Nitric Oxide is Involved in Methyl Jasmonate-induced Defense Responses and Secondary Metabolism Activities of *Taxus* Cells

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Methyl jasmonate (MeJA), a methyl ester of jasmonic acid (JA), is a well-established signal molecule in plant defense responses and an effective inducer of secondary metabolite accumulation in plant cell cultures such as the valuable anticancer diterpenoid taxol (paclitaxel) in *Taxus* spp. This work examines the involvement of nitric oxide (NO) in MeJA-induced plant defense responses and secondary metabolism in *Taxus chinensis* cell cultures. Exogenously supplied MeJA at 100 µM induced rapid production of NO in the *Taxus* cell cultures, reaching a maximum within 6 h of MeJA supply. Several other responses occurred concomitantly, including the production of hydrogen peroxide (H₂O₂), and the increases in intracellular malondialdehyde (MDA) content, lipoxygenase (LOX) and phenylalanine ammonium-lyase (PAL) activities. The MeJA-induced H₂O₂ production was suppressed by an NO donor, sodium nitroprusside (SNP), but enhanced by NO inhibitors, N⁶-nitro-L-arginine (L-NNa) and 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO). In contrast, the MeJA-induced MDA, LOX and PAL were all enhanced by the NO donor but suppressed by the NO inhibitors. The NO inhibitors also suppressed MeJA-induced taxol accumulation. These results are suggestive of a role for NO as a signal element for activating the MeJA-induced defense responses and secondary metabolism activities of plant cells.

Keywords: Methyl jasmonate — Nitric oxide — Oxidative responses — Paclitaxel production — Phenylalanine ammonium-lyase — *Taxus* cell culture.

Abbreviations: CHS, chalcone synthase; DAF-2 DA, 4,5-diaminofluorescein diacetate; IBU, ibuprofen; JA, jasmonic acid; LOX, lipoxygenase; MDA, malondialdehyde; MeJA, methyl jasmonate; L-NNa, N⁶-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PAL, phenylalanine ammonium lysers; PTIO, 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside; TBA, thilobarbituric acid; TCA, trichloroacetic acid.

Introduction

Nitrogen monoxide or nitric oxide (NO) is a free radical gas formed endogenously in many biological systems including animals, plants and microbes, in which it performs a wide range of biological activities. Although the physiological functions of NO in plants remain to be characterized, evidence is emerging that NO plays a regulatory role in plant growth, development and defense responses (McDowell and Dangl 2000, Beligni and Lamattina 2001). As NO production in plant tissue and cells usually occurs in response to pathogen invasion (Delledonne et al. 1998) and challenges by fungal elicitors (Forissner et al. 2000) and abiotic stresses (Leschem and Haramaty 1996, Pedroso et al. 2000, Garcia et al. 2001), its most possible and prominent role is signaling and regulating plant defense or stress responses. Indeed, the chemical properties of NO, i.e. small molecule, short half-life, absence of charge and high diffusivity, would serve an ideal inter- and intracellular signaling molecule in plant defense responses. In particular, NO may be implicated in some of the defense responses mediated by reactive oxygen species (ROS), such as defense gene activation, the hypersensitive cell death and phytoalexin biosynthesis (Delledonne et al. 1998, Durner et al. 1998). It has been suggested that ROS alone are not always sufficient to mediate a strong disease resistance response in plants, and their combination with NO can act synergistically to activate a stronger response.

Jasmonic acid (JA) and its methyl ester methyl jasmonate (MeJA) are well-established signal molecules belonging to an integral part of a general signal transduction system in plant defense responses (Mueller et al. 1993, Sembdner and Parthier 1993). In plants, JA is synthesized endogenously via the octadecanoid pathway initiated with the peroxidation of linolenic acid by lipoxygenase (LOX), and usually stimulated by biotic and abiotic stresses such as fungal elicitors (Gundlach et al. 1992) and wounding (Creelman et al. 1992). The activation of LOX activity and JA biosynthesis during elicitation has also been found to be important for the induction of phytoalexin and secondary metabolite accumulation (Alami et al. 1999, Wu and Ge 2004). Exogenous JA or MeJA applied to plant tissue and cells has been shown to mimic the effects of wounding and elicitors to induce the related responses such as the synthesis of secondary metabolites and proteinase inhibitors (Creelman et al. 1992, Gundlach et al. 1992) and rapid production of H₂O₂ (Krupa and Maksymiec 2002). Recent studies have suggested that NO is involved in some of the JA-induced or mediated defense responses in plants, such as the inhibition of wound-
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Inducing H₂O₂ production and proteinase inhibitor synthesis in tomato leaves (Orozco-Cárdenas and Ryan 2002), and MeJA-induced accumulation of H₂O₂ and malondialdehyde (MDA) in rice leaves (Hung and Kao 2004). Moreover, a strong NO burst has been induced by JA as well as wounding in Arabidopsis thaliana plants (Huang et al. 2004).

Plant secondary metabolites are major defense elements of plants against pathogen and herbivore attacks. In addition, plant secondary metabolites constitute the most important class of natural products with diverse and valuable chemical properties and biological activities. Paclitaxel (Taxol®, Bristol-Myers Squibb, New Brunswick, NJ, USA), a diterpenoid secondary metabolite in various Taxus (yew) species, is an excellent anticancer drug which has been widely used in the treatment of breast, ovarian and lung cancers as well as AIDS-related Kaposi’s sarcoma (Patel 1998). Plant cell cultures of Taxus spp. have been developed for renewable and sustainable production of taxol and related taxanes. MeJA has been one of the most effective elicitors for enhancing taxol production in Taxus cell cultures (Yukimune et al. 1996, Ketchum et al. 1999, Wu and Lin 2003). On the other hand, endogenous JA biosynthesis has been stimulated in Taxus cell cultures by a fungal elicitor (Mueller et al. 1993). Oxidative burst, i.e. transient and rapid production of ROS, has been reported as an early event during the elicitation of taxol production in T. chinensis cell cultures (Yuan et al. 2001), and the ROS such as H₂O₂ and O₂·− from the oxidative burst have been found (through ROS inhibitor experiments) to play a signaling role in the elicitation of taxol production in T. chinensis cell cultures (Wu and Ge 2004).

However, there is still insufficient knowledge of JA- or MeJA-induced NO production and the role of NO in JA-induced defense responses in plants, and there is no published study on JA-induced NO in Taxus spp. This work was carried out to examine MeJA-induced NO production and its relationship with other elicitor responses including H₂O₂ production, lipid peroxidation, and the activation of phenylalanine ammonia-lyase (PAL) and taxol production in T. chinensis cell cultures.

**Results**

**MeJA-induced NO burst in Taxus cell cultures**

The MeJA-induced NO production (intracellular) in the T. chinensis cell cultures could be directly observed by fluorescence microscopy, showing the green fluorescence of 4,5-diaminofluorescein diacetate (DAF-2 DA)-stained cells (Fig. 1). Fig. 2 shows the time courses of fluorescence intensity of culture medium in the DAF-2 DA-stained cultures after various treatments by MeJA and NO-related reagents. In the culture treated by MeJA only, a higher fluorescence intensity over that of the control was detected within 15 min after MeJA addition, and reached a maximum in 6 h, which was about 10.6-fold higher than that of the control. There was no further increase in the fluorescence intensity detected thereafter (data not shown). The MeJA-induced fluorescence increase was effectively blocked by the NO scavenger, 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO; 100 µM), proving that the fluorescence increase detected in the cultures was mainly a result of NO production and release by the MeJA-treated cells. The culture treated with the NO donor sodium nitroprusside (SNP) also exhibited a gradual increase in the fluorescence intensity, but to a lower level than that induced by MeJA. The combination of SNP with MeJA (MeJA + SNP in Fig. 2) had an almost negligible effect on the fluorescence...
intensity of the MeJA-treated culture. The rapid and transient production of NO in the culture after MeJA treatment is indicative of an NO burst. Moreover, the induced NO production was significantly suppressed by the NO synthase (NOS) inhibitor, \(N\)-\(\omega\)-nitro-L-arginine (\(L\)-NNA; 100 \(\mu\)M, Fig. 2), suggesting that NOS was a source of the induced NO production in the \(T. chinensis\) cells.

The MeJA-induced NO production increased with the concentration of MeJA from 50 to 300 \(\mu\)M, indicative of a dose-dependent stimulation (Fig. 3). The MeJA dose of 100 \(\mu\)M was chosen for all subsequent experiments because of its significant stimulation of NO and taxol production and negligible inhibition of cell growth (compared with larger doses).

**MeJA-induced \(H_2O_2\) production**

The MeJA treatment induced rapid production of \(H_2O_2\) in the \(T. chinensis\) cell culture, which evolved through a biphasic time course, reaching the first and smaller peak in about 0.5 h (3.7 \(\mu\)M high), and the second and much larger peak (12.5 \(\mu\)M high) around 4 h post-treatment (Fig. 4). This time course is characteristic of an oxidative burst in elicitor-treated plant cell cultures. The MeJA-induced \(H_2O_2\) production was enhanced by both NO inhibitors, \(L\)-NNA and PTIO, but inhibited by the NO donor SNP. SNP alone in the absence of MeJA did not induce any \(H_2O_2\). These results are suggestive of an adverse effect of NO on the MeJA-induced oxidative burst.

**MeJA-induced LOX activity and lipid peroxidation**

MeJA treatment of \(T. chinensis\) cells induced a rapid increase in the intracellular LOX activity (Fig. 5A) and malondialdehyde (MDA) content (Fig. 5B), which were detected within 1–2 h of treatment. The MeJA-induced LOX activity rose to its peak around 3 h after MeJA addition to the culture medium and then dropped back to the control level about 3 h later; MDA rose to its peak around 4 h after MeJA addition, and then dropped steadily but less rapidly than LOX. At the peak levels, the LOX activity was increased about 5-fold and MDA content increased about 12-fold compared with the control levels.

The MeJA-induced LOX activity and MDA accumulation in the cells was suppressed by both the NO synthase inhibitor \(L\)-NNA and the NO scavenger PTIO (Fig. 6) (e.g. LOX activity 0.66 U g\(^{-1}\) FW with MeJA vs. 0.47 U g\(^{-1}\) FW with MeJA + \(L\)-NNA and 0.23 U g\(^{-1}\) FW with MeJA + PTIO in Fig. 6A; MDA content 4.41 \(\mu\)g g\(^{-1}\) FW with MeJA vs. 1.96 \(\mu\)g g\(^{-1}\) FW with MeJA + \(L\)-NNA, and 1.10 \(\mu\)g g\(^{-1}\) FW with MeJA + PTIO in Fig. 6B). The NO donor SNP itself stimulated the LOX
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Activity by about 2-fold and also slightly enhanced the MeJA-induced LOX activity, by about 20% (SNP and MeJA + SNP vs. MeJA in Fig. 6A). However, SNP had no obvious influence on the MDA production, and only slightly enhanced MeJA-induced MDA (Fig. 6B). In general, most of the results here are indicative of an association of NO with the MeJA-induced LOX activity and lipid peroxidation (MDA production).

Fig. 6 The influence of NO donor and inhibitors (with the same procedure and dosage as specified in Fig. 2) on MeJA-induced increases in LOX activity (A) and MDA content (B) in T. chinensis cells (LOX measured at 3 h and MDA at 4 h after treatment with 100 µM MeJA).

MeJA-induced PAL activity and taxol production

MeJA treatment induced a gradual increase in the intracellular PAL activity in T. chinensis cell cultures, reaching a peak in 12 h of treatment, which was >3-fold the control level (Fig. 7). The NO donor SNP significantly stimulated the PAL activity by itself, and also enhanced the MeJA-induced PAL activity (MeJA + SNP vs. MeJA). On the other hand, the MeJA-induced PAL activity was suppressed by both NO inhibitors, L-NNa and PTIO. All these results show a positive effect of NO on the MeJA-induced PAL activity.

MeJA treatment stimulated taxol production in the Taxus cell cultures, leading to a rapid increase in the taxol content or accumulation in the cells between 2.5 and 7 d after the treatment (Fig. 8). At peak level on day 7 after MeJA treatment, the taxol content of the cell increased by >12-fold compared with that of the control (0.19 vs. 0.015 mg g⁻¹ DW). Unlike the MeJA-induced responses shown above which reached their peak levels within hours, the MeJA-induced taxol accumulation lasted for several days before reaching the peak. Similarly, MeJA-induced taxol production in other Taxus cell cultures also lasted for several to >10 d (Yukimune et al. 1996, Ketchum et al. 1999).

The NO donor SNP slightly but significantly stimulated taxol production by itself as well as in combination with MeJA (MeJA + SNP) (Fig. 9). The MeJA-induced taxol production was significantly decreased by the NO scavenger PTIO. In addition, the MeJA-induced taxol production was suppressed by the LOX inhibitor, ibuprofen (IBU; about 90%), indicative of the implication of LOX activity in the elicitation of taxol biosynthesis.

The amount of taxol released from cells to medium was no more than 25% of the total under all conditions, and the related data were not presented as they would have negligible influence on the trends of treatment effects as shown.

Discussion

The experimental results have shown several characteristic defense responses and taxol accumulation induced by exogenously supplied MeJA in T. chinensis cultures, including the
were added 30 min before the addition of MeJA. The taxol content was measured 7 d after MeJA addition to the culture. SNP, PTIO and IBU and mechanical stress (Garcês et al. 2001). The induction of H$_2$O$_2$ production, lipid peroxidation (MDA) and enhanced PAL activity. More importantly, our study has shown the MeJA-induced NO burst, and its quantitative and temporal relationships with the other defense responses in Taxus cell cultures. The kinetics of MeJA-induced NO production is similar to that induced by a fungal elicitor in tobacco leaves (Foissner et al. 2000) and mechanical stress in various Taxus brevifolia tissues (Pedroso et al. 2000). Therefore, NO production is an early event in the elicitation of plant defense responses. Moreover, the MeJA-induced NO production in Taxus cell cultures was enhanced by the NO generator (SNP) and inhibited by the NOS inhibitor (1-NNA) and NO scavenger (PTIO). Similar effects of the NO donor and inhibitors have also been observed on the NO production in other plant species induced by microbial pathogens (Delledonne et al. 1998) and mechanical stress (Garcês et al. 2001).

The induction of H$_2$O$_2$ production by MeJA is suggestive of the involvement of MeJA in signaling the activation of oxidative burst during elicitation. The biphasic time course of H$_2$O$_2$ accumulation after MeJA induction in the Taxus cell cultures shown in our study (MeJA in Fig. 5) closely matches that induced by microbial pathogens or their constituent-derived elicitors (Baker et al. 1993, Levine et al. 1994, Low and Merida 1996). It has been shown that wounding and systemin induce systemic H$_2$O$_2$ production in tomato leaves via the octadecanoid pathway (Orozco-Cárdenas and Ryan 1999), and act as a downstream signal following MeJA for systemin-induced defense genes in response to wounding (Orozco-Cárdenas et al. 2001). The stimulation of MeJA-induced H$_2$O$_2$ production by the NO inhibitors in our present study with T. chinensis cells is consistent with the blockage of JA-induced H$_2$O$_2$ production by an NO donor in tomato leaves as reported previously (Orozco-Cárdenas and Ryan 2002). It suggests that endogenous NO is implicated in the regulation of oxidative stress responses of plant cells.

PAL is a key enzyme in the first step of the phenylpropanoid pathway responsible for the synthesis of plant phenylpropanoids or phenolics, many of which play important roles in plant defense against pathogens and herbivores (Dixon and Paiva 1995). The activation of PAL activity is a common response of plant cells to biotic and abiotic stresses. It has been shown that fungal elicitors, pathogen infection and wounding (Lawton and Lamb 1987) as well as MeJA (Gundlach et al. 1992) can induce the PAL activity. In our present study, MeJA-induced PAL activity in the T. chinensis cell cultures was potentiated by the NO donor but inhibited by the NO inhibitors, suggesting that the activation of PAL may be regulated by the NO produced endogenously during elicitation. It has been reported (Delledonne et al. 1998) that the NO donor SNP induced the expression of genes encoding PAL and chalcone synthase (CHS; the first enzyme in a branch of the phenylpropanoid pathway) in soybean (Glycine max) cell cultures. In addition, the NO inhibitor l-NNA prevented the accumulation of PAL and CHS transcripts in response to an avirulent bacterium. Transcriptional induction of PAL has also been observed in tobacco plants and cell cultures (Durner et al. 1998). In an early study, however, exogenous H$_2$O$_2$ only triggered a weak accumulation of the PAL and CHS transcripts in soybean cells (Levine et al. 1994).

The involvement of LOX enzymes, which catalyze the oxidation of fatty acids to fatty acid hydroperoxides, in plant response to environmental stimuli such as wounding and pathogen attack has been established based on biochemical studies on the modulation of LOX protein and activity levels in response to both biotic and abiotic stresses (Melen et al. 1993). The results from our present study have shown that LOX activity was induced by exogenous MeJA applied to T. chinensis cells (Fig. 5A), and was enhanced further by the NO donor but suppressed by the NO inhibitors (Fig. 6A). These results suggest that the LOX level may be regulated by endogenous NO during MeJA treatment. LOX may play an important role in plant–pathogen interactions by initiating membrane damage.

**Fig. 9** Effect of NO donor and inhibitors (the same dosage as specified in Fig. 2), and LOX inhibitor (IBU at 1 mM) on MeJA-induced taxol production in T. chinensis cell cultures. The taxol content was measured 7 d after MeJA addition to the culture. SNP, PTIO and IBU were added 30 min before the addition of MeJA.

**Fig. 10** A simplified hypothetical model for MeJA-induced NO production and the involvement of NO in the signal transduction of MeJA-induced responses of T. chinensis cells, such as lipid peroxidation, LOX and PAL activities, and Taxol synthesis (dashed lines indicate the more uncertain steps; —, point of inhibitor application).
during the hypersensitive response through direct or indirect promotion of lipid peroxidation (Brash 1999, Maccarrone et al. 2000). The suppression of MeJA-induced LOX and lipid peroxidation (MDA production) by the NO inhibitor and scavenger (Fig. 6B) provides further support for the involvement of NO in the lipid peroxidation of cell membranes.

Based on our experimental results and the above discussion, we propose a simplified hypothetical model as shown in Fig. 10 for the network of MeJA-induced responses in the T. chinensis cell culture and the signal pathways. The model chart highlights the multiple signal pathways through which MeJA, ROS and NO induce and mediate the defense responses and the biosynthesis of secondary metabolites such as taxol. According to this model, MeJA is the initial trigger of the responses; NO (from NOS) and ROS are at the upstream of the pathways, which may function independently or synergistically to signal further downstream responses, including lipid peroxidation, LOX activity and activation of defense genes, followed by PAL activity and secondary metabolite (and taxol) accumulation. A possible cause of the decreased \( \text{H}_2\text{O}_2 \) production with increased NO production is the consumption of ROS for the production of reactive nitrogen species (RNS) from NO.

MeJA has been a proven and effective stimulant for enhancing the production of taxol and other taxane relatives in cell cultures of several Taxus species including T. chinensis (Yukimune et al. 1996, Ketchum et al. 1999, Wu and Lin 2003). Our present study has shown the strong dependence of MeJA-induced taxol accumulation on NO production as well as the ROS and LOX activity (Fig. 8). This finding, together with the effect of NO on LOX and PAL activity mentioned above, indicates a role for NO as a signal molecule and its interplay with other signal elements, JA and ROS, to regulate the MeJA-induced secondary metabolism responses in Taxus spp. Since elicitation has been a successful strategy for enhancing the production of many bioactive secondary metabolites in plant tissue cultures, the characterization of elicitor-induced NO production and its relationship with other elicitor responses will bring about not only new insights into the physiological mechanisms of elicitation but also more effective strategies for the production of desired secondary metabolites.

**Materials and Methods**

**Taxus cell cultures**

A T. chinensis cell line induced from the young stems of a T. chinensis tree was used in this study, which was routinely maintained on solid MS medium. Cell suspension culture was maintained in liquid MS medium in 125 ml Erlenmeyer flasks on an orbital shaker at 110–120 rpm and 25 ± 1°C in the dark (referred to as shake-flask culture). Each of the culture flasks was filled with 25 ml of medium and inoculated with 3.0 g of fresh weight of cells from the solid culture. More details of the culture medium and conditions have been given elsewhere (Wu and Ge 2004). The experiments for studying the elicitor responses of T. chinensis cells were all conducted in, or with cells collected from the shake-flask culture. The fresh weight of cells in the suspension culture was obtained by filtration through a Whatman filter paper under vacuum, and the dry weight by drying the fresh cell mass at 50°C in an oven until constant weight.

**Study of MeJA-induced responses**

MeJA (catalog no. 08529TU, Aldrich, Milwaukee, WI, USA) was first dissolved in ethanol at about 44 µmol ml\(^{-1}\), and a small volume (<70 µl) of the MeJA solution was added to the culture flasks to make up the desired final concentration. MeJA was added to the culture on day 7 post-inoculation which was in the middle of a rapid growth phase, a favorable time for elicitor treatment. SNP (catalog no. 30190, BDH, Poole, UK) was used as an NO donor, t-NTA (catalog no. N-5501, Sigma, St Louis, MO, USA), a NOS inhibitor, as an NO inhibitor, and PTIO (catalog no. P-5084, Sigma) as an NO scavenger. These NO reagents and their dosage used in the experiments were chosen based on previous studies (Delledonne et al. 1998, Pedroso et al. 2000). They were all pre-dissolved in distilled water at 50–100 times the final concentrations in culture and sterilized by filtration. Both t-NTA (100 µM) and PTIO (100 µM) were added to the culture at 30 min prior to, and SNP (10 µM) simultaneously with the MeJA. IBU (catalog no. I-4883, Sigma) was used as a LOX inhibitor in some of the experiments as described previously (Wu and Ge 2004), and were added to the culture 30 min before MeJA treatment.

All the following MeJA-induced responses were measured in the shake-flask cultures except the NO production described below. All treatments were performed in triplicate and the results represented by their mean ± SE. All results presented represent the typical and reproducible treatment effects based on at least two independent repeat experiments.

**Measurement of NO production**

The NO concentration in the culture medium was quantified by binding DAF-2 DA in a fluorometric assay (Garcês et al. 2001, Tun et al. 2001). Each 100 mg of fresh weight of cells from the shake-flask culture was transferred into 0.95 ml of culture medium containing 10 µM DAF-2 DA (catalog no. D-225, Sigma) in a 2 ml microcentrifuge tube and incubated for 2 h before the MeJA and NO treatments as described above. Following the treatments, the tubes were harvested at selected time intervals and the culture supernatant was collected by centrifugation, and the fluorescence intensity measured using a PerkinElmer LS50B luminescence spectrophotometer at 495 nm excitation and 515 nm emission. The relative fluorescence value shown in the Results is the ratio of fluorescence intensity at a given time to that at 0 min.

NO production in the cells (intracellular) was also observed by fluorescence microscopy on a Leica DMBR fluorescence microscope mounted with an I\(_{2}\) filter, at 470 nm excitation and 515 nm emission.

**Measurement of \( \text{H}_2\text{O}_2 \) production**

\( \text{H}_2\text{O}_2 \) released into the culture medium was quantified by luminol chemiluminescence (Glazener et al. 1991). Briefly, 50 µl of the sample medium was added to 750 µl of phosphate buffer (50 mM potassium phosphate, pH 7.9) prior to automated injection of 200 µl of luminol (0.3 mM in phosphate buffer) and 100 µl of K\(_{3}\)[Fe(CN)\(_6\)] (14 mM in \( \text{H}_2\text{O} \)) by a TD-20/20 luminometer (Turner Designs, Sunny Vale, CA, USA). The luminescence was recorded after the last injection at an integration time of 5 s and the intensity was calibrated to \( \text{H}_2\text{O}_2 \) concentration with pure \( \text{H}_2\text{O}_2 \) liquid.

**Measurement of MDA content**

MDA content, a quantitative index of lipid peroxidation in the cells, was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh cell mass (200 mg) from
culture was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12,000 g for 20 min. The supernatant (1 ml) obtained was mixed with an equal volume of TCA (10%) containing 0.5% (w/v) TBA (or no TBA as the blank), and heated at 95°C for 30 min and then cooled in ice. The reaction product was centrifuged at 12,000 g for 15 min and the supernatant absorbance was measured at 400, 532 and 600 nm. The MDA equivalent was derived from the absorbance according to Hodges et al. (1999).

Intracellular LOX assay
Details of the LOX extraction and activity assay have been given elsewhere (Wu and Ge 2004). In brief, LOX was extracted from fresh cell mass with 50 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpyrrolidone and 10 mM mercaptoethanol. LOX activity was determined by measuring the formation of (13(S)-hydroperoxylinolenic acid at 25°C, using linolenic acid as the substrate. One unit of LOX activity is equal to the increase in one absorbance unit at 234 nm per min.

Intracellular PAL assay
PAL was extracted from fresh cell mass (300 mg FW) with 6.5 ml of 50 mM pH 8.8 Tris–HCl buffer containing 15 mM of β-mercaptoethanol in an ice-cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged at 12,000 g for 30 min, and the supernatant was collected for enzyme assay. PAL activity was determined based on the rate of cinnamic acid production as described by Ochoa-Alejo and Gómez-Peralta (1993). Briefly, 1 ml of the extrac- tion buffer, 0.5 ml of 10 mM pH 7.0 containing 1% (w/v) polyvinylpyrrolidone and 10 mM mercaptoethanol. PAL activity was determined by measuring the formation of (13(S)-hydroperoxylinolenic acid at 25°C, using linolenic acid as the substrate. One unit of PAL activity is equal to the increase in one absorbance unit at 234 nm per min.

Analysis of taxol content
The extraction and analysis of taxol from cells and the culture medium was based on the procedure as described by Wu and Ge (2004). In brief, intracellular taxol was extracted from dry cell mass with methanol, and taxol in the medium was extracted by methylene chloride. Taxol content in the extract solution was analyzed by reverse-phase high-performance liquid chromatography (HPLC) with UV detection at 227 nm, using a 250 mm × 4.6 mm Alttech Econosphere 5 μm C18 column (Alttech, Deerfield, IL, USA), and a mobile phase consisting of methanol : acetonitrile : water at 25 : 35 : 45 by vol. Taxol content was quantified with a genuine standard (catalog no. T-7402, Sigma).

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