

Methyl jasmonate represses growth and affects cell cycle progression in cultured *Taxus* cells

Rohan A. Patil · Sangram K. Lenka ·
Jennifer Normanly · Elsbeth L. Walker ·
Susan C. Roberts

Received: 20 February 2014 / Revised: 18 April 2014 / Accepted: 26 April 2014 / Published online: 16 May 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract

Key message Methyl jasmonate elicitation of *Taxus* cultures enhances paclitaxel accumulation, but represses growth by inhibition of cell cycle progression. Growth repression is evident both at the culture level and transcriptional level.

Abstract Methyl jasmonate (MeJA) elicitation is an effective strategy to induce and enhance synthesis of the anticancer agent paclitaxel (Taxol[®]) in *Taxus* cell suspension cultures; however, concurrent decreases in growth are often observed, which is problematic for large-scale bioprocessing. Here, increased accumulation of paclitaxel in *Taxus cuspidata* suspension cultures with MeJA elicitation was accompanied by a concomitant decrease in cell growth, evident within the first 3 days post-elicitation. Both MeJA-elicited and mock-elicited cultures exhibited similar viability with no apoptosis up to day 16 and day 24 of the cell culture period, respectively, suggesting that

growth repression is not attributable to cell death. Flow cytometric analyses demonstrated that MeJA perturbed cell cycle progression of asynchronously dividing *Taxus* cells. MeJA slowed down cell cycle progression, impaired the G1/S transition as observed by an increase in G0/G1 phase cells, and decreased the number of actively dividing cells. Through a combination of deep sequencing and gene expression analyses, the expression status of *Taxus* cell cycle-associated genes correlated with observations at the culture level. Results from this study provide valuable insight into the mechanisms governing MeJA perception and subsequent events leading to repression of *Taxus* cell growth.

Keywords Cell cycle · Next-generation sequencing · Methyl jasmonate · Paclitaxel · Plant cell culture · *Taxus*

Introduction

Dedifferentiated plant cell suspension cultures provide a regulated environment independent of geographical and external environmental factors for the synthesis of plant-based secondary metabolites. Cell suspension culture offers a distinct advantage over tissue or organ culture as existing bioprocess technology developed for microbial and mammalian cells can be easily adapted to plant cells (Kieran et al. 1997). The use of elicitors in plant cell suspension culture can both increase product yields and consequently decrease the long fermentation times, facilitating the use of plant cell culture technology in commercial applications. Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA) have been widely used as elicitors to induce secondary metabolite production in a variety of plant cell culture systems (Gundlach et al. 1992; Lijavetzky et al.

Communicated by K. K. Kamo.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-014-1632-5) contains supplementary material, which is available to authorized users.

R. A. Patil · S. C. Roberts (✉)
Department of Chemical Engineering, Institute for Cellular
Engineering, University of Massachusetts, 686 North Pleasant
Street, Amherst, MA 01003, USA
e-mail: sroberts@ecs.umass.edu

S. K. Lenka · E. L. Walker
Department of Biology, University of Massachusetts, Amherst,
MA 01003, USA

J. Normanly
Department of Biochemistry and Molecular Biology, University
of Massachusetts, Amherst, MA 01003, USA

2008; Pauwels et al. 2008; Yazaki et al. 1997). In particular, jasmonates have been effective at enhancing production of the anticancer drug paclitaxel (Taxol[®]) in a variety of *Taxus* species and cell cultures (Bonfill et al. 2006; Ketchum et al. 1999; Yukimune et al. 1996). Paclitaxel is widely used for treatment of breast, ovarian and lung cancers as well as AIDS-related Kaposi's sarcoma, and is being investigated for use in the treatment of neurological disorders and in post-surgery heart patients (Vongpaseuth and Roberts 2007). Paclitaxel titers of up to 900 mg/L have been achieved in industrial environments using a combination of MeJA elicitation and cell culture optimization strategies (Bringi et al. 2007).

Increased secondary metabolite accumulation upon MeJA elicitation is often accompanied with concurrent decreases in culture growth (Kim et al. 2005; Thanh et al. 2005; Zhang and Turner 2008; Sun et al. 2013). MeJA has been shown to broadly induce defense responses and secondary metabolism in plants (Farmer and Ryan 1990; Reymond and Farmer 1998; Seo et al. 2001), which diverts carbon resource allocation from primary metabolism (Logemann et al. 1995; Pauwels et al. 2009). Recent studies indicate that MeJA-mediated growth inhibition is associated with perturbations in mitochondrial membrane integrity along with decreases in the biosynthesis of ATP (Ruiz-May et al. 2011) and proteins related to energy metabolism (Cho et al. 2007).

At a mechanistic level, MeJA has demonstrated an inhibitory effect on growth at the level of the cell cycle (Pauwels et al. 2008; Swiatek et al. 2002). Most studies to understand the effect of jasmonates on the cell cycle have been done in angiosperms, such as *Arabidopsis thaliana* and tobacco BY-2 cell suspension cultures (Pauwels et al. 2008; Swiatek et al. 2002). Exogenously applied MeJA blocks the G1/S and G2/M transitions in the cell cycle of cultured tobacco BY-2 cells (Swiatek et al. 2002). Micromolar concentrations of MeJA added to *A. thaliana* suspension cultures repressed the activation of M phase genes, arresting cells in G2 phase (Pauwels et al. 2008). Genomic information and established protocols for synchronizing cell cultures (Kumagai-Sano et al. 2006; Menges et al. 2002) to understand cell cycle events are readily available for these plant species, facilitating mechanistic studies. In contrast, gymnosperms such as *Taxus* have not been as well studied with regard to cell cycle progression and the mechanism of MeJA-repressed growth.

While a number of studies have reported increased taxane biosynthetic pathway gene products upon MeJA elicitation (Jennewein et al. 2004; Nims et al. 2006; Patil et al. 2012; Li et al. 2012), there have been few reports regarding the role of MeJA on growth inhibition and cell cycle progression in *Taxus* cultures (Kim et al. 2005; Naill and Roberts 2005a). In the present study, we investigate the

influence of MeJA on both cell growth and viability of *Taxus* cells in batch culture. The effect of MeJA on cell cycle progression was determined using asynchronous *Taxus cuspidata* cells. Actively dividing cells were quantified and cell cycle kinetics were determined by cumulative and pulse labeling using 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analog of thymidine. Recently obtained 454 and Illumina transcriptome sequencing data for both MeJA-elicited and mock-elicited cultures were used to obtain the expression status of cell cycle-associated genes in the asynchronous *T. cuspidata* cultured cells. There is currently minimal sequence information on cell cycle-regulated genes derived from this division of the plant kingdom (Li et al. 2012; Sun et al. 2013), and these studies provide the first insight into cell cycle control upon elicitation with MeJA. Because the mechanism of action of MeJA has not been investigated to date for gymnosperms such as *Taxus*, strategies to promote growth, while still enhancing secondary metabolite synthesis for bioprocessing, have not been identified or tested. The results here show that MeJA-induced growth repression in *Taxus* growth occurs at the level of cell cycle, providing important mechanistic information on the influence of MeJA on *Taxus* cell proliferation.

Materials and methods

Cell culture maintenance and MeJA elicitation

The *T. cuspidata* P93AF cell line was provided by the U.S. Plant Soil and Nutrition Laboratory (Ithaca, NY), and maintained in our laboratory on gyratory shakers as described previously (Kolewe et al. 2010). For elicitation, 40 μ L of 100 % methyl jasmonate (MeJA) was added to 460 μ L of 95 % (v/v) ethanol and 500- μ L nanopure water. This solution was vortexed and then filtered through a 0.2- μ m Gelman PVDF filter into a sterile container. This solution was then added to the cultures on day 7 post-transfer to yield a final concentration of 150 μ M. The foam closures were covered with aluminum foil to prevent evaporation. Mock-elicited cultures were generated using equivalent amounts of sterile water instead of MeJA. All chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Biomass and taxane content measurements

A Multisizer 3TM Coulter counter equipped with a 2,000 μ m aperture (Beckman Coulter, Brea, CA, USA) was used to determine total biomass dry weight based on previously published correlations (Kolewe et al. 2010). For analysis, 2 \times 2 mL samples of well-mixed culture broth

(cells plus media) were taken from each flask. Taxane content in mock-elicited and MeJA-elicited cultures were analyzed at several time points post-elicitation using Ultra Performance Liquid Chromatography (UPLC), as described previously (Patil et al. 2012).

Viability analysis

Qualitative analysis of viability was performed by staining with fluorescein diacetate (FDA) and propidium iodide (PI). 500 μL of well-mixed culture was sampled, to which 10 μL of a 0.5 mg/mL FDA stock solution and 4 μL of a 1 mg/mL PI stock solution was added. After a 10-min incubation in the dark, supernatant was removed, and 500 μL of fresh Gamborg's B5 medium was added. 100 μL of cell suspension was observed under a fluorescence microscope. A Zeiss Axiovert 200 inverted microscope fitted with a blue filter set (excitation at 450–490 nm and emission above 515 nm) for FDA fluorescence and a green filter set (excitation at 530–560 nm and emission above 580 nm) for PI fluorescence was used. FDA detects living cells, whereas PI detects non-viable cells. Note that esterases released from non-viable cells can result in background fluorescence.

DNA laddering assay

Total genomic DNA was extracted following the method of Dellaporta et al. (1983) with a slight modification. Fresh cells (0.2 g) were ground in liquid nitrogen with mortar and pestle. Ground cells were transferred to a sterilized Eppendorf tube and dissolved in 600- μL buffer (pH 8.0) consisting of NaCl (100 mM), Tris/HCl (50 mM), EDTA (25 mM), sodium dodecyl sulfate (1 %, w/v), and β -mercaptoethanol (10 mM). The mixture was shaken vigorously through inversion and incubated in a water bath at 65 °C for 10 min. 250 μL of potassium acetate (5 M) was added to the mixture, which was incubated on ice for 30 min followed by centrifugation at 10,000 $\times g$ for 10 min at 4 °C. The supernatant was collected and mixed with an equal volume of isopropanol (\sim 600 μL). The precipitate formed was centrifuged, washed with 70 % (v/v) ethanol, and redissolved in 200 μL of buffer (10 mM Tris/HCl, 5 mM EDTA, pH 8.0). Further precipitation was achieved by addition of 20- μL sodium acetate (3 M, pH 5.2), followed by 500 μL of 100 % (v/v) ethanol and gentle inversion to completely mix the two phases. Precipitates were centrifuged, washed with 70 % (v/v) ethanol and then dried at 25 °C. Pellets were resuspended in 40- μL buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). RNase A (100 $\mu\text{g}/\text{mL}$) was added to digest RNA at 37 °C for 30 min. DNA concentrations were quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific,

Wilmington, DE, USA). For analysis of DNA laddering, equal amounts of DNA were run on a 1.5 % (w/v) agarose gel stained with ethidium bromide (0.5 $\mu\text{g}/\text{mL}$), and observed under a UV transilluminator. 1-kbp and 100-bp DNA ladders (New England Biolabs) were used as molecular weight markers.

Isolation and fixation of intact nuclei

Intact nuclei were isolated from *Taxus* cells in Galbraith's buffer [45 mM MgCl_2 , 30 mM sodium citrate, 20 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 0.3 % (w/v) Triton X-100, pH 7.0] as described previously (Patil et al. 2013; Gaurav et al. 2010; Galbraith et al. 1983). Post isolation, nuclei were fixed with 1 % paraformaldehyde (dissolved in Galbraith's buffer) at 4 °C for 30 min. Fixed nuclei were washed twice with Galbraith's buffer by centrifuging at 700 $\times g$ for 4 min at 4 °C. After washing, nuclei were resuspended in 1 mL of Galbraith's buffer and stored at 4 °C until further analysis.

Distribution of cells in different phases of the cell cycle

Triplicate samples of MeJA-elicited and mock-elicited cultures were sampled and nuclei were isolated at several time points post-elicitation as described above. 1 mL of the nuclei solution were aliquoted, and 50 μL of 1 mg/mL RNase and 50 μL of 1 mg/mL PI were added. Samples were stained for at least 30 min on ice before flow cytometric analysis (see below).

EdU incorporation assay

MeJA-elicited and mock-elicited *Taxus* cell cultures were maintained in a medium containing 10 μM EdU for the required incubation period (see “Cumulative and pulse labeling” below). Nuclei were isolated from the EdU-labeled cultures, fixed and incubated with 250- μL EdU detection cocktail (Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay kit, cat no: C35002, Invitrogen, Carlsbad, CA) at 25 °C for 30 min as per manufacturer's protocol, with slight modifications. For one sample reaction (250 μL), the following amounts of kit components were used: 219 μL of 1X Click-iT Reaction buffer (Component G, diluted to 1X in Galbraith's buffer), 5 μL Copper (II) sulfate solution (Component H, 100 mM aqueous CuSO_4), 1.25 μL Alexa Fluor 488 azide (Component B) and 25 μL 1X buffer additive (component I, diluted to 1X in Galbraith's buffer). Post incubation, 2 mL of Galbraith's buffer were added to the samples as a wash; samples were centrifuged at 700 $\times g$ and 4 °C for 4 min, and resuspended in 0.5-mL Galbraith's buffer for subsequent staining and analysis. 1 mg/mL of RNase A was added followed by

1 mg/mL 7-aminoactinomycin D (7-AAD) (Invitrogen, Carlsbad, CA). Samples were incubated for 60 min at 25 °C before flow cytometric analysis (see below).

Pulse labeling of MeJA-elicited and mock-elicited cultures

MeJA-elicited and mock-elicited cultures on day 5 of the culture period were pulse labeled for 4 h with 10 μ M EdU. Cells were collected by centrifugation at 800 \times g for 5 min, and washed with conditioned medium. Conditioned medium was obtained by decanting settled MeJA-elicited and mock-elicited cell suspensions incubated without EdU under the same conditions. The wash procedure was repeated two more times to eliminate excess EdU. The volume was then adjusted with conditioned medium to that before washing, and cultures were continued in the absence of EdU. Samples of pulse-labeled cells were taken periodically over 48 h and processed for EdU analysis. A series of bivariate DNA–EdU distributions at various times after pulse labeling was obtained using flow cytometry (see below).

Cumulative labeling of MeJA-elicited and mock-elicited cultures

MeJA-elicited and mock-elicited cultures on day 7 of the culture period were incubated with 10 μ M EdU. After EdU addition, nuclei were isolated and fixed every 24 h for the following 5 days. Isolated nuclei were stained for EdU (Alexa Fluor 488) and DNA content (7-AAD) (see above) and analyzed via flow cytometry (see below).

Flow cytometry

For nuclei analysis, a Becton–Dickinson (San Jose, CA) LSRII analytical flow cytometer equipped with an argon laser tuned to 488 nm with the standard filter setup was used. A minimum of 5,000 events were collected in the gated region of a forward scatter and side scatter plot corresponding to nuclei. The scatter plots were manually gated to exclude debris and doublets. For cell cycle analysis, forward scatter and side scatter were collected on a logarithmic scale, and PI fluorescence was collected on a linear scale. For cumulative- and pulse-EdU labeling analysis, a bivariate plot of DNA–EdU was obtained. Alexa Fluor 488 EdU intensity was detected between 515 and 545 nm. For detection of 7-AAD intensity (DNA content), the 663–677 nm emission range was used with the standard filter sets available on the LSRII. The boundary of EdU-labeled nuclei in biparametric plots was obtained by subtracting the background using a non-EdU-treated culture.

Cell cycle-associated contig generation, annotation and expression analysis

RNA was isolated from *T. cuspidata* P93AF cells (MeJA-elicited and mock-elicited) every 24 h post-elicitation over a time period spanning 22 days of the culture period. Equal amounts of total RNA from each culture were pooled from each time point for 454 sequencing. After rRNA depletion and fragmentation, a transcriptome library was generated by sequencing the pooled RNA sample on one full PicoTiter-Plate (PTP) using the 454 Genome Sequencer FLX Titanium SystemTM, following the manufacturer's instructions (Roche, Branford, CT). In addition, 50-bp paired end mRNA-sequencing libraries were prepared from both MeJA-elicited and mock-elicited *T. cuspidata* P93AF cells at 18- and 72-h time points using the Illumina HiSeq 2000 platform (Illumina, Inc. San Diego, CA). Reads from both 454 and Illumina sequencing libraries were used in *de novo* assembly to generate contigs using CLC genomics workbench (CLC Bio, Aarhus, Denmark) by setting A, C, G, T voting method for conflict resolution. A total of 48,614 contigs were generated (>200 bp, avg. >100 reads/contig, >50X coverage) with N50 contig length of 873 bp. These contigs were annotated using Blast2GO default parameters (Conesa et al. 2005). Based on the Blast2GO annotation, 149 contigs representing known cell cycle-associated genes were identified (Online Resource 2). Paired end reads from each Illumina library were mapped onto the contigs using CLC Genomics Workbench software. Gene expression for both MeJA-elicited and mock-elicited P93AF cells at the 18 and 72 h post-elicitation time points was calculated using the RPKM (Reads per kb per million reads) method (Mortazavi et al. 2008). To identify differentially expressed genes, the proportion-based test was used between any two RNA-seq libraries under comparison with a *P* value < 0.05 (Kal et al. 1999). To calculate the fold change between any two conditions, quantile normalization of the RPKM values was used.

Results

MeJA represses cell growth without significant changes in necrosis and apoptosis

Inhibition of growth after MeJA elicitation has been observed in a variety of *Taxus* species and cell lines (Bonfill et al. 2006; Kim et al. 2004; Laskaris et al. 1999; Yukimune et al. 1996), as well as other plant cell culture systems (Goossens et al. 2003; Thanh et al. 2005). Although most reports indicate a decrease in *Taxus* cell growth upon MeJA elicitation, some data show no difference in cell growth between MeJA-elicited and

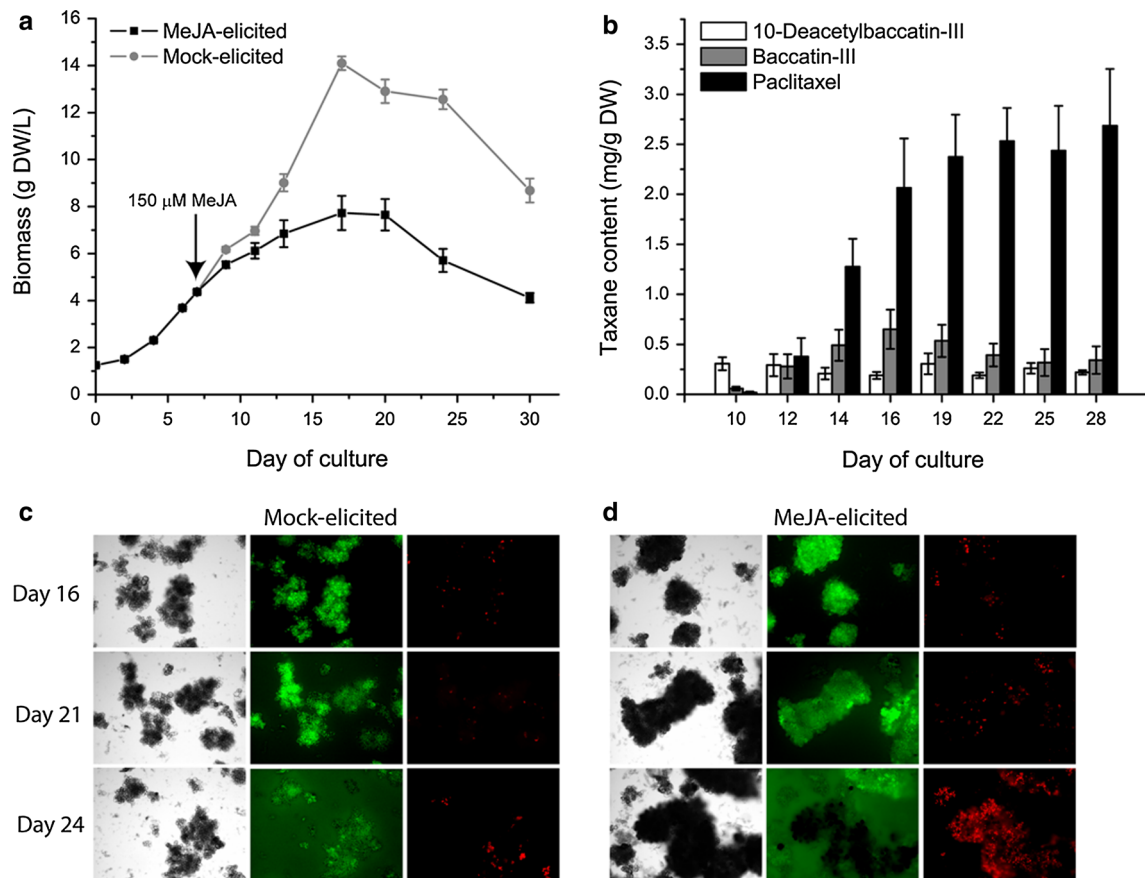


Fig. 1 Effect of MeJA elicitation on *T. cuspidata* P93AF cultures growth, taxane production and viability. **a** Biomass concentrations in MeJA-elicited cultures as compared to mock-elicited cultures. Reported values are the average of three biological replicates, and *error bars* represent standard error of the mean (*SEM*). **b** Taxane levels as determined by UPLC in MeJA-elicited cultures. Note that mock-elicited cultures did not produce detectable levels of taxanes.

Reported values are the average of three biological replicates, and *error bars* represent SEM. **c, d** Viability of mock-elicited and MeJA-elicited cultures on day 16 (Row 1), day 21 (Row 2), and day 24 (Row 3) of the culture period. Columns indicate brightfield (Column 1), FDA-stained (Column 2) and PI-stained (Column 3) images. Cultures were either mock-elicited or elicited with 150 μ M MeJA on day 7 of the culture period

non-elicited *Taxus* cell lines (Bonfill et al. 2007; Ketchum et al. 1999). Upon addition of MeJA on day 7 of the culture period, *T. cuspidata* P93AF cell cultures clearly demonstrate repressed growth (Fig. 1a) and increased taxane production (Fig. 1b) as compared to mock-elicited cultures.

Subsequently, we examined the viability of cultures using fluorescein diacetate (FDA) and propidium iodide (PI), to indicate both viable and non-viable cells, respectively. Eight time points were examined spanning 24 days of the culture period; representative data are shown in Fig. 1c, d. Both MeJA-elicited and mock-elicited cultures exhibited ~90–95 % viability until day 16 of the culture period (9d post-elicitation) (Fig. 1c, d). At later time points, the FDA fluorescence intensity decreased in both MeJA-elicited and mock-elicited cultures, implying a decrease in metabolic activity for all cultures (Li et al. 2011). More PI fluorescent cells were observed in MeJA-elicited cultures as compared to mock-elicited cultures on

days 21 and 24 of the culture period (14 and 17 days post-elicitation) (Fig. 1c, d), indicating compromised cell membrane integrity and the beginning of cell necrosis in MeJA-elicited cultures. There was a time lag between evidence of reduced viability and a measurable decrease in biomass (dry weight), as has been observed with other *Taxus* cell lines (Kim et al. 2005; Naill and Roberts 2005a).

A hallmark feature of apoptotic cell death is the fragmentation of DNA into oligonucleosomal fragments of ~180–200 bp or multiples thereof, giving rise to a ladder during gel electrophoresis of genomic DNA (Ryerson and Heath 1996; Yuan et al. 2002). Genomic DNA was isolated from MeJA-elicited and mock-elicited cultures on day 11, day 24 and day 30 of the culture period. A DNA laddering pattern was not observed until day 30 of the culture period (23 days post-elicitation) in mock-elicited and MeJA-elicited cultures (Fig. 2), indicating that apoptosis is not a direct consequence of MeJA elicitation. Repression of cell

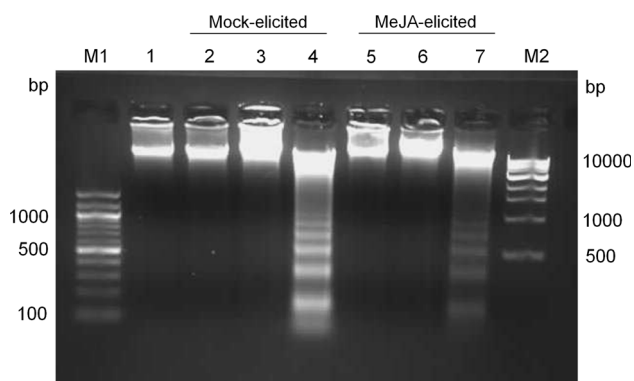


Fig. 2 Effect of MeJA elicitation on induction of oligonucleosomal fragmentation in cultured *T. cuspidata* P93AF cells. The agarose gel shows DNA extracted from mock-elicited and MeJA-elicited cultures. M1, 100-bp marker; Lane 1 mock-elicited (day 7), Lanes 2, 3 and 4, mock-elicited cultures on day 11, 24 and 30 of culture period, respectively; Lanes 5, 6, and 7, MeJA-elicited cultures on day 11, 24 and 30 of the culture period, respectively; M2, 1-kbp marker. Cultures were either mock-elicited or elicited with 150 μ M MeJA on day 7 of the culture period

growth thus occurs before significant necrosis and apoptosis begins in MeJA-elicited *Taxus* cultures, which necessitates further investigation into the cell cycle to understand the role of MeJA in growth inhibition.

MeJA causes a transient increase in G2 phase cells and a decrease in S phase cells, followed by an arrest at G0/G1 in asynchronous *Taxus* suspension cultures

Nuclei were isolated from mock-elicited and MeJA-elicited cultures and stained with PI for flow cytometric-based DNA quantification. A flow cytometric DNA histogram of *Taxus* nuclei is shown in Fig. 3d for reference. All data presented were extracted from similar histograms. The percentage of cells in the different cell cycle phases was calculated using the Watson Pragmatic Model of FlowJo software (v 7.6, Tree Star, Inc.). The proportion of cells in each cell cycle phase in mock-elicited and MeJA-elicited cultures is shown in Fig. 3a–c. Within the first 24 h post-elicitation, MeJA-elicited cultures had more cells in G2 phase (18 % in MeJA-elicited, 13 % in mock-elicited cultures) and fewer cells in S phase (10 % in MeJA-elicited, 14.5 % in mock-elicited cultures) when compared to mock-elicited cultures. This trend was confirmed by further analyzing cultures at shorter time increments before 24 h post-elicitation (Online Resource 1). After 72 h post-elicitation, the percentage of cells in both G2 and S phases decreased and the distributions shifted towards a higher percentage of cells in G0/G1. An increased G0/G1 cell population post-MeJA elicitation has also been observed with another *Taxus* cell suspension line (Naill and Roberts 2005a).

MeJA slows down the cell cycle

5-Ethynyl-2'-deoxyuridine (EdU) pulse labeling was used to investigate the effect of MeJA on cell cycle kinetics. A 4-h EdU pulse was provided to both MeJA-elicited and mock-elicited cultures. As cells only in S phase are able to incorporate EdU, labeled cells were quantified as they pass through different phases of the cell cycle (S to G2/M to G0/G1) using the bivariate DNA–EdU distributions obtained with flow cytometry. Approximately 10 % EdU incorporation was used for pulse labeling, which allows enough cells to be labeled for accurate quantification using flow cytometry. A similar percentage of 5-bromo-2'-deoxyuridine (BrdU) incorporation was used successfully in pulse-labeling studies of *Solanum aviculare* cells (Yanpaisan et al. 1998). The G0/G1 peak in mock-elicited cultures appeared 9 h after the EdU pulse; whereas it took 24 h after the EdU pulse to observe the G0/G1 peak in MeJA-elicited cultures (Fig. 4). This later appearance of the G0/G1 peak in MeJA-elicited cultures clearly demonstrates that MeJA repressed cell cycle progression in *Taxus* cultures. However, the appearance of the G0/G1 peak in MeJA-elicited cultures indicates that the cell cycle is not arrested at the G2/M transition.

MeJA decreases the number of cycling cells

Cumulative EdU incorporation in MeJA-elicited and mock-elicited *Taxus* cultures is shown in Fig. 5. 5 days after incubation with EdU, the number of cells incorporating EdU in mock-elicited cultures was \sim 45 % of the total cell population, whereas in MeJA-elicited cultures it was only 12 % of the total cell population. These data indicate that MeJA addition results in fewer cells participating in DNA synthesis, hence a lower number of actively cycling cells in culture. Repression of DNA synthesis and blockage of cells in G1 and G2 phases of the cell cycle upon MeJA elicitation have also been observed in synchronized tobacco BY-2 cell cultures (Swiatek et al. 2002).

MeJA represses a number of genes participating in cell cycle progression

Using a combination of deep sequencing technologies and Blast2GO annotation, 149 cell cycle-associated contigs (referred to as cell cycle-associated genes from here onwards) were identified in the transcriptome of mRNA isolated from cultured *Taxus* cells (Online Resource 2). A comparison of gene expression between mock-elicited and MeJA-elicited cultures was done at 18 and 72 h post-elicitation. At both 18 and 72 h post-elicitation, none of the 149 cell cycle-associated genes were upregulated in MeJA-elicited cultures relative to the mock-elicited cultures.

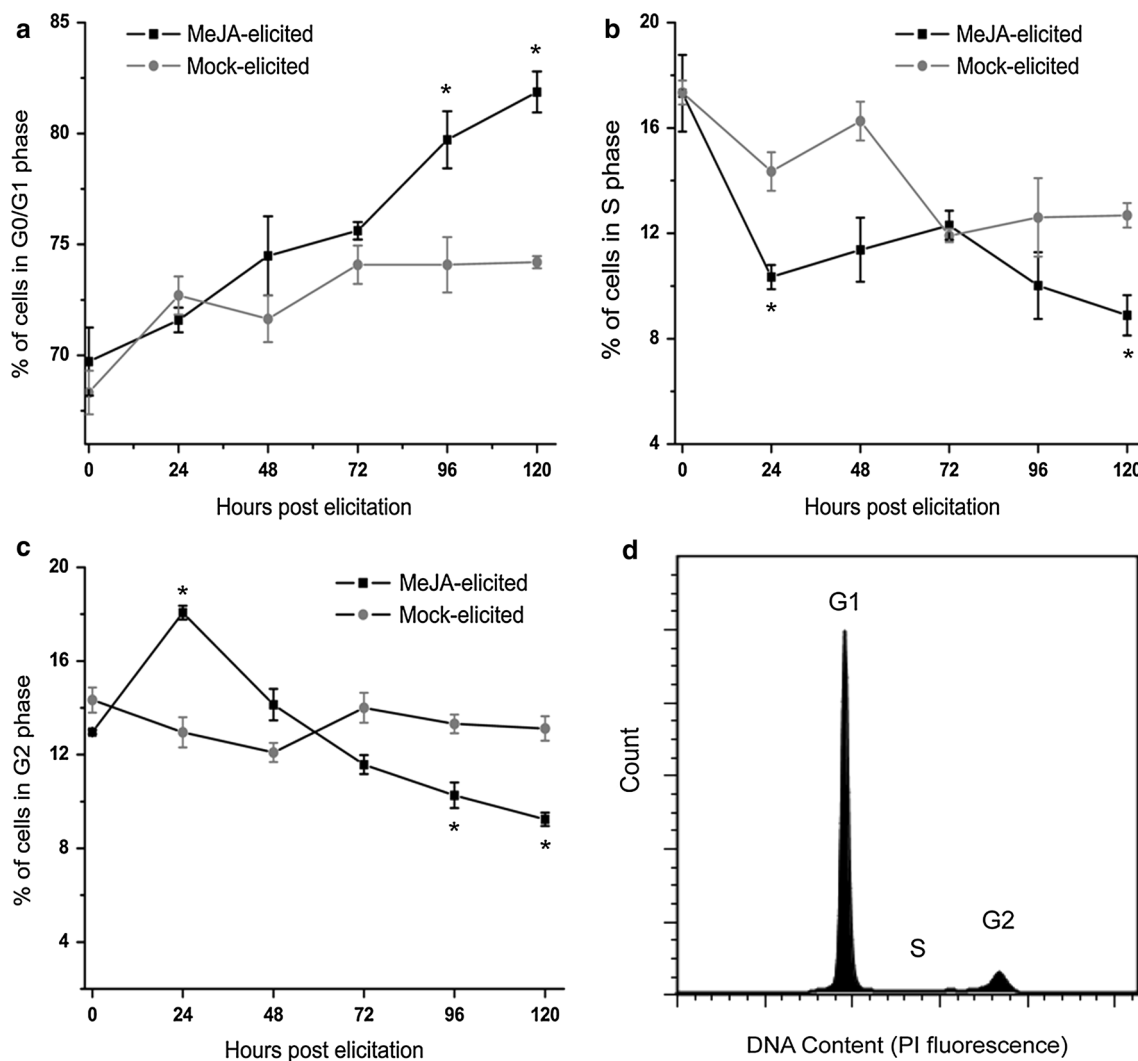


Fig. 3 Cell cycle distribution in MeJA-elicited and mock-elicited *T. cuspidata* P93AF cultures. The percentage of cells in (a) G0/G1, (b) S, and (c) G2/M phases is shown. (d) Cell cycle analysis using the Watson Pragmatic Model of the FlowJo (v7.6) software. RNAse A (50 $\mu\text{g}/\text{mL}$) treatment, followed by staining with propidium iodide (PI) (50 $\mu\text{g}/\text{mL}$), was performed to obtain DNA histograms. Reported

values are the average of three biological replicates, and error bars represent SEM. The asterisk indicates a statistically significant difference ($P < 0.05$; paired Student's *t* test) between MeJA-elicited and mock-elicited conditions. Cultures were either mock-elicited or elicited with 150 μM MeJA on day 7 of the culture period

However, a total of 52 cell cycle genes were significantly downregulated ($>$ twofold downregulation, $P < 0.05$) at 18 and 72 h post-elicitation in MeJA-elicited cultures relative to mock-elicited cultures. Figure 6 shows the hierarchical clustering of the 52 cell cycle genes that were downregulated in MeJA-elicited cultures. At 18 h post-elicitation, 49 genes that were homologous to genes involved in the G2/M and G1/S transition in other plant species were downregulated in MeJA-elicited cultures (Table 1). In particular, the gene representing E2F target protein 1 (ETG1), which is a component of the replisome complex and needed for DNA replication (Takahashi et al. 2008), was drastically downregulated (~ 160 fold). The transcription factor E2F, which in *Arabidopsis* governs expression of ETG1 and

other cell cycle genes [about 70 target genes, (Vandepoel et al. 2005)] involved in the G1/S transition was also downregulated. Genes representing CDC6 (Castellano et al. 2001), CDC45 (Stevens et al. 2004), CDC48 and D-type cyclins (Meijer and Murray 2000), whose expression typically peaks during G1 or early S phase, and some of the genes representing B-type cyclins and other G2 and M phase-specific genes that facilitate progression through mitosis were also downregulated.

At 72 h post-elicitation, about 20 cell cycle genes were significantly downregulated in MeJA-treated cultures (Table 2). Most of these genes (17 out of 20) were also downregulated at the 18-h time point when compared to mock-elicited cultures. However, in contrast to the 18-h

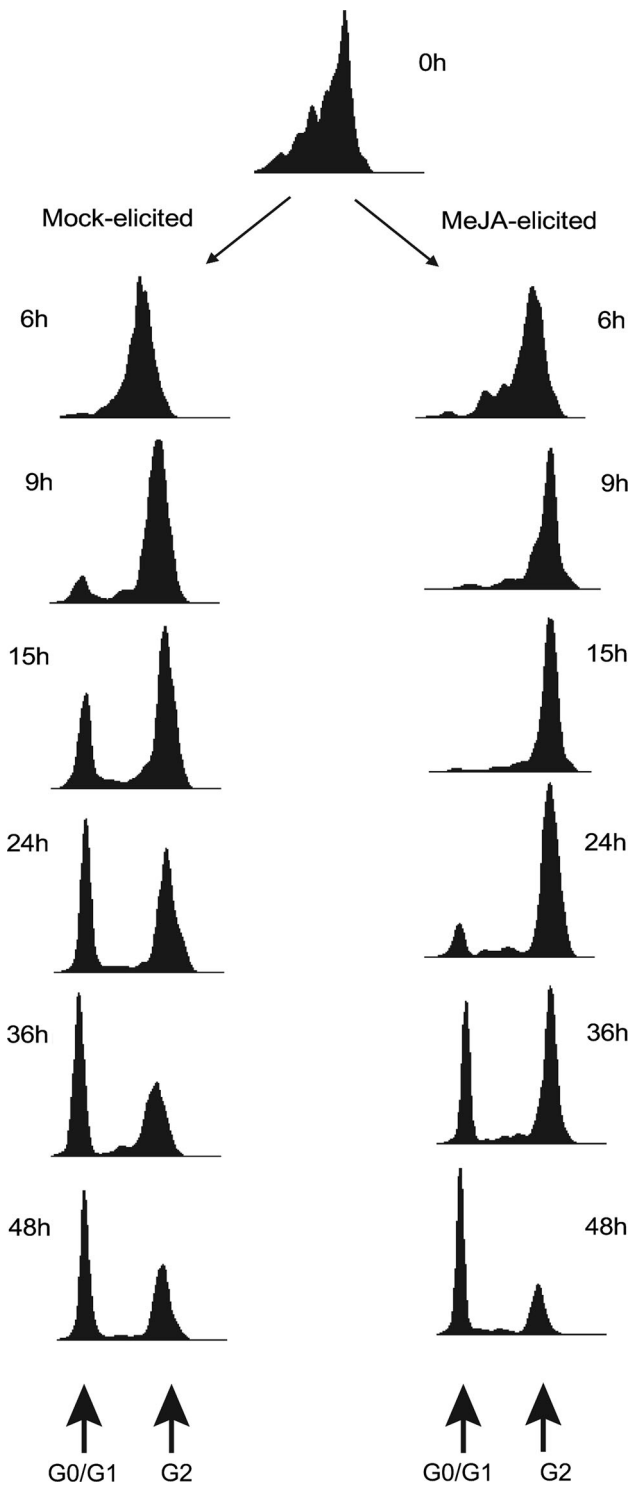


Fig. 4 Progression of EdU pulse-labeled cells in mock-elicited and MeJA-elicited cultures. Only EdU positive cells were selected from bivariate histograms of EdU/DNA content. Cultures were either mock-elicited or elicited with 150 μ M MeJA on day 7 of the culture period. 4 h later, 10 μ M EdU was added to all cultures

time point, which had genes representing both the G1/S and G2/M transition, the majority of the downregulated genes at 72 h were those whose expression typically peaks during

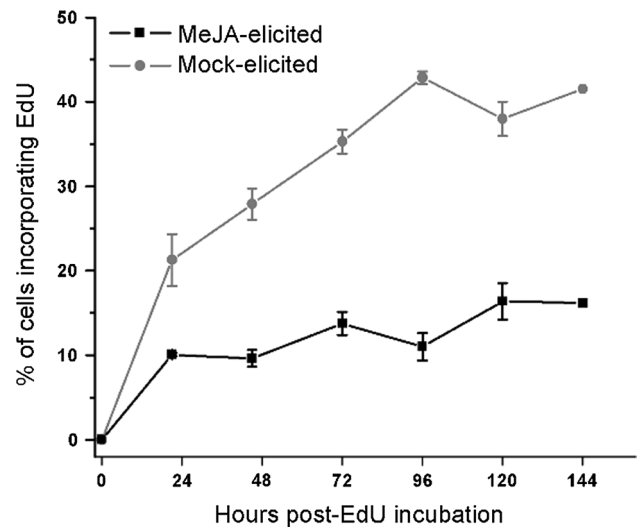


Fig. 5 Total EdU incorporation in mock-elicited and MeJA-elicited cultures. *Circles* represent mock-elicited cultures. *Squares* represent MeJA-elicited cultures. Cultures were either mock-elicited or elicited with 150 μ M MeJA on day 7 of the culture period. 4 h later 10 μ M EdU was added to all cultures

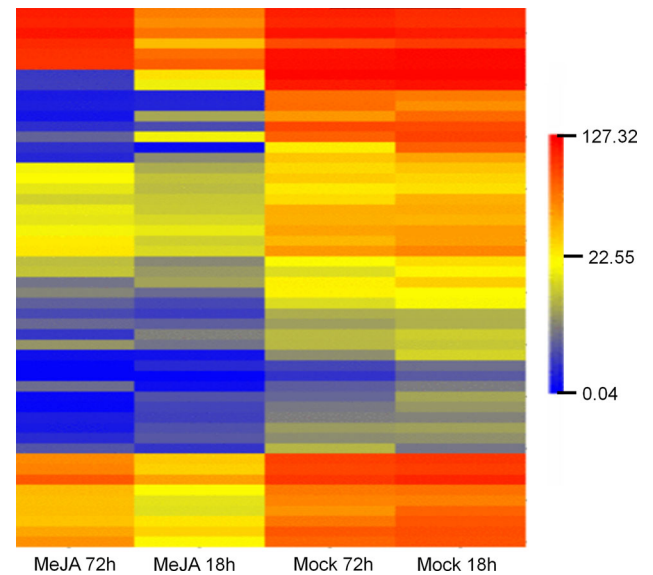


Fig. 6 Heat map showing expression patterns of significantly down-regulated cell cycle-related genes in MeJA-elicited cultures as compared to mock-elicited cultures. Hierarchical clustering was performed using average linkage and Euclidean distance as a measurement of similarity. Changes in gene expression were calculated based on the RPKM values of corresponding genes

G2 and M phase. For example, cyclin A1, B1, B2, cyclin-dependent kinase B (CDKB) and other G2/M-specific cyclins, whose transcripts are known to accumulate during G2 and M phases (Inze and De Veylder 2006), were found to be the most downregulated (>tenfold downregulation, Table 2). This result implies that in MeJA-elicited cultures,

Table 1 Contigs expressed in *Taxus* cell cultures and annotated as cell cycle-related genes downregulated in MeJA-elicited cultures relative to mock-elicited cultures at 18 h post-elicitation

Contig no.	Sequence description	Fold change (normalized)
140962	E2F target protein 1	-163.64
121259	Cell division control protein 6 homolog	-143.52
65828	ccb22_orysj ame: G2 mitotic-specific cyclin-B2-2	-36.41
12355	Probable G2 mitotic-specific cyclin	-33.23
10406	Cyclin-dependent kinase regulator	-26.23
11366	D2 4-type cyclin	-19.75
68975	G2 mitotic-specific cyclin S13-6	-14.18
61510	D3-type cyclin	-6.19
119383	Cyclin-dependent kinase B	-5.46
118076	Cell division control protein 45 homolog	-5.21
19091	Cell division cycle-associated 7-like isoform 2	-5.03
68106	B1-type cyclin-dependent kinase	-5.00
66244	Cyclin-dependent kinases regulatory subunit	-4.41
141136	Cyclin A1	-4.35
18492	Cyclin B 2	-4.17
62294	E2F protein	-3.49
142510	Antagonist of E2F-DP complex	-3.26
9782	Cyclin-dependent kinases regulatory subunit	-3.14
11005	Cell division cycle protein	-3.05
12010	Cyclin-dependent kinases regulatory subunit	-2.89
75242	Cyclin B3-1	-2.88
108329	Cell division cycle protein	-2.81
57179	Cell division	-2.74
105427	Cell division cycle protein 48 homolog	-2.72
91147	Cell division cycle protein 48 homolog	-2.60
113240	Cell division control protein	-2.57
134490	Cell division cycle protein	-2.56
99133	Cell division cycle protein 48 homolog	-2.55
100939	Cell division cycle protein 48 homolog	-2.51
10476	Transcription factor E2F	-2.50
103306	Cell division cycle protein 48 homolog	-2.43
110709	Cell division cycle protein 48 homolog	-2.41
97765	Cell division control protein	-2.39
134513	Cell division cycle protein	-2.37
127620	Cell division cycle protein 48 homolog	-2.37
109918	Cell division cycle protein 48 homolog	-2.35
112279	Cell division cycle protein 48 homolog	-2.35
105698	Cell division cycle protein 48 homolog	-2.34
106161	Cell division control protein	-2.33
59549	D2 4-type cyclin	-2.31
116949	Cell division cycle protein 48 homolog	-2.26

Table 1 continued

Contig no.	Sequence description	Fold change (normalized)
111055	Cell division cycle protein 48 homolog	-2.18
107757	Cell division cycle protein 48 homolog	-2.18
109405	Cell division cycle protein 48 homolog	-2.11
102800	Cell division cycle protein	-2.07
13227	Cyclin-dependent kinase A	-2.05
109272	Cell division cycle protein 48 homolog	-2.03
18149	Cyclin-dependent kinase F-1	-2.00
100185	Cell division cycle protein 48 homolog	-2.00

Table 2 Contigs expressed in *Taxus* cell cultures and annotated as cell cycle-related genes downregulated in MeJA-elicited cultures relative to mock-elicited cultures at 72 h post-elicitation

Contig no.	Sequence description	Fold change (normalized values)
141136	Cyclin A1	-49.32
119383	Cyclin-dependent kinase B	-44.40
65828	ccb22_orysj ame: G2 mitotic-specific cyclin-B2-2	-42.36
12355	Probable G2 mitotic-specific cyclin	-40.96
142510	Antagonist of E2F-DP complex	-40.53
68975	G2 mitotic-specific cyclin S13-6	-32.15
68106	B1-type cyclin-dependent kinase	-27.65
118076	Cell division control protein 45 homolog	-23.96
66244	Cyclin-dependent kinases regulatory subunit	-23.03
11005	Cell division cycle protein	-20.99
10406	Cyclin-dependent kinase regulator	-14.94
140962	E2F target protein 1	-12.52
75242	Cyclin B3-1	-9.86
9782	Cyclin-dependent kinases regulatory subunit	-8.81
79802	Cell division cycle cofactor of APC complex	-6.64
10476	Transcription factor E2F	-5.79
81095	Anaphase-promoting complex subunit CDC20	-5.69
61510	D3-type cyclin	-3.68
12010	Cyclin-dependent kinases regulatory subunit	-3.51
125598	Cell division control protein	-3.25
19091	Cell division cycle-associated 7-like isoform 2	-3.12
18492	Cyclin B 2	-2.99

Table 3 Contigs expressed in *Taxus* cell cultures whose expression is downregulated 72 h post-elicitation as compared to 18 h post-elicitation in MeJA-elicited cultures

Contig no.	Sequence description	Fold change (normalized values)
119383	Cyclin-dependent kinase B	-17.90
141136	Cyclin A1	-13.62
66244	Cyclin-dependent kinases regulatory subunit	-13.34
68975	G2 mitotic-specific cyclin S13-6	-8.09
68106	B1-type cyclin-dependent kinase	-7.96
75242	Cyclin B3-1	-6.74
12355	Probable G2 mitotic-specific cyclin	-5.50
79802	Cell division cycle cofactor of APC complex	-4.97
65828	ccb22_orysj ame: G2 mitotic-specific cyclin-B2-2	-4.79
81095	Anaphase-promoting complex subunit CDC20	-4.06
9782	Cyclin-dependent kinases regulatory subunit	-3.62

Table 4 Contigs expressed in *Taxus* cell cultures whose expression is upregulated 72 h post-elicitation as compared to 18 h post-elicitation in MeJA-elicited cultures

Contig no.	Sequence description	Fold change (normalized values)
11366	D2 4-type cyclin	16.25
11041	Cyclin-dependent protein	3.50
59549	D2 4-type cyclin	2.55
107757	Cell division cycle protein 48 homolog	2.19
100185	Cell division cycle protein 48 homolog	2.18
112647	Cell division cycle protein 48 homolog	2.15
109272	Cell division cycle protein 48 homolog	2.13
145394	Cell division control protein	2.12
113719	Cell division cycle protein 48 homolog	2.04
92332	Cell division cycle protein 48 homolog	2.04
101973	Cell division cycle protein 48 homolog	2.04
107852	Cell division cycle protein 48 homolog	2.01

fewer cells are going through mitosis at the 72-h time point as compared to mock-elicited cultures.

A comparison was made between the gene expression levels at 18 and 72 h for both mock-elicited and MeJA-

elicited cultures (18-h mock-elicited vs. 72-h mock-elicited and 18-h MeJA-elicited vs. 72-h MeJA-elicited). There was no change in expression of cell cycle-related genes in the mock-elicited cultures. However, comparison between 18-h MeJA-elicited and 72-h MeJA-elicited cultures showed that genes that are usually expressed during G2 and M phase (e.g., cyclin-dependent kinase B, cyclin A1, cyclin B2 and B3, etc.) were downregulated at 72 h as compared to 18 h (Table 3). Some G1 phase-specific genes (e.g., D-type cyclins and CDC 48 homologs) were upregulated at 72 h as compared to 18 h (Table 4).

Apart from the cell cycle-specific genes, genes representing the subclasses of core histones, H2A, H2B, H3 and H4, which are essential for cell proliferation and required for the packaging of DNA into chromatin (Gutierrez 2009; Yi et al. 2006) were also repressed in MeJA-elicited cultures at both 18 and 72 h (Online Resource 3 and Online Resource 4).

Discussion

In the present study, we quantified the effect of MeJA, a widely used enhancer of plant secondary metabolism, on asynchronously dividing *T. cuspidata* cell cultures. Biomass measurements showed that MeJA repressed culture growth (Fig. 1a). There was a time lag between growth inhibition and cell death, indicating that MeJA-mediated growth inhibition was not due to necrosis and/or rupturing of cell membranes (Fig. 1c, d). Biotic (fungal derived) and abiotic [Cerium (Ce^{4+})] elicitors induce apoptotic cell death in *Taxus* cultures within a few hours to few days post-elicitation (Ge et al. 2002; Qiao et al. 2003; Yuan et al. 2002). Here, we observed DNA fragmentation, a hallmark of apoptotic cell death, only at a very late time point (day 30 of the culture period), implying that apoptosis is not directly linked to MeJA elicitation (Fig. 2). Similar results have been reported for *T. cuspidata* P991 and *T. canadensis* C093D cell lines upon MeJA elicitation (Kim et al. 2005). One explanation for this anomalous behavior for MeJA is that MeJA elicitation in *Taxus* suspensions did not markedly increase the phosphatic acid (PA) levels, which is associated with regulating cell death response (Yang et al. 2008). Ce^{4+} caused both increased paclitaxel levels and increased PA levels, leading to cell death (Yang et al. 2008). Repression of growth without cell death and a delayed onset of apoptosis suggest that MeJA is repressing growth by altering cellular metabolism and/or affecting the cell cycle. We previously demonstrated that MeJA upregulates paclitaxel biosynthetic pathway genes (Lenka et al. 2012; Nims et al. 2006; Patil et al. 2012), and thus metabolism is shifted towards synthesis of paclitaxel.

Here, the influence of MeJA on the *Taxus* cell cycle and the mechanism of growth inhibition have been elucidated.

MeJA addition to asynchronously dividing *Taxus* cultures resulted in four effects on the cell cycle, as revealed by flow cytometric analyses: (1) transient increase in G2 phase cells, (2) transient decrease in S phase cells, (3) increase in G0/G1 phase cells at later stages post-elicitation, and (4) decreases in G2 and S phase cells at later stages post-elicitation (Fig. 3 and Online Resource 1). At a mechanistic level, the effect of jasmonates has been shown to be dependent on the phase of the cell cycle in synchronized tobacco BY-2 cells (Swiatek et al. 2002). When JA was applied before the G1/S transition it prevented DNA replication, keeping BY-2 cells in G1 phase. When JA was applied during S phase, it prevented cells from entering mitosis without directly affecting their DNA synthesis (Swiatek et al. 2002, 2004). Though our cultures were not synchronized, a similar effect was observed upon MeJA elicitation, with a drop in the percentage of cells in S phase and an increase in the percentage of cells in G2 phase immediately following elicitation. The decreased percentage of cells in G2 and S and increased percentage of cells in G0/G1 at 96 h post-elicitation suggest that the cells were not permanently arrested in G2/M, but rather moving slowly. An EdU pulse label assay confirmed these data showing clearly that MeJA addition slowed progression through the cell cycle (Fig. 4).

The transcription of several cell cycle genes at 18 and 72 h post-elicitation with MeJA also correlated with the flow cytometric data trends. Concomitant with the decrease in S phase cells (Fig. 3b), significant downregulation of expression of ETG1 and CDC6 genes at the 18-h time point was observed. Expression of ETG1 and CDC6 genes has been shown to be necessary for the G1/S transition in *Arabidopsis* (Castellano et al. 2001; Takahashi et al. 2008). Genes representing CDC45 (Stevens et al. 2004) and CDC48 homologs (Feiler et al. 1995), which play a role in the G1/S transition were also downregulated. Plant cyclins are known to interact with cyclin-dependent kinases (CDKs), and these CDK-cyclin complexes regulate the key G1/S and G2/M transition points responsible for DNA replication and mitosis, respectively. Plant cyclins, especially the A and B types, show oscillatory behavior at the transcription level, where transcript levels peak during certain phases of the cell cycle. Generally, D-type cyclins are known to regulate the G1/S transition, A-type cyclins regulate the S/M transition, and B-type cyclins regulate both the G2/M transition and M phase control (Breyne and Zabeau 2001; Inze and De Veylder 2006); though a number of exceptions to these functional assignments have been reported (Inze and De Veylder 2006; Kawamura et al. 2006). Genes representing all these three classes of cyclins were downregulated at the 18-h time point in MeJA-

elicited cultures relative to mock-elicited cultures. Also, cyclin-dependent kinase B (CDKB) genes, which are regulated at the transcript level and are necessary for the G2/M transition (Inze and De Veylder 2006), were downregulated at 18 h in MeJA-elicited cultures. The expression patterns of some of these cell cycle genes are thus consistent with the increase in G2 phase cells within 24 h post-elicitation with MeJA (Fig. 3c). At 72 h post-elicitation, the majority of downregulated genes were those for which expression peaked during G2 and M phases (e.g., cyclin A1, CDKB, cyclin B2, etc.). Some of the genes that are involved in G1/S transition (e.g., ETG1, CDC45, etc.) were also downregulated at the 72-h time point (Table 2), indicating that cell proliferation and progression of cells from G1 for division is hindered.

Comparison of cultures at 18 and 72 h post-elicitation with MeJA shows that G2/M-specific cell cycle genes (e.g., G2 mitotic-specific cyclin, cyclin A1, CDKB, etc.) are downregulated at 72 h as compared to 18 h (Table 3). This result implies that the downregulation of genes required to drive cells through G2 and M phases causes more cells to remain in G2 phase. Some G1 phase genes [e.g., cyclin D (d2–4 type cyclin) and CDC 48; Table 4] are also upregulated at 72 h as compared to 18 h post-elicitation, implying that more cells are present in G0/G1 phase at 72 h relative to 18 h in MeJA-elicited cultures. These results correlate well with flow cytometric data (Fig. 3), where both a decrease in G2 and S phase cells and an increase in G0/G1 phase cells were observed after 72 h post-elicitation.

Moreover, along with the cell cycle-specific genes, histone-encoding genes were also downregulated upon MeJA elicitation. Histones are highly conserved across eukaryotic species and have been classified into four subcategories of core histones (H2A, H2B, H3, and H4) and linker histones (H1). For most species, each core histone protein is encoded by a multigene family (Piontkivska et al. 2002). Biotic and abiotic stresses can repress expression of histone genes in plants. For example, in cultured parsley cells, UV radiation and fungal elicitors repressed expression of several genes essential for cell cycle progression including histones H2A, H2B, H3, and H4 (Logemann et al. 1995), leading to growth inhibition. Similar results were observed here in MeJA-elicited *T. cuspidata* cultures, where all the genes representing histones were downregulated relative to mock-elicited cultures at both 18 and 72 h post-elicitation. Thus, histone gene repression can be correlated to decreased cell division in *Taxus*, analogous to observations in parsley cells (Logemann et al. 1995).

The number of cells incorporating EdU was significantly lower in MeJA-elicited cultures when compared to mock-elicited cultures (Fig. 5). This result suggests that over time the number of actively dividing cells (i.e., synthesizing

DNA) decreased in MeJA-elicited cultures. This result is supported by the increase in G0/G1 phase cells observed in MeJA-elicited cultures (Fig. 3a). One explanation is that cells remain in G0 phase and are specialized for accumulation of paclitaxel and potentially other secondary metabolites (Naill and Roberts 2005a). Total metabolic activity inferred from total cellular protein content is relatively uniform in MeJA-elicited cultures (Naill and Roberts 2005b), indicating that non-cycling cells are still metabolically active, but potentially redirect carbon flux away from primary metabolism towards secondary metabolism. Alkaloid accumulation increased in cultures of *Solanum aviculare* where cell cycle progression was inhibited using a cell cycle arrest agent, again suggesting that the metabolic flux may be directed towards secondary pathways in non-proliferating cells (Mak and Doran 1993). Research to date in plant systems has been able to identify a significant G0 population in culture and presents indirect evidence to suggest a function of this population, but has not been able to explicitly correlate the non-cycling cell population to other metabolic information. A multi-parametric flow cytometry study to simultaneously analyze non-cycling cells (G0 phase cells) and paclitaxel-accumulating cells can reveal this relationship in the *Taxus* cell culture system, and warrants further investigation.

In summary, the MeJA-mediated repression of cell growth in *Taxus* cultures was shown to correlate with inhibition of cell cycle progression as evident both at the culture level through flow cytometric analyses and at the transcriptional level by repression of key cell cycle-associated genes. The newly annotated *Taxus* cell cycle-associated genes will provide an importance resource for future cell cycle studies of both *Taxus* and related gymnosperms. The cell cycle progression patterns in the culture closely parallel the transcriptional regulation of cell cycle-associated genes in MeJA-elicited and mock-elicited *Taxus* cell cultures. The results from this study advance fundamental understanding of the mechanism of action of secondary metabolite elicitors such as MeJA on repression of plant cell division. This result is especially important for species such as *Taxus*, where most research has been focused on improving paclitaxel synthesis with less attention paid to the negative effect of MeJA on growth and implications on bioprocessing.

Acknowledgments This work was funded by National Institute of Health (Grant No. GM070852). We acknowledge the National Science Foundation-sponsored Institute for Cellular Engineering IGERT Program (DGE-0654128) for facilities and financial support. We thank Dr. Donna Gibson of the USDA, Agricultural Research Service, for the *Taxus* cell cultures. We also thank the UMass Amherst Flow Cytometry Facility and Prof. Shelly Peyton for the use of her Zeiss Axiovert 200 microscope.

Conflict of interest The authors declare that they are no conflicts of interest.

References

- Bonfill M, Exposito O, Moyano E, Cusido RM, Palazon J, Pinol MT (2006) Manipulation by culture mixing and elicitation of paclitaxel and baccatin III production in *T. baccata* suspension cultures. *In Vitro Cell Dev Biol Plant* 42:422–426
- Bonfill M, Bentebibel S, Moyano E, Palazón J, Cusidó R, Eibl R, Piñol M (2007) Paclitaxel and baccatin III production induced by methyl jasmonate in free and immobilized cells of *Taxus baccata*. *Biol Plant* 51:647–652
- Breyne P, Zabeau M (2001) Genome-wide expression analysis of plant cell cycle modulated genes. *Curr Opin Plant Biol* 4:136–142
- Bringi V, Kaddake P, Prince CL, and Roach B. (2007) Enhanced production of taxol and taxanes by cell cultures of *Taxus* species. US Patent-7264954 B1
- Castellano MM, del Pozo JC, Ramirez-Parra E, Brown S, Gutierrez C (2001) Expression and stability of *Arabidopsis* CDC6 are associated with endoreplication. *Plant Cell* 13:2671–2686
- Cho K, Agrawal GK, Shibato J, Jung YH, Kim YK, Nahm BH et al (2007) Survey of differentially expressed proteins and genes in jasmonic acid treated rice seedling shoot and root at the proteomics and transcriptomics levels. *J Proteome Res* 6:3581–3603
- Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21:3674–3676
- Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA miniprep- aration: version II. *Plant Mol Biol Rep* 1:19–21
- Farmer EE, Ryan CA (1990) Interplant communication—airborne methyl jasmonate induces synthesis of proteinase-inhibitors in plant leaves. *Proc Natl Acad Sci USA* 87:7713–7716
- Feiler HS, Desprez T, Santoni V, Kronenberger J, Caboche M, Traas J (1995) The higher-plant *Arabidopsis thaliana* encodes a functional Cdc48 homolog which is highly expressed in dividing and expanding cells. *EMBO J* 14:5626–5637
- Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell-cycle in intact plant-tissues. *Science* 220:1049–1051
- Gaurav V, Kolewe ME, Roberts SC (2010) Flow cytometric methods to investigate culture heterogeneities for plant metabolic engineering. *Methods Mol Biol* 643:243–262
- Ge ZQ, Yuan YJ, Wang YD, Ma ZY, Hu ZD (2002) Ce⁴⁺-Induced apoptosis of *T. cuspidata* cells in suspension culture. *J Rare Earths* 20:139–144
- Goossens A, Hakkinen ST, Laakso I, Seppanen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Soderlund H, Zabeau M, Inze D, Oksman-Caldentey KM (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc Natl Acad Sci USA* 100:8595–8600
- Gundlach H, Muller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor induced plant cell cultures. *Proc Natl Acad Sci USA* 89:2389–2393
- Gutierrez C (2009) The *Arabidopsis* cell division cycle. *Arabidopsis Book* 7:e0120
- Inze D, De Veylder L (2006) Cell cycle regulation in plant development. *Annu Rev Genet* 40:77–105

- Jennewein S, Wildung MR, Chau M, Walker K, Croteau R (2004) Random sequencing of an induced *Taxus* cell cDNA library for identification of clones involved in Taxol biosynthesis. *Proc Natl Acad Sci USA* 101:9149–9154
- Kal AJ, van Zonneveld AJ, Benes V, van den Berg M, Koerkamp MG, Albermann K, Strack N, Ruijter JM, Richter A, Dujon B, Ansoorge W, Tabak HF (1999) Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources. *Mol Biol Cell* 10:1859–1872
- Kawamura K, Murray JAH, Shinmyo A, Sekine M (2006) Cell cycle regulated D3-type cyclins form active complexes with plant-specific B-type cyclin-dependent kinase in vitro. *Plant Mol Biol* 61:311–327
- Ketchum REB, Gibson DM, Croteau RB, Shuler ML (1999) The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnol Bioeng* 62:97–105
- Kieran PM, MacLoughlin PF, Malone DM (1997) Plant cell suspension cultures: some engineering considerations. *J Biotechnol* 59:39–52
- Kim BJ, Gibson DM, Shuler ML (2004) Effect of subculture and elicitation on instability of Taxol production in *Taxus* sp suspension cultures. *Biotechnol Progr* 20:1666–1673
- Kim BJ, Gibson DM, Shuler ML (2005) Relationship of viability and apoptosis to taxol production in *Taxus* sp suspension cultures elicited with methyl jasmonate. *Biotechnol Progr* 21:700–707
- Kolewe ME, Henson MA, Roberts SC (2010) Characterization of aggregate size in *Taxus* suspension cell culture. *Plant Cell Rep* 29:485–494
- Kumagai-Sano F, Hayashi T, Sano T, Hasezawa S (2006) Cell cycle synchronization of tobacco BY-2 cells. *Nat Protoc* 1:2621–2627
- Laskaris G, Bounkhay M, Theodoridis G, van der Heijden R, Verpoorte R, Jaziri M (1999) Induction of geranylgeranyl diphosphate synthase activity and taxane accumulation in *T. baccata* cell cultures after elicitation by methyl jasmonate. *Plant Sci* 147:1–8
- Lenka SK, Boutaoui N, Paulose B, Vongpaseuth K, Normanly J, Roberts SC, Walker EL (2012) Identification and expression analysis of methyl jasmonate responsive ESTs in paclitaxel producing *T. cuspidata* suspension culture cells. *BMC Genom* 13:148
- Li J, Ou D, Zheng L, Gan N, Song L (2011) Applicability of the fluorescein diacetate assay for metabolic activity measurement of *Microcystis aeruginosa* (Chroococcales, Cyanobacteria). *Phycol Res* 59:200–207
- Li S, Zhang P, Zhang M, Fu CH, Zhao CF et al (2012) Transcriptional profile of *Taxus chinensis* cells in response to methyl jasmonate. *BMC Genom* 13:295
- Lijavetzky D, Almagro L, Belchi-Navarro S, Martinez-Zapater J, Bru R, Pedreno M (2008) Synergistic effect of methyljasmonate and cyclodextrin on stilbene biosynthesis pathway gene expression and resveratrol production in *Monastrell* grapevine cell cultures. *BMC Res Notes* 1:132
- Logemann E, Wu SC, Schroder J, Schmelzer E, Somssich IE, Hahlbrock K (1995) Gene activation by UV light, fungal elicitor or fungal infection in *Petroselinum crispum* is correlated with repression of cell cycle-related genes. *Plant J* 8:865–876
- Mak YX, Doran PM (1993) Effect of cell-cycle inhibition on synthesis of steroidal alkaloids by *Solanum aviculare* plant cells. *Biotechnol Lett* 15:1031–1034
- Meijer M, Murray JAH (2000) The role and regulation of D-type cyclins in the plant cell cycle. *Plant Mol Biol* 43:621–633
- Menges M, Hennig L, Gruijsem W, Murray JAH (2002) Cell cycle-regulated gene expression in *Arabidopsis*. *J Biol Chem* 277:41987–42002
- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 5:621–628
- Naill MC, Roberts SC (2005a) Cell cycle analysis of *Taxus* suspension cultures at the single cell level as an indicator of culture heterogeneity. *Biotechnol Bioeng* 90:491–500
- Naill MC, Roberts SC (2005b) Flow cytometric analysis of protein content in *Taxus* protoplasts and single cells as compared to aggregated suspension cultures. *Plant Cell Rep* 23:528–533
- Nims E, Dubois CP, Roberts SC, Walker EL (2006) Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering. *Metab Eng* 8:385–394
- Patil RA, Kolewe ME, Normanly J, Walker EL, Roberts SC (2012) Contribution of taxane biosynthetic pathway gene expression to observed variability in paclitaxel accumulation in *Taxus* suspension cultures. *Biotechnol J* 7:418–427
- Patil RA, Kolewe ME, Roberts SC (2013) Cellular aggregation is a key parameter associated with long term variability in paclitaxel accumulation in *Taxus* suspension cultures. *Plant Cell Tissue Organ Culture* 112:303–310
- Pauwels L, Morreel K, Witte ED, Lammertyn F, Montagu MV, Boerjan W, Inzé D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells. *Proc Natl Acad Sci USA* 105:1380–1385
- Pauwels L, Inzé D, Goossens A (2009) Jasmonate-inducible gene: what does it mean? *Trends Plant Sci* 14:87–91
- Piontkivska H, Rooney AP, Nei M (2002) Purifying selection and birth-and-death evolution in the histone H4 gene family. *Mol Biol Evol* 19:689–697
- Qiao JJ, Yuan YJ, Zhao H, Wu JC, Zeng AP (2003) Apoptotic cell death in suspension cultures of *T. cuspidata* co-treated with salicylic acid and hydrogen peroxide. *Biotechnol Lett* 25:387–390
- Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr Opin Plant Biol* 1:404–411
- Ruiz-May E, De-la-Peña C, Galaz-Ávalos RM, Lei Z, Watson BS, Sumner LW, Loyola-Vargas VM (2011) Methyl jasmonate induces ATP biosynthesis deficiency and accumulation of proteins related to secondary metabolism in *Catharanthus roseus* (L.) G. hairy roots. *Plant Cell Physiol* 52:1401–1421
- Ryerson DE, Heath MC (1996) Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell* 8:393–402
- Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci USA* 98:4788–4793
- Stevens R, Grelon M, Vezon D, Oh JS, Meyer P, Perennes C, Domenichini S, Bergounioux C (2004) A CDC45 homolog in *Arabidopsis* is essential for meiosis, as shown by RNA interference-induced gene silencing. *Plant Cell* 16:99–113
- Sun G, Yang Y, Xie F, Wen J-F, Wu J, Wilson IW, Tang Q, Liu H, Qiu D (2013) Deep sequencing reveals transcriptome reprogramming of *Taxus* × media cells to the elicitation with methyl jasmonate. *PLoS One* 8(4):1
- Swiatek A, Lenjou M, Van Bockstaele D, Inze D, Van Onckelen H (2002) Differential effect of jasmonic acid and abscisic acid on cell cycle progression in *Tobacco* BY-2 cells. *Plant Physiol* 128:201–211
- Swiatek A, Van Dongen W, Esmans EL, Van Onckelen H (2004) Metabolic fate of jasmonates in tobacco bright yellow-2 cells. *Plant Physiol* 135:161–172
- Takahashi N, Lammens T, Boudolf V, Maes S, Yoshizumi T, De Jaeger G, Witters E, Inze D, De Veylder L (2008) The DNA

- replication checkpoint aids survival of plants deficient in the novel replisome factor ETG1. *EMBO J* 27:1840–1851
- Thanh NT, Murthy HN, Yu KW, Hahn EJ, Paek KY (2005) Methyl jasmonate elicitation enhanced synthesis of ginsenoside by cell suspension cultures of *Panax ginseng* in 5-l balloon type bubble bioreactors. *Appl Microbiol Biotechnol* 67:197–201
- Vandepoele K, Vlieghe K, Florquin K, Hennig L, Beemster GTS, Grissem W, Van De Peer Y, Inze D, De Veylder L (2005) Genome-wide identification of potential plant E2F target genes. *Plant Physiol* 139:316–328
- Vongpaseuth K, Roberts SC (2007) Advancements in the understanding of Paclitaxel metabolism in tissue culture. *Curr Pharm Biotechnol* 8:219–236
- Yang S, Lu SH, Yuan YJ (2008) Lipidomic analysis reveals differential defense responses of *T. cuspidata* cells to two elicitors, methyl jasmonate and cerium (Ce^{4+}). *Biochimica Et Biophysica Acta Mol* 1781:123–134
- Yanpaisan W, King NJC, Doran PM (1998) Analysis of cell cycle activity and population dynamics in heterogeneous plant cell suspensions using flow cytometry. *Biotechnol Bioeng* 58:515–528
- Yazaki K, Takeda K, Tabata M (1997) Effects of methyl jasmonate on shikonin and dihydroechinofuran production in *Lithospermum* cell cultures. *Plant Cell Physiol* 38:776–782
- Yi H, Sardesai N, Fujinuma T, Chan CW, Veena, Gelvin SB (2006) Constitutive expression exposes functional redundancy between the *Arabidopsis* histone H2A gene HTA1 and other H2A gene family members. *Plant Cell* 18:1575–1589
- Yuan Y-J, Li C, Hu Z-D, Wu J-C, Zeng A-P (2002) Fungal elicitor-induced cell apoptosis in suspension cultures of *T. chinensis* var. *mairei* for taxol production. *Process Biochem* 38:193–198
- Yukimune Y, Tabata H, Higashi Y, Hara Y (1996) Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. *Nat Biotechnol* 14:1129–1132
- Zhang Y, Turner JG (2008) Wound-induced endogenous jasmonates stunt plant growth by inhibiting mitosis. *PLoS One* 3:e3699