltration (Amicon centrifugal filter devices MWcut off 5000 Da) and dialysis (Spectra/Por MWcut off 500 Da). Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) spectrometry [49] was used to determine XGO composition. The mass spectrum showed the presence of XGO ions with m/z of 791, 953, 1085, 1247 and 1409 corresponding to $(M+Na)^+$ adduct ions of XXG, XXGG, XXXG, XXLG/XLXG (XGO isomers are not distinguished by MALDI-TOF analysis), and XLLG [**Online Resource** Figure 1]. The mixture was predominantly XLLG and XXLG, and a lower proportion of XXXG XXGG and XXG, as classified by Fry *et al.* [29] [**Online Resource** Table 1]. The relative proportion of xyloglucan oligosaccharides obtained by MALDI and HAEC-PAD analysis (data not show) were similar. The profiles and relative proportions of xyloglucan oligosaccharides were in good agreement with those reported previously for this plant species [79, 7].

Culture of Tobacco BY-2 Cells and Experimental Treatments

The tobacco (*Nicotiana tabacum* L.) BY-2 cell line was cultured on BY-2 medium [43] and subcultured at 7 d intervals as described previously [27]. To assess the effect of XGOs on BY-2 fresh weight, 10 mL of a cell suspension was transferred to 95 mL of fresh medium supplemented with XGOs at 0.1, 10 or 100 mg L^{-1} (0.8, 8 or 80 µM), or fresh medium as a control. Cell mass (fresh weight) was determined after 7 days of culture by centrifugation and weighing the pellet of three independent cultures. For determination of mitotic index and cell area, 20 µL of cells was removed from the culture and mixed immediately with 1 µL Hoechst stain (Bisbenzimide Sigma, 100 µg mL^{-1} in 2 % (v/v) Triton X-100) and analysed with an Olympus BH2 fluorescence microscope (UV λ = 420 nm). The mitotic index (the sum of prophase, metaphase, anaphase, and telophase mitotic figures as a percentage of all cells) was measured daily for a minimum of 300 cells per slide on random transects across the coverslip on one slide from each of three independent cultures per sampling time per treatment. Interphase and mitotic cell areas were measured using SigmaScan® (Jandel Scientific, San Rafael, CA, USA). All the measurements were performed daily throughout the 7 d culture period.

RNA extraction

For RNA extraction, BY-2 cells were sampled 1 h following subculture into BY2 medium (day 0) and then on day 2 (log phase) grown with or without 0.1 mg L^{-1} of XGO. Cells were collected by centrifugation, frozen in liquid nitrogen, and stored at -80 °C until required. Total RNA was extracted using the Ambion® RNAqueous-Micro Kit (Ambion, Austin, USA), according to the manufacturer's instructions. RNA was extracted from replicate cultures separately for use in the microarray and real-time PCR analysis thus providing biological replicates for the experiment.

Real time PCR analysis

Total RNAs were isolated as described above and then treated with DNase I (Ambion, Austin, USA). They were then converted to cDNAs using a First Strand Synthesis Kit for RT-PCR, RETROscript™ (Ambion, Austin, USA), according to the manufacturer's instructions. Quantitative RT-PCR was performed with the use of ABsolute™ QPCR SYBR® Green Mix which is optimised for SYBR® Green I assays (Thermo Fisher Scientific Inc., ABgene®, UK). Gene specific primers designed and used to analyze transcript abundance are shown in [**Online Resource** Table 2]. All the primers were designed using the programme Primer3: available online (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) [62].

Real-time amplification was carried out in a 20 µL total volume containing 300-400 nM of each primer and 10 µL SYBR Green Mix (ABsolute™ QPCR Thermo Fisher Scientific Inc., ABgene®, UK or PowerSYBR Green PCR Master Mix Applied Biosystems). Thermal cycling conditions were set at 15 min at 95 °C, followed by 45 cycles consisting of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C in a Real-Time PCR Detection System Rotor-Gene 6000 (Corbett Life Science, QIAGEN) or 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a StepOne™ Real-Time PCR System (Applied Biosystems). The mean of triplicate reactions was used to estimate transcript copy number. To utilize the comparative Ct method of relative quantitation of gene expression, validation experiments were performed on all target gene primers [primer pairs listed in **Online Resource** Table 2). To test primer specificity, melting curve analysis (from 60 °C to 95 °C with an increasing heat rate of 0.5 °C s^{-1}) was performed following amplification. Relative quantification of gene expression was carried out using 2^{-DDCT} or comparative Ct method [44]. Expression levels were normalized using the elongation factor 1-alpha mRNA [18] [**Online Resource** Table 2].

Microarray Analysis

For microarray analysis, total RNA was isolated as described above from two biological replicates on day 2 of culture (log phase) for both treatments, 0.1 mg L^{-1} of XGO and control (no XGO). Array analysis was performed at the Nottingham Arabidopsis Stock Centre (The University of Nottingham, UK) using the Affymetrix service for Tobacco Transcriptomics. Samples from the two independent biological replicates for each treatment were subjected to hybridization with the Probe Array Type ATCTOBa520488 of Tobacco Expression Atlas (TobEA), containing 43768 genes [24]. Unigenes were previously annotated using BLASTX based on the best hit (e-value $<1 \times 10^{-10}$) against a database of protein sequences from *Arabidopsis thaliana* (Arabidopsis Information Resource (TAIR) (http://arabidopsis.org/index.jsp)) and also using the program Blast2GO [15] against a database of non-redundant proteins from Genbank [24].

Statistical analyses

Growth data were evaluated statistically using t-tests (GraphPad Software, Inc.) available online (http://www.graphpad.com/quickcalcs/ttest1.cfm). All microarray data were processed by the NASC's Affymetrix Service using the MAS5 algorithm [33]. Statistical tests were carried out using the program GeneSpring GX ver 11.0 (Agilent, Technologies, Inc. 2009, Santa Clara, CA, USA) with Benjamini and Hochberg false discovery rate multiple testing correction MTC [5]. Array data are expressed as FCA Absolute (fold change) with associated p value.

Gene Ontology (GO) analysis (GeneSpring GX 11.0.1) was carried out using a custom Perl script based on GO annotation from the TAIR 8 release (as of May 2012). A Contingency χ^2 test and t-tests were performed using Minitab15 (Minitab Inc., PA, USA).

Results

Exogenous XGOs stimulated growth and mitotic activity in tobacco BY-2 cell cultures

XGO treatment altered fresh weight of 7-day old cell cultures; the most significant increase was obtained with a 0.1 mg L^{-1} XGO treatment (Fig. 1A). This concentration was therefore selected for all further experiments. BY-2 cells treated with 0.1 mg L^{-1} XGO showed a peak in the mitotic index on day 2 of culture whereas in untreated control cultures the mitotic index peaked on day 3 (Fig. 1B). Indeed on day 2 the mitotic index in the XGO treated cells was significantly higher than in the control cells confirming the known promotion of cell proliferation by these XGOs [31].

Mitotic cell size data conformed to an inverse temporal pattern compared with mitotic indices, regardless of treatment. It was large on day 1, smaller on days 2-4 and large once more on day 6 (Fig. 1C). When treated with 0.1 mg L^{-1} XGO the size of mitotic cells on day 2 of culture was significantly smaller compared to controls, although on all other days cell size was not significantly changed compared to untreated controls.

The effect of 0.1 mg L^{-1} XGO treatment on the expression of *CDKB1;2*, as a marker for mitotic activity, was investigated (Fig. 1D). The pattern of *CDKB1;2* expression was similar in 0.1 mg L^{-1} XGO treated and control cells; expression in both cultures peaked between day 1 and day 2, partly coinciding with the peak in mitotic index.

Global gene expression in BY-2 cells is modified by exogenous XGO treatment

Having confirmed a positive effect on cell proliferation elicited by the 0.1 mg L^{-1} XGO treatment, an Affymetrix array representing 43,768 genes was screened to identify changes in global gene expression associated with XGO treatment. The second day of cell culture was selected as the point when this treatment elicited the greatest difference in mitotic index and cell area. Changes in gene expression on day 2 of culture with and without the treatment with 0.1 mg L^{-1} XGO were therefore compared. A total of 591 genes were differentially expressed (more than a 2-fold change relative to the reference, with a *P*-value of less than 0.05) [25] [**Online Resource** Table 3]. Principal Component Analysis (PCA) revealed that the XGO treatment replicates were tightly clustered, and were well separated from the untreated controls indicating a clear difference in overall transcriptional profile (Fig. 2).

Of the 591 differentially expressed genes the number whose expression was up-regulated (334 genes) was higher than those down-regulated (257 genes). Putative functions, processes or responses could only be defined for 146 of these genes, due to incomplete annotation of the tobacco genome to date. Of these, 89 were up-regulated and 63 down regulated [**Online Resource** Table 3]. Based on gene ontology (GO) annotations and homology to genes of known function in The Arabidopsis Information Resource (TAIR) (http://arabidopsis.org/index.jsp), a putative protein function could be ascribed to 140 of these genes (Table 1), dividing them into 28 different functional groups as listed in Table 1.

The largest group was related to proteolysis, of which substantially more were up- rather than down-regulated. Cytoskeletal and transferase-related genes were more highly represented amongst genes that were down-regulated whereas chromatin remodelling, proteolysis-related, oxidoreductases and transporters were more highly represented amongst the up-regulated genes. However the overall pattern of differentially expressed genes between the different classes did not differ significantly between those that were up-or down-regulated (analysed by a contingency χ^2 test).

Not all the genes could be confidently categorised in relation to a biological or cellular function. However of particular functional significance in relation to the mechanism of action of XGOs, were the three genes related to cell wall metabolism, a group of 33 genes with functions related to signal transduction and stress responsiveness, and four genes related to cell division ([**Online Resource** Table 3]; Table 2 and Table 3). The 10 genes related to chromatin remodelling and transcriptional control (Table 1) are also of interest in relation to the effects of XGOs on other down-stream processes.

The expression of selected genes, showing significant changes in expression on the microarrays, was further tested by real time RT-PCR. These were selected to represent functional groups of specific interest in relation to the role of XGOs: cell wall remodelling, signal transduction and auxin responses, and defence responses. For this experiment, expression with and without XGO treatment was compared both after 2 d to confirm the array result and also after only 1 h treatment to establish whether the XGOs elicited any very rapid transcriptional responses (Fig 3). The individual results are described below.

Cell wall metabolism

Three of the differentially expressed genes have putative functions in cell-wall architecture. Expression of genes encoding a putative XTH-related protein and a cell wall invertase were both up-regulated by 3.4- and 2.9-fold respectively, while a gene encoding a putative (1-4)-beta-mannan endohydrolase was down-regulated by 2.9-fold. The closest match to the *XTH*-like gene (CV020867) in *Arabidopsis thaliana* was to *XTH9* (AT4G03210), encoding an enzyme involved in loosening and rearrangement of the cell wall and maximally expressed in vegetative and floral shoot apices [34]. The tobacco Expressed Sequence Tag (EST) used to design oligos for the microarray was 64% homologous to the Arabidopsis *XTH9* at the amino acid level and includes the XTH conserved active site motif [61]. Real time RT-PCR confirmed the up-regulation of the tobacco *XTH*-like gene in the XGO treated cultures compared to controls on day 2 of culture (Fig 3A). Furthermore it also revealed a very rapid up-regulation of the *XTH*-like gene expression within 1 h of XGO addition. Expression of this gene fell in both control and XGO treated cells from day 0 to day 2 of culture.

Signal transduction and responses

Nineteen genes putatively related to signal transduction or signal responses showed differential expression (Table 2), ten were up-regulated whereas nine were down-regulated. Up-regulated genes included those with putative functions in calcium mediated signalling, and responses to auxin and jasmonic acid (JA). Down-regulated genes included those with putative functions in development and phototropism, and signal transduction of brassinosteroids.

Ten genes with homology to kinases were differentially expressed; four were up-regulated, while the other six were down-regulated. Two of the kinase genes showed closest homology to phosphofructokinase B type family (PFKB-type), putatively involved in metabolic functions [56] and one is related to cytoskeletal functions. The remaining genes showed homology to receptors such as RLKs, serine/threonine protein kinase family and leucine rich repeat family, all of which may have roles in signalling [64].

A gene with homology to an *Arabidopsis* GTP-binding family protein (AT5G54840) was down-regulated on the arrays (3.4-fold). In *Arabidopsis* this gene (*AtSGP1*) is expressed in the quiescent centre of the root apical meristem, columella of the root cap, guard cells and stele, and may play an important role in signalling of cell fate/ cell differentiation [4]. Real time PCR confirmed the down-regulation of expression with XGO treatment (Fig. 3B) both after the 2 d time period tested in the arrays and also less dramatically, but still significantly after just 1h of XGO treatment. In contrast, expression of this gene increased significantly in the two days of culture in the untreated control cells.

Auxin-induced genes not specifically related to signalling also included a gene with homology to *Medicago truncatula NODULIN21* (*MtN21*) (up-regulated by 2.4-fold), and genes encoding proteins with functions in carbohydrate metabolism, e.g., β-galactosidase (up-regulated by 2.6-fold), which are regulated by auxin in other plant species [11]. Expression of *DW001943*, a gene showing 79% homology to an *Arabidopsis* auxin-responsive gene (AT2G04850, [55]) at the amino acid level was also up-regulated in the arrays (3.1-fold). This was confirmed by real-time PCR where the expression of this gene on day 2 of culture was significantly higher when grown in the presence of XGO than without XGO (Fig. 3C). Furthermore, expression of this gene was strongly induced following the 1 h exposure to XGO on day 0 suggesting a very rapid response, but then fell during continuous exposure to XGO over the 2 d culture period. Conversely in control cells cultured without XGO, expression rose between day 0 and day 2 of culture.

Stress responsive genes

Several of the differentially expressed genes also have putative functions in stress responses, both biotic (seven genes) and abiotic (11 genes) (Table 3). Differentially expressed genes related to elevated biotic stress included a chitinase-like gene with closest homology to

AT3G12500, a gene involved in the ethylene/ JA mediated signalling pathway during systemic acquired resistance [75]. Two tobacco targets were homologous to this gene, one of which was up- and the other-down regulated. Expression of a gene with homology to *Arabidopsis JAZ8* (CQ809070; jasmonate-zim-domain protein 8, AT1G30135), was also up-regulated (by 2.7-fold). Although the overall homology to the *Arabidopsis* gene is low, the tobacco EST contains the TIFY sequence which is required as part of the ZIM domain for protein-protein interactions between JAZ family proteins [13]. In *Arabidopsis*, JAZ proteins act as repressors of JA signalling and mediate various jasmonate-regulated processes, including defence [12]. Up-regulation of the tobacco *JAZ8*-like gene with XGO treatment compared to untreated control cells on day 2 of culture was verified by real time RT-PCR. In control cells expression was undetectable at either time point, but was rapidly induced by the 1 h XGO exposure on day 0. Expression levels then fell in continuous exposure to XGO after 2 d of culture [**Online Resource** Fig 2].

A gene with closest homology to *Arabidopsis LOL1* (AT1G32540) was down-regulated 3.4-fold. The homology between the tobacco EST (EB428982) and *LOL1* covers one of the three *LOL1* zinc finger domains [23]. *LOL1* encodes a DNA binding protein which promotes cell death and is involved in the hypersensitive response. Reduced *LOL1* expression was reflected by the real-time PCR results (Fig. 3D). Remarkably, expression of this gene was highly induced by the 1 h XGO treatment on day 0 indicating a rapid response to the XGO treatment but fell between day 0 and day 2 of culture in both control and XGO treated cells.

Genes relating to iron deficiency, heat, including two heat shock proteins (HSPs), cold and hypoxia were all up-regulated (Table 3). However, genes related to dehydration, cold, DNA repair and wounding, were all down-regulated.

Cell cycle related genes

Six genes on the array showing altered expression with XGO treatment have putative functions in cell cycle control. Three were up- and three were down-regulated. One of the up-regulated genes shows homology to *Arabidopsis TSK* (*TONSOKU*, AT3G18730) (3.7-fold), which encodes a protein necessary for cell cycle progression at G2/M phase [71]. Also there was a 4.7-fold up regulation of a microtubule motor gene (encoding a kinesin-like protein), and a 2-fold up regulation of a gene with homology to *Arabidopsis GAMMA-H2AX* (gamma histone variant *H2AX*, AT1G54690. Interestingly, the gene encoding the kinesin-like protein is preferentially expressed in mitotic BY-2 cells and appears to function mainly in cell division [47]; γ-H2AX in *Arabidopsis* plays a role in meiotic processes [9].

All three of the down-regulated genes with putative functions in cell division showed homology to kinesin-like proteins. One of these (BP130115, down-regulated by 2.5-fold) was most highly expressed in the log phase of BY-2 cells [48] and may be involved in cytokinesis. The other two: EB448475 and BP527174 show closest homology to an *Arabidopsis* kinesin motor protein (AT5G65460) involved in cytokinesis [77] and actin mediated chloroplast movement [70].

Chromatin remodelling, histone associated and transcriptional control

Three up-regulated genes had putative functions related to histone modification and chromatin remodelling. These included a histone deacetylase (BP529582) (2.1-fold) and a gene with homology to a meiosis specific histone protein (EB449808) (*H2AX*), up-regulated by 2-fold already discussed above. In addition, a tobacco gene (U01961) with homology to *Arabidopsis HAC1* (AT1G79000), was also up-regulated by 4.5-fold. *HAC1* is a H3/H4 histone acetyltransferase involved in the regulation of flowering time [21].

Seven transcription factors were differentially expressed (Table 4). Four with homology to *MADS5, MYB68, DUO1* and *ZAT6* were up-regulated, whereas three with homology to a

C3HC4-type RING finger, *BHLH093* and *NAC* domain transcription factors were down-regulated. Two of the up-regulated transcription factors show homology to *Arabidopsis* genes involved with root development: *MYB68* is maximally expressed in roots [54] and *ZAT6* helps regulation of phosphate homeostasis during root development [22]. Less is known about the *Arabidopsis* homologues to the down-regulated transcription factors, although *BHLH093* may have a role in stomatal development [57].

Discussion

XGOs stimulate growth

Changes in fresh weight and the higher and anticipated mitotic index peak with XGO treatment of tobacco BY-2 cells confirm previous reports [31, 38] that XGOs stimulate cell division both as individual compounds and as the natural extract containing a mixture of XGOs used here. The fall in mitotic cell size in both control and XGO treated cells during the peak of mitotic index, regaining original size by the end of the culture period, is in line with previous observations in our lab [65]. The null effect of XGO on cell size is also in agreement with the finding that XGO treatment of BY-2 cells shortens G1 whilst mitotic cell size remained constant [31]. Kaida *et al.* [38] found a reduction in cell size associated with XGO treatment of a different tobacco cell culture system (XD-6 derived from *Nicotiana tabacum* L. var. Xanthi). This is in agreement with the significant reduction in cell area at day 2 of culture in the XGO treated cells found here.

The coincidence between timing of the increase in the mitotic index and peak in *CDKB1;2* expression are consistent with a previous report of *CDKB1* RNA expression during the complete BY-2 cell growth cycle [68], where this gene was highly expressed within the exponential growth phase and then declined substantially as cells exited the cell cycle and entered stationary phase. *CDKB1* transcripts and protein accumulate during S, G2, and M phases and their associated kinase activity peaks during mitosis [36].

Microarray analysis reveals changes in the expression of genes with putative functions in cell wall metabolism, the cell cycle, auxin and stress responses

The clear differentiation between expression profiles of XGO treated and untreated BY-2 cells shown by PCA and the similar proportions of up- or down-regulated genes indicate that the cellular effects seen with XGO treatment involve complex changes in gene expression.

In a previous microarray analysis characterizing gene expression during normal growth of BY-2 cells, Matsuoka et al [48] found that log phase cells predominantly expressed DNA/chromosome duplication gene homologues. In addition, many genes for basic transcription and translation machineries, as well as proteasomal genes, were up-regulated at this growth phase. Our findings are consistent with these previous results. However, we show here that when challenged with XGOs differentially expressed genes include those related to cell wall metabolism, the cell cycle, auxin responses as well as stress responses: both biotic and abiotic.

A putative XTH-related gene was up-regulated by XGO treatment

The up-regulation of a gene with close homology to an *XTH* by XGO treatment is consistent with an increase in xyloglucan endotransglycosylase activity in response to XGOs in *Azuki* bean hypocotyls [39], which correlated with increased cell wall extensibility and xyloglucan breakdown. Kaku et al. [39] suggested that the XGOs may stimulate endotransglycosylation by acting as acceptor substrates. Data presented here show increased transcription of an *XTH*-like gene in response to XGOs. The very rapid transcriptional up-regulation of the *XTH*-like expression following only 1 h of XGO treatment shown here suggests a direct effect of the XGOs on transcription, stimulating increased enzyme production in addition to effects on enzyme activity [39]. The fall in transcript levels with continuous XGO treatment is likely due to a feedback system ensuring homeostasis of cell wall turnover.

XGO treatment results in changes in the expression of genes related to cell division and differentiation

Part of the positive growth effects seen in previous studies [1, 2, 31] in response to XGO treatment can be attributed to increased competence of cells to enter mitosis shown here and in Kaida et al [38], although they did not report on changes in the mitotic index peak or effects on gene expression. Of significance in this context is the up-regulation of *TSK* (*TONSOKU*) reported here, which is required during the cell cycle. *tsk* mutants are delayed in G2/M progression [71] which may be caused by activation of the G2/M checkpoint, or defects in mitosis. TSK localizes to the ends of spindle microtubules during mitosis, and defects in TSK cause disruption of the cell division plane [72]. Thus TSK is probably required for correct organisation of the spindle structure.

Up-regulation of a gene encoding a kinesin-like protein, *TBK1*, is consistent with its preferential expression in mitotic BY-2 cells [47] suggesting a role during cell division. Thus the treatment with XGOs may also be affecting cell division through up-regulation of genes that are required for mitosis. Moreover, down-regulation of the *AtSGP1*-like gene, involved in cell fate/ cell differentiation signalling in *Arabidopsis* [4], is consistent with an effect of XGOs in promoting mitosis and repressing differentiation.

XGO's treatment affects the expression of genes related to signalling by and responses to plant growth regulators

Since exogenous XGOs elicit clear cellular effects, directly or indirectly, it follows that this signal must be perceived and transduced within the cell. The finding that four genes with homology to serine/threonine (Ser/Thr) kinases and four with homology to leucine rich repeat (LRR) proteins were differentially expressed in response to the XGO treatment is thus consistent with a signalling role for XGOs.

A class of Ser/Thr protein kinases that are tightly bound to the cell wall, named wall-associated kinases (WAKs), are candidate receptors for oligogalacturonides (OGs) released from the plant cell wall. Notably WAKs bind these oligosaccharides *in vitro* [6, 8, 20]. Possibly other members of the WAK family also bind XGOs. Thus future work to characterise the Ser/Thr receptor-like genes that are differentially expressed in response to exogenous XGOs will be an important step towards understanding the mode of action of these oligosaccharides in plants.

Another class of receptors that could be mediating the signal transduction of xyloglucans comprises leucine-rich repeat transmembrane protein kinases (LRRs) as they are involved in response to several plant growth regulators; e.g., brassinosteroids [41], ethylene [78] and gibberellins [76].

Given the early reports suggesting an interaction between XGOs and auxins [51, 52] we noted here the differential expression of several auxin responsive genes which supports this interaction. The complexity of the interaction between XGOs and auxin [51, 52] is reflected in the transcript levels of an auxin-responsive gene (DW001943), which was very rapidly up-regulated following just 1 h of treatment with XGOs but then fell during the following 2 d.

The up-regulation of this gene in control cultures, mirroring the rise in the mitotic index, is consistent with the expression of another auxin-responsive gene (*arcA*) in cultured BY-2 cells [37] whose expression fell in parallel with a fall in the mitotic index.

XGOs as elicitors of plant defences and responses to stress

One notable finding from the microarray analysis was the differential expression of several stress responsive genes, which supports earlier reports that XGOs may have a role in acting as elicitors of plant defence [67, 69]. The differential expression of chitinase genes supports previous reports of the effects of xyloglucan fragments prepared from tamarind seeds and pea stems. Increased activity of peroxidase, beta-1,3-glucanase and chitinase occurred in the extracellular fluid of cucumber cotyledons which relates to the hypersensitive response of cucumber to Tobacco Necrosis Virus (TNV) [67]. The rapid up-regulation of two defence-response related genes, *JAZ8* and *LOL1*- like genes in response to the XGOs, followed by a decline over the 2 d culture period is similar to the wounding response of *JAZ8* in *Arabidopsis* which is rapidly induced by wounding [13] with maximal levels after 1 h thereafter falling off.

Also of interest were the class of differentially expressed genes that have putative roles in abiotic stress responses, including genes that respond to all the stress related plant growth regulators. To our knowledge, although other oligosaccharins have been associated with responses to abiotic stress [81, 82], a link between XGO treatment and abiotic stress had not previously been made. Effects of XGOs on abiotic stress responses require further work.

Conclusions

We show for the first time, that XGO treatment of tobacco BY-2 cells with a natural admixture of XGOs, thus representing more closely XGOs in vivo, elicits substantial changes in gene expression. These changes cover several important biological processes which are probably related to XGO function in whole plants. Of particular significance is the finding that XTH activity may be promoted through transcriptional activation. Up-regulation of genes promoting mitosis, and down regulation of genes promoting differentiation with XGO treatment explains the increase in mitotic cells. Our data also support reports of positive effects of XGOs as elicitors, and further suggest that XGOs may be involved in abiotic stress responses.

Supplementary information (online resources)

Supplementary Fig 1 MALDI-TOF mass spectra of xyloglucan oligosaccharides (NB XGO isomers are not distinguished by MALDI-TOF analysis).

Supplementary Fig 2 Comparative expression (by real-time PCR) of *JAZ8*-like gene in BY2 cells treated with 0.1 mg L^{-1} XGO at day 0 (X-0) and day 2 (X-2) of culture (mean ± S.E., n= 3, different letters indicate statistically different means $P < 0.05$).

Supplementary Table 1: XGO composition in cellulase hydrolysates of *Tamarindus indica* L. xyloglucan as determined my MALDI-TOF mass spectrometry.

Supplementary Table 2: Primers used for real time PCR analysis. The gene identification corresponds to the sequence annotated in the GeneBank database of NCBI (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and used as template for primer design. F: forward and R: reverse primers.

Supplementary Table 3: Results of microarray analysis: probes that were up or down regulated by ≥2-fold and putative functions where data are available.

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FIGURE LEGENDS

Fig. 1 Effects of XGO treatment in the tobacco BY2 cell line on: (A) growth (fresh weight of cell culture after 7 d culture, mean ± S.E. n ≥ 3), (B) mitotic index (% frequency of cells in division; mean ± S.E., n= 3), (C) mitotic cell area (μm^2, mean ± S.E. n= 15-20) and (D) expression of CDKB1;2 ± XGO by real-time PCR; (±S.E., n=2) over 3 days of culture * $= P < 0.05$, *** $= P < 0.001$ compared to 0 mgL^{-1} XGO on each day).

Fig. 2 Principal Components Analysis (PCA) of the microarray data (two replicates each of XGO treated and control). All genes are plotted with respect to first and second principal components. Samples occupying similar position in PC space share similar gene expression trends.

Fig. 3 Expression pattern (by real-time PCR) of (A) *XTH*-like gene (CV020867), (B) a GTP binding protein, (C) a putative auxin-responsive gene (DW001943), (D) a *LOL1*-like gene (EB428982) in BY2 untreated cells on day 0 (C-0) and day 2 (C-2) of culture and in cells treated with 0.1 mg L^{-1} XGO (X-0 and X-2) (mean ± S.E., n= 3, different letters indicate statistically different means $P < 0.05$).

TABLES

Table 1: Functional groups of genes whose expression was up- or down-regulated (>2-fold) in response to XGO treatment

Predicted protein function	up	down	total	%
ATPase	0	2	2	1.5
biosynthesis	2	2	4	3.0
calcium binding	1	1	2	1.5
carbohydrate binding	2	3	5	3.7
cell wall metabolism	2	1	3	2.2
chaperonin/HSPs	3	0	3	2.2
chitinase	1	1	2	1.5
chromatin /histone associated	4	0	4	3.0
cyt P450	2	0	2	1.5
cytoskeleton	1	5	6	4.5
Glutathione-S-transferase	1	0	1	0.7
GTPase	1	1	2	1.5
hydrolase	4	2	6	4.5
kinase	4	5	9	6.7
lipid binding	2	2	4	3.0
metabolism	1	3	4	3.0
nucleic acid binding	6	6	12	9.0
oxidoreductase	5	1	6	4.5
phosphatase	0	2	2	1.5
photosynthesis	4	5	9	6.7
proteolysis	12	5	17	12.7
protein binding	4	6	10	7.5
receptor	2	0	2	1.5
ribosomal	1	1	2	1.5
secondary metabolism	3	0	3	2.2
transcription factor	3	3	6	4.5
transferase	0	5	5	3.7
transporter	6	0	6	4.5
unknown	6	0	6	4.5

Table 2: Differentially expressed genes related to signal transduction and responses, whose expression was up- or down-regulated >2-fold in response to XGO treatment.

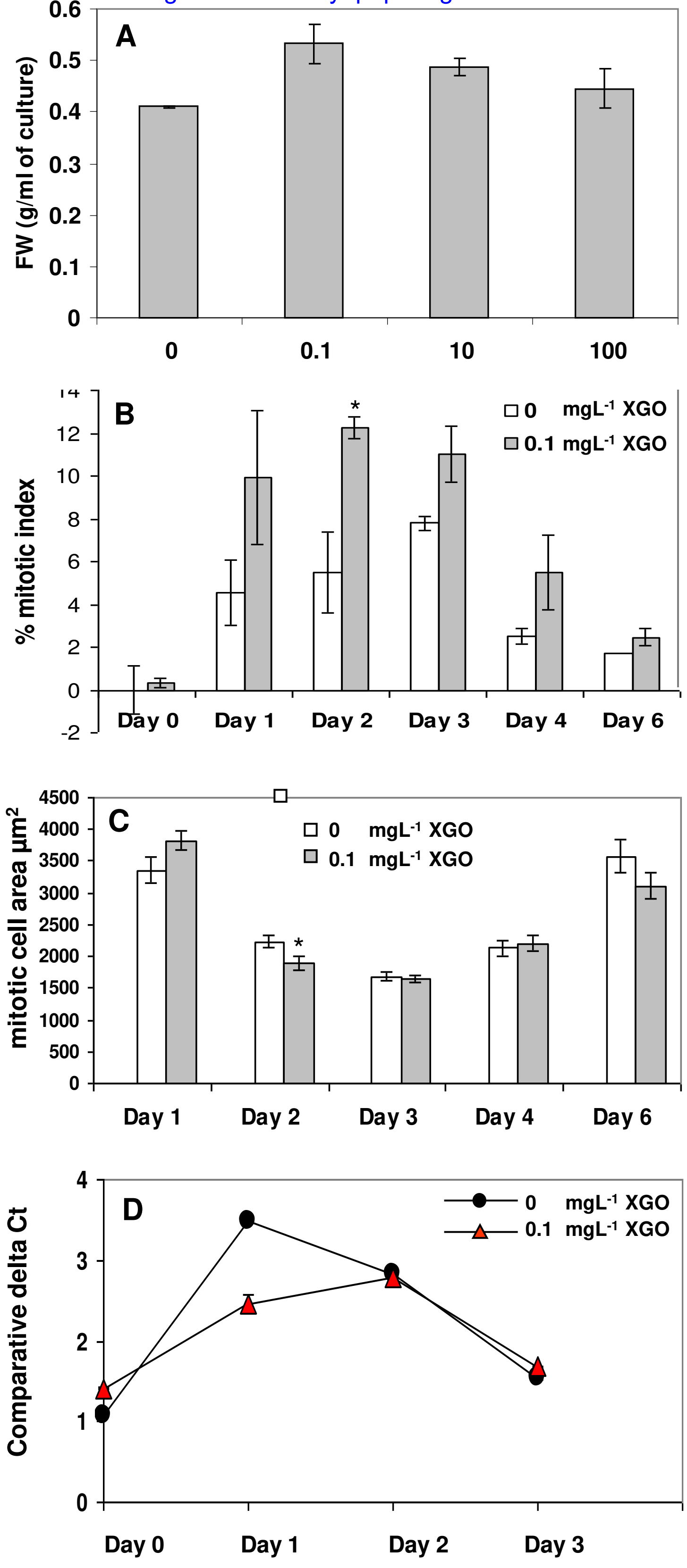
Probe Set ID	FCA absolute (Fold change on array)	Closest Arabidopsis homologue AGI code	Description
Up-regulated			
DW001943_at	3.1	AT2G04850	auxin-responsive protein-related
BP134562_at	2.2	AT5G38210	serine/threonine protein kinase family protein
BP526893_at	3.4	AT5G43020	leucine-rich repeat transmembrane protein kinase, putative
C4219_at	2.5	AT4G28950	*ROP9 (RHO-RELATED PROTEIN FROM PLANTS 9*)
BP526893_at	3.4	AT5G43020	leucine-rich repeat transmembrane protein kinase, putative
BP192587_at	2.5	AT4G24480	serine/threonine protein kinase, putative
MT203B_at	2.7	AT1G30135	*JAZ8/TIFY5A (JASMONATE-ZIM-DOMAIN PROTEIN 8)*
BP529083_at	2.7	AT3G63060	circadian clock coupling factor *ZGT*
BP136882_at	2.9	AT3G01400	armadillo/beta-catenin repeat family protein
EB442205_at	3.8	AT2G26190	calmodulin-binding family protein
Down regulated			
BP526151_at	2.1	AT2G16250	leucine-rich repeat transmembrane protein kinase, putative
BP128689_at	2.2	AT1G15750	*TPL/WSIP1 (WUS-INTERACTING PROTEIN 1);* protein binding
BP129606_at	3.1	AT1G24650	leucine-rich repeat family protein / protein kinase family protein
C2467_at	2.3	AT2G30520	*RPT2 (ROOT PHOTOTROPISM 2);* protein binding
C9904_at	2.4	AT5G60900	*RLK1 (RECEPTOR-LIKE PROTEIN KINASE 1)*
C2929_at	3.4	AT5G54840	GTP-binding family protein
BP131989_at	2.6	AT4G31160	transducin family protein / WD-40 repeat family protein
BP130215_at	2.6	AT3G13670	serine/threonine protein kinase family protein
C4477_at	4.8	AT1G32130	involved in brassinosteroid-regulated gene expression.

Table 3: Differentially expressed genes related to stress responses whose expression was up- or down-regulated >2-fold in response to XGO treatment.

Probe Set ID	FCA absolute (Fold change on array)	Closest Arabidopsis homologue AGI code	Description	Response to/ function
Up-regulated				
DQ131889_x_at	2.1	AT4G31970	*CYP82C2* (cytochrome P450)	hypoxia
C9400_at	2.8	AT4G31940	*CYP82C4* (cytochrome P450)	low Fe
C6549_at	3.5	AT3G12500	*ATHCHIB (BASIC CHITINASE);* chitinase	biotic stress
C2859_s_at	2.6	AT1G78380	*ATGSTU19 (GLUTATHIONE TRANSFERASE 8)*	drought, oxidative stress
C5973_at	3.4	AT1G53540	17.6 kDa class I small heat shock protein (*HSP17.6C-CI*)	heat shock protein
BP132586_at	2.2	AT2G26890	*GRV2 (KATAMARI2*); binding / heat shock protein binding	heat shock protein
C2748_at	3.3	AT1G72860	disease resistance protein (TIR-NBS-LRR class), putative	biotic stress
MT203B_at	2.7	AT1G30135	*JAZ8/TIFY5A (JASMONATE-ZIM-DOMAIN PROTEIN 8)*	biotic stress
CV016057_at	2.6	AT2G15970	*COR413-PM1* (cold regulated 413 plasma membrane 1)	cold
EB447067_s_at	3.6	AT1G65870	disease resistance-responsive family protein	biotic stress
CN498873_s_at	6.1		induced by the bacterial effector protein AvrPto	biotic stress
Down-regulated				
EB425750_at	3.2	AT1G70670	caleosin-related family protein	drought, ABA
C8646_at	2.9	AT3G12500	*ATHCHIB (BASIC CHITINASE);* chitinase	biotic stress
EB428982_at	3.4	AT1G32540	*LOL1 (LSD ONE LIKE 1)*	biotic stress
BP528192_at	3.7	AT1G80210	*BRCA1/BRCA2-CONTAINING COMPLEX 36 HOMOLOG A*	DNA repair
BP192659_at	3.2	AT5G53000	*TAP46 (2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 KD)*	cold
DW001183_at	2.1	AT1G55480	*ZKT*, phosphorylated at Thr and Ser residues after wounding	wounding
BP132027_at	2.5	AT4G18030	SAM methyl transferase family protein	dehydration

Table 4: Differentially expressed genes with homology to transcription factors whose expression was up- or down-regulated >2-fold in response to XGO treatment.

Probe Set ID	FCA absolute (Fold change on array)	Closest Arabidopsis homologue AGI code	Description	Response to/ function
Up-regulated				
AF068724_at	2.9	AT1G69120	MADS box protein *MADS5*	MADS domain transcription factor
BP526619_at	2.2	AT5G65790	*MYB 68* (myb domain protein 68)	response to gibberellin stimulus, response to salicylic acid stimulus
EB643472_x_at	2.7	AT3G60460	*DUO1 MYB* transcription factor	required for male gamete formation
BP528590_at	3.5	AT5G04340	*C2H2 (ZINC FINGER OF ARABIDOPSIS THALIANA 6; ZAT6)*	Root development and phosphate homeostasis
Down-regulated				
C249_at	2.1	AT3G14320	zinc finger (*C3HC4*-type RING finger) family protein	Highly expressed in seed
EB678910_at	3.1	AT5G65640	*BHLH093 (BETA HLH PROTEIN 93)*	Maximal expression in floral apex and hypocotyl, role in stomatal development
EB683185_at	2.1	AT2G24430	*ANAC038/ANAC039*	NAC domain, seed-specific expression



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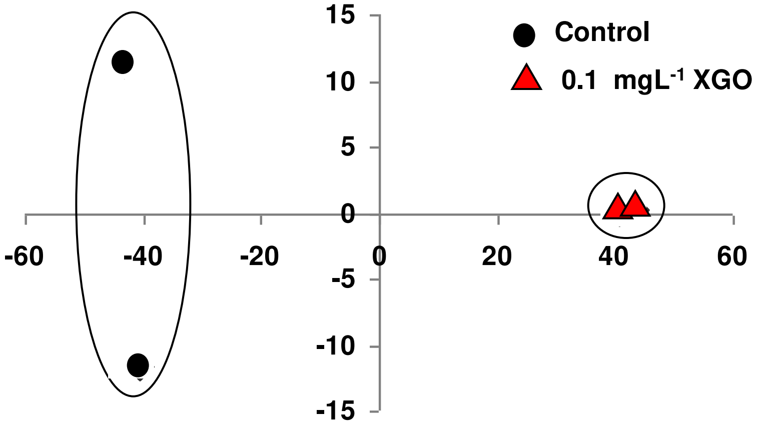
González-Pérez et al. Fig. 1



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González-Pérez et al. Fig. 2

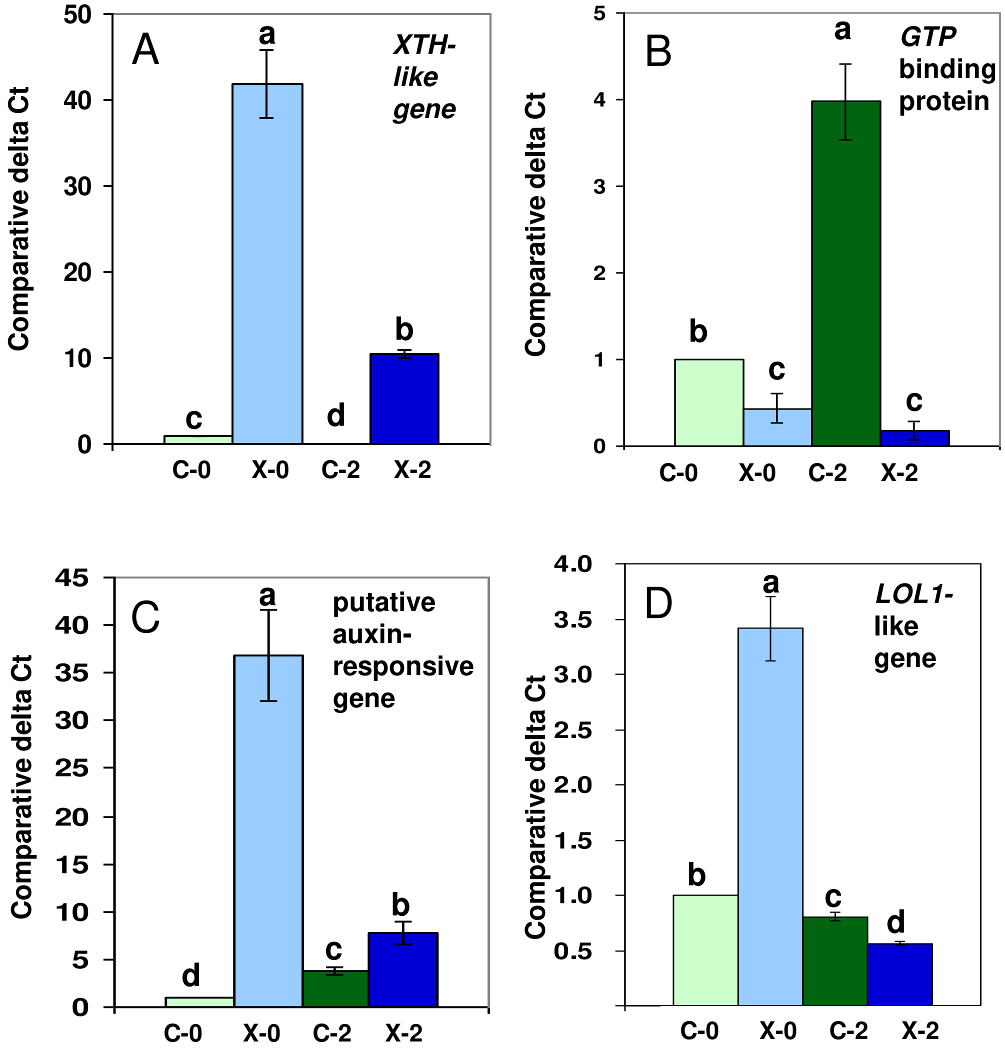

Fig. 3 Expression pattern (by real-time PCR) of (A) *XTH*-like gene (CV020867), (B) a GTP binding protein (C) a putative auxin-responsive gene (DW001943) (D) a *LOL1*-like gene (EB428982) in BY2 cells extracted from untreated cells at day 0 (C-0) and day 2(C-2) ± 0.1 mg L^{-1} XGO (X-0 and X-2) (mean ± S.E., n= 3, different letters indicate statistically different means $P < 0.05$).

González-Pérez et al. Fig. 3

Supplementary Figs

Supplementary Tables 1-2

Supplementary Table 3