Hydrogen Peroxide from the Oxidative Burst is Not Involved in the Induction of Taxol Biosynthesis in Taxus chinensis Cells

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In cell suspension cultures of Taxus chinensis, 40 mg/l fungal elicitor from Aspergillus niger and 20 µm HgCl2 elicited 5.7 and 3.6 mg/l taxol, which was a 9-fold and 5-fold increase vs. compared with the control, respectively. The fungal elicitor induced hydrogen peroxide (H2O2) accumulation but HgCl2 did not, indicating that H2O2 was not necessary for enhancement of taxol induced by elicitor. Compared with the treatment with fungal elicitor alone, exogenous catalase, ascorbic acid, diphenylene iodonium and superoxide dismutase induced a 0.45, 0.4, 0.7 and 1.4-fold H2O2, but elicited taxol production, which was 0.98, 1.2, 1.1 and 0.9-fold, respectively, vs. non-treated cells. Elicitor-induced taxol production was not accorded with the amount of H2O2 production.

Key words: Elicitor, Taxol, Taxus chinensis

Introduction

Oxidative burst, a transient increase in the production of reactive oxygen species (ROS), generally occurs in a number of plant pathogen interactions at the early stage. Hydrogen peroxide (H2O2), the most stable compound among ROS, has been implicated in plant disease resistance (Lamb and Dixon, 1997). However, reports linking H2O2 from the oxidative burst to biosynthesis of second metabolites, such as phytoalexin, have been contradictory, even with respect to experiments performed on the same plant species, such as soybean (Levine et al., 1994; Mithöfer et al., 1997; Guo et al., 1998) and tobacco (Lamb and Dixon, 1997; Doreyl et al., 1999).

Presently there has been increasing interest in exploiting Taxus spp. cell cultures to produce the anticancer drug, taxol. A wide variety of elicitors such as fungal elicitors (Zhang et al., 2000; Yuan et al., 2001; 2002; Yu et al., 2002), methyl jasmonate (Zhang et al., 2000; Spela et al., 2002) and heavy metal ion (Zhang et al., 2000) have been employed to induce the biosynthesis of taxol in Taxus cell cultures. Before the activation of de novo synthesis of taxol, the oxidative burst is also observed in elicitor-induced Taxus chinensis cultures (Yuan et al., 2001, 2002; Yu et al., 2002). Yuan et al., (2001, 2002) concluded that the dependence of taxol production on the intensity of H2O2 from oxidative burst followed a modified logistic curve, and inferred that the syntheses of the side chain and nucleus of taxol were enhanced by low and high intensities of H2O2, respectively. However, it is not clear whether inhibition or enhancement of H2O2 from the oxidative burst would affect taxol biosynthesis, which is the main subject of the present paper.

Materials and Methods

Plant materials and culture conditions

Taxus chinensis cell lines, isolated from Taxus chinensis zygote embryos, were maintained in modified MS medium as previously described (Zhang et al., 2000). With 10 g (fresh weight) of cells inoculated into a 250 ml Erlenmeyer flask containing 100 ml liquid modified MS media, 40 mg/l fungal elicitor, and 20 µm HgCl2 were added to the 10-day-old cultures. The flasks were shaken at 120 ± 5 rpm at 25 ± 1 °C.

Fungal elicitor preparation

The fungal strain Aspergillus niger was isolated from the inner bark of Taxus chinensis. Preparation of fungal elicitor was according to the method described by Zhang et al. (2000). The elicitor dose was measured by the total carbohydrate content of the fungal homogenate, which was determined by the phenol-sulfuric acid method using glucose as standard.

H2O2 measurement

Contents of H2O2 were measured by monitoring the A415 of the titanium-peroxide complex according to Brennan and Frenkel (1977). Absorbance values were calibrated to the standard curve.
Taxol determination

The cells after elicitation were washed with de-ionized water to remove residual medium and filtrated under vacuum. The cells were then freeze-dried for 30 h for dry wt determination. Dried samples (100 mg) were mashed and extracted with 4 ml methanol/dichloromethane (1:1, v/v) with sonication for 1 h at room temperature for 3 times. The extract was evaporated to dryness with a rotary evaporator equipped with a condenser for solvent recovery, then redissolved in 2 ml methanol. The methanol extracts were centrifuged at 3400 × g for 5 min prior to HPLC analysis. Samples of 5 ml from the cell free medium were extracted with 2 ml of dichloromethane for 3 times. The combined dichloromethane fraction was vacuum dried and redissolved in 2 ml methanol for HPLC analysis after centrifugation. Taxol was analyzed by HPLC on a reverse-phase C18 column at 227 nm at 25 °C using a mobile phase of methanol/acetonitrile/water (25:30:30, v/v/v). The elution rate was kept at 1 ml min⁻¹. Throughout the experiment, all injection volumes were 10 µl. Taxol concentration (mg/l) in the samples was the combination of taxol in cells and medium.

Results and Discussion

Effect of elicitor on H₂O₂ production

Several suspension cultures plant cell have been reported produce H₂O₂ during stimulation by elicitors from the fungus (Levine et al., 1994; Lamb and Dixon, 1997; Mithöfer et al., 1997; Guo et al., 1998; Doreyl et al., 1999). Fig. 1 shows the effect of the fungal elicitor from Aspergillus niger on H₂O₂ production in Taxus chinensis cell cultures. The elicitor-induced H₂O₂ production started to increase 1 to 2 h after treatment, and reached a maximum of 240 nmol/gFW at about 4 h, and decreased thereafter (Fig. 1). Treatment of Taxus chinensis cultures with fungal elicitor stimulated the H₂O₂ accumulation, which agrees with the observations by Yuan et al. (2001; 2002) and Yu et al. (2002). Taxus chinensis cultures without elicitor treatment accumulated little H₂O₂. 20 µM HgCl₂ only induced a slight increase in H₂O₂ accumulation; however, the increase vs. free elicitor treatment was not statistically significant (P < 0.05) (Fig. 1). Indeed, a low concentration of HgCl₂ had little effect on H₂O₂ accumulation in white clover (Trifolium repens L.) suspension cultures (Devlin et al., 1992).

Effect of elicitor on taxol production

The time courses of taxol accumulation in Taxus chinensis cultures treated with fungal elicitor and HgCl₂ were demonstrated in Fig. 2. Significant dif-

![Fig. 1. H₂O₂ accumulation induced by fungal elicitor and HgCl₂ in Taxus chinensis suspension cells. The rate of H₂O₂ accumulation was determined in cultures at various times after addition of 40 mg/l fungal elicitor and 20 µM HgCl₂, and in the untreated control. Elicitor was added into 10-d old cultures. FW represents fresh weight. Each value is the mean ± SE from seven independent experiments.](image1)

![Fig. 2. Taxol accumulation induced by fungal elicitor and HgCl₂ in Taxus chinensis suspension cells. The rate of taxol accumulation was determined in cultures at various times after addition of 40 mg/l elicitor and 20 µM HgCl₂, and in the untreated control. Elicitor was added to 10-d old cultures. Each value is the mean ± SE from five independent experiments.](image2)
ferences in taxol concentrations between elicited cultures and the control appeared within 2 d. The highest taxol concentrations under the influence of fungal elicitor (5.7 mg/l) and HgCl₂ (3.6 mg/l) appeared on day 10 after treatment, which was 10-fold and 6-fold of the control, respectively.

Although H₂O₂ acts as a second messenger for the induction of some defense genes (Lamb and Dixon, 1997), Levine et al. (1994) and Doreyl et al. (1999) observed that manipulation of H₂O₂ of the oxidative burst did not modify transcripts of phenylalanine ammonia-lyase and chalcone synthase, respectively. Moreover, phytoalexin and terpenes, secondary metabolites generally present in plant-pathogen interactions, were independent on hydrogen peroxide in elicitor-treated tobacco and soybean cell cultures (Devlin et al., 1992; Levine et al., 1994; Mithöfer et al., 1997; Guo et al., 1998; Doreyl et al., 1999). The present results showed that HgCl₂ did not induce H₂O₂ accumulation, but enhanced taxol production similarly to what the fungal elicitor did. Therefore, we postulate that H₂O₂ was not required for enhancement of taxol induced by elicitor.

Effect of scavengers and inhibitors of H₂O₂ generation on H₂O₂ accumulation and taxol production induced by fungal elicitor

To further examine the relationship between H₂O₂ accumulation and taxol biosynthesis, we analyzed these responses in the presence of some scavengers and inhibitors of ROS generation. All chemicals at the concentration used were not toxic to the cells and had little effect on taxol production. Fig. 3 shows the effect of various chemicals on H₂O₂ accumulation at 4 h and taxol production on 10 d after fungal elicitor treatment. Addition of 200 U/ml catalase (CAT) and 60 mg/l ascorbic acid (ASA), scavengers of ROS (Mithöfer et al., 1997; Yu et al., 2002), and diphenylene iodonium (DPI; 500 µm), an inhibitor of NAD(P)H oxidase (Levine et al., 1994; Lamb and Dixon, 1997; Mithöfer et al., 1997; Guo et al., 1998), induced a 0.45, 0.4, and 0.7-fold H₂O₂ accumulation, and elicited a 0.98, 1.2, and 1.1-fold taxol production, higher than found with fungal elicitor alone (Fig. 3 A). Similarly, phytoalexin production was little inhibited when H₂O₂ level was decreased by DPI or ASA (Mithöfer et al., 1997; Doreyl et al., 1999). For reasons, yet unknown, we even found that superoxide dismutase (SOD; 300 U/ml), which alleviates the superoxide radical anion, increased the amount of H₂O₂ accumulation (1.4-fold), but decreased the taxol production (0.9-fold) compared with the treatment of fungal elicitor alone did (Fig. 3 B). These results show that elicitor-induced taxol production did not depend on the intensity of H₂O₂ from oxidative burst, which is in contrast to the observations of Yuan et al. (2001; 2002).

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