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Cell Biology International 31 (2007) 1179–1183

Cell Biology International

www.elsevier.com/locate/cellbi

Salicylic acid-induced taxol production and isopentenyl pyrophosphate biosynthesis in suspension cultures of *Taxus chinensis* var. *mairei*

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Received 10 January 2007; revised 25 February 2007; accepted 23 March 2007

Abstract

The influences of salicylic acid (SA) on taxol production and isopentenyl pyrophosphate (IPP) biosynthesis pathways in suspension cultures of *Taxus chinensis* var. *mairei* were investigated by adding SA and mevastatin (MVS), a highly specific inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase in the mevalonate pathway for IPP biosynthesis, into the culture systems. The cell death and taxol production were induced upon the introduction of SA, and 20 mg/l was proved to be the optimal SA concentration in terms of the less damage to *Taxus* cells and marked activation of phenylalanine ammonia lyase (PAL). In the coexistence of SA (20 mg/l) and MVS (100 nmol/l), the taxol content (1.626 mg/g dry wt) was higher than that (0.252 mg/g dry wt) of the MVS-treated system but almost equal to that (1.581 mg/g dry wt) of the SA-treated system. It is thus inferred that the activated non-mevalonate pathway should be responsible for the formation of IPP in taxol biosynthesis in the presence of SA.

Keywords: *Taxus chinensis* var. *mairei*; Suspension culture; Taxol; Salicylic acid; Isopentenyl pyrophosphate

1. Introduction

Taxol, a diterpene found in various tissues of *Taxus* species, and relevant taxanes have received considerable attention as promising anticancer agents owing to their unique action mode on the microtubular cell system (Eisenhauer and Vermorken, 1998). Due to the very limited supply of taxol from nature, plant cell cultures have been recognized as a potential alternative of producing taxol in a large scale (Yukimune et al., 2000; Khosroushahi et al., 2006). However, owing to the low yield and titer of taxol, further improvement in taxol production by plant cell cultures is essential, which should be based on an effective regulation of taxol biosynthesis in *Taxus* cells.

A number of articles have reported the strategies to increase secondary metabolite production from plant cell cultures. Among them eliciting secondary metabolism by adding various kinds of abiotic and biotic elicitors is frequently used (Wang et al., 2004; Naill and Roberts, 2005; Kim et al., 2005). It has been shown that salicylic acid (SA), one of the plant hormones, plays an important signaling role in activation of various plant defense responses and strongly induces secondary metabolisms in plants (Sirvent and Gibson, 2002; Bulgakov et al., 2002; Kang et al., 2004). The induction mechanism of elicitors is generally regarded as inducing the expression of defense-related genes and activating the pathways of defense-related secondary metabolisms (Ebel and Mithorer, 1998; Qian et al., 2006). It is thus inferred that the induction

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doi:10.1016/j.cellbi.2007.03.038

of SA on secondary metabolisms may be relevant to the activation of biosynthesis pathways.

Isopentenyl pyrophosphate (IPP) is an essential precursor for taxol biosynthesis (Connolly and Hill, 1992). It has been shown that IPP biosynthesis might be involved in the mevalonate and non-mevalonate pathways at the late growth phase of *Taxus* cells (Srinivasan et al., 1996; Wang et al., 2003a,b). Thus, the effective regulation of the two IPP biosynthesis pathways may improve taxol production depending on the induction of elicitors. However, little is known on the mechanism of activating the IPP biosynthesis pathways by elicitors.

In the present work, the effects of SA on taxol production and IPP biosynthesis pathways were studied by adding SA and a metabolic inhibitor into the suspension cultures of *Taxus chinensis* var. *mairei* to shed some light on the induction mechanism of SA on taxol biosynthesis from the viewpoint of activating the IPP biosynthesis pathways.

2. Materials and methods

2.1. Chemicals

SA and MVS were purchased from Sigma. All other chemicals were of analytical grade and obtained commercially.

2.2. Cell line and culture conditions

Taxus chinensis var. *mairei* (T) was from the Botany Institute of Chinese Academy of Sciences. The callus and cell line preparation was done as previously described (Li et al., 1999). The cell line was grown in modified solid B5 medium (pH 5.8) supplemented with inositol (0.1 g/l), vitamins B_1 (10 mg/l), B_6 (1 mg/l), nicotinic acid (1 mg/l), sucrose (25 g/l), 6-benzyladenine (0.5 mg/l) and casein acid (1 g/l). Fresh cells (4 g) from the solid medium were collected, inoculated into 50 ml fresh modified liquid B5 medium in a 250 ml Erlenmeyer flask and subcultured at 25 °C in dark with continuous shaking at 110 rpm for 15 days. To ensure the cell uniformity, 6–8 flasks each containing 50 ml cultures of the 5th generation were combined as the inoculum in a 500 ml Erlenmeyer flask containing 150 ml fresh liquid medium. After being incubated for 15 days, cells were transferred into 250 ml fresh medium in a 1000 ml Erlenmeyer flask. These cells were collected by vacuum filtration after being cultured 15 days. Cell samples (4 g) were transferred successively to 50 ml fresh liquid medium in 250 ml flasks and divided into 3 groups for triplicate experiments.

2.3. Experimental procedures

Appropriate amounts of SA or MVS were added into the culture medium at day 7 or 14 of the cell growth through a sterilized filter. Samples of the treated and control cultures were taken at predetermined time intervals for analysis. All data were the average of triplicate experiments and the standard deviations (SD) were determined using the Microsoft Excel program. *P* values calculated by Student's *t*-test less than 0.05 were considered statistically significant.

2.4. Evens blue staining

Evans blue staining was performed following the procedures of Shirasu et al. (1997) with a slight modification. Fresh cells (1 g) were washed three times with phosphate buffer (0.05 M, pH 5.8) and incubated for 10 min with 0.05% (w/v) Evans blue. The unbound dye was washed three times with phosphate buffer (0.05 M, pH 5.8). The dye bound to dead cells was dissolved in 50% (v/v) methanol containing 1% (w/v) SDS at 50 °C for 30 min and then quantified with a Model 9100 UV-Vis spectrophotometer (Beijing Ruili Co., Beijing, China) at 600 nm.

2.5. Medium conductivity measurement

Suspension cultures (10 ml) were filtered and the filtrate was collected for conductivity measurement using a conductivity meter (Model DDS-307, Shanghai, China).

2.6. Phenylalanine ammonia-lyase (PAL) assay

PAL extraction and activity assay were performed as described in a previous publication (Yuan et al., 2002).

2.7. Extraction of taxol and analysis by HPLC

Taxol was extracted and analyzed as reported previously (Wang et al., 2003a).

3. Results

3.1. Evans blue staining

Cell death after the addition of SA was detected by Evans blue staining (Mazel and Levine, 2001) (Fig. 1). For the control culture, the ratio of numbers of dead cells to total cells decreased with time within the first 7 days due to the rapid growth of cells at the exponential phase and changed less afterwards as the cells entered into a steady growth phase. In the case of 10 and 20 mg/l SA the ratio changed less with culture time while it increased in the case of 30 and 40 mg/l SA. The ratio increased with increasing SA concentration, indicating that lower SA concentration caused less damage to the cells.

3.2. Conductivity measurement

The change of culture medium conductivity was measured as an indicator of plant cell death (Mazel and Levine, 2001) to quantify the kinetics of lesion development after SA addition in *Taxus* cells (Fig. 2). The conductivity of the control cultures

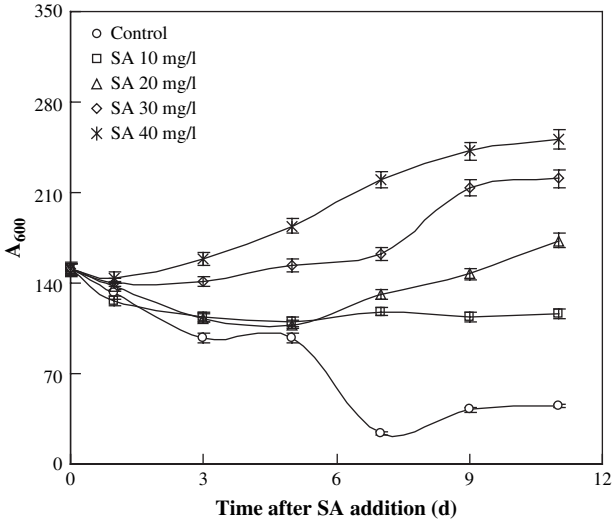

Fig. 1. Cell death (assayed by Evans blue staining) in suspension cultures of *Taxus chinensis* var. *mairei* in the presence of salicylic acid (SA) added at day 7 of cultivation. Error bars represent standard deviation.