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ANTIOXIDANT ENZYMES IN *NICOTIANA* CELLS CONTAINING AN *IPOMOEA* PEROXIDASE GENE

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Key Word Index—*Ipomoea batatas*; Convolvulaceae; *Nicotiana tabacum*; Solanaceae; transgenic plants; transgenic cell lines; peroxidase; superoxide dismutase; catalase; glutathione reductase.

Abstract—The levels of the antioxidant enzymes, peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), were investigated in seven cell lines (referred to as transgenic tobacco cell lines) derived from transgenic tobacco (*Nicotiana tabacum*) plants carrying a sweet potato (*Ipomoea batatas*) anionic POD cDNA (swpa1). Transgenic tobacco cell lines were induced from the leaf tissues of transgenic plants on MS medium supplemented with 1 mg l^{-1} 2,4-D. A novel isoenzyme encoded by the swpa1 cDNA was detected on the native-PAGE in all transgenic tobacco cell lines. Average POD activity in transgenic cell lines was ca 1.3 times higher than that of control cell lines, whereas the other three antioxidant enzymes showed a slightly lower level in transgenic cell lines. The POD specific activity in each transgenic cell line had a high correlation with that of leaves in the original transgenic plants ($r = 0.63$). The ratio of *in vitro* cell lines to plant leaves in specific activities of four antioxidant enzymes showed a considerable difference: 2.5 in POD activity, 2.1 in SOD activity, 0.05 in CAT activity and 0.9 in GR activity. These results suggest that the antioxidative mechanism between *in vitro* cultured cells and intact plants may be differently regulated.

INTRODUCTION

Peroxidase (POD, EC 1.11.1.7) is a ubiquitous enzyme that reduces hydrogen peroxide in the presence of an electron donor. POD plays significant roles in many physiological processes including lignification and is widely used for industrial applications [1–3]. POD activity in plants increases in response to a variety of physical, chemical, and biological stresses [4–6]. In all of these physiological processes the POD isoenzyme patterns are often complex. This complexity has caused difficulty in understanding the specific function of these enzymes *in vivo* and their specific roles in plant growth and in adaptation to environmental stress [7]. Therefore, it is important to isolate the genes that encode each isoenzyme, and characterize their expression and properties *in vivo*. Cultured plant cells might be good material for POD production and study of antioxidative mechanism, because they are considered to be grown under high oxidative stress conditions [8–11].

In previous reports, we have characterized three acidic POD isoenzymes and two POD cDNAs (anionic swpa1 and neutral swpn1) from suspension cultured cells of sweet potato (*Ipomoea batatas*), and developed transgenic tobacco (*Nicotiana tabacum*) plants of two cultivars (cv Bel W3 and cv Samsun) carrying two POD cDNAs [11–13]. Among transgenic plants developed, Bel W3 transgenic plants carrying anionic POD cDNA (swpa1) showed the highest POD activity in young fully expanded leaves [13]. In this study, we describe the callus induction from the leaves of seven Bel W3 transgenic tobacco plants carrying swpa1, and the levels of four antioxidant enzymes, peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR), in transgenic cell lines.

RESULTS AND DISCUSSION

Transgenic tobacco cell lines carrying a sweet potato anionic POD

Calli were formed on leaves of seven transgenic tobacco plants carrying a sweet potato anionic POD

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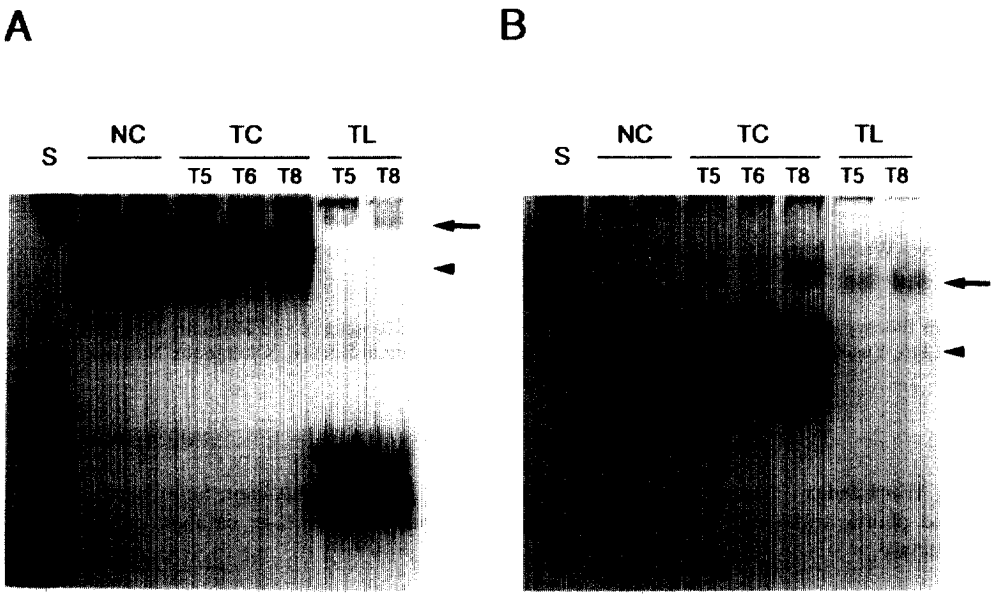

Fig. 1. Native gel stained for the POD activity from transgenic tobacco cell lines. 10 μg of protein in cell lines and 40 μg of protein in leaves were loaded on the gel on a 9.8% non-denaturing polyacrylamide gel for 3 h (A) and 5 h (B) at 20 mA. S: sweet potato suspension cultured cells (SP-47 cell line) [10]; NC: nontransgenic cell lines; TC: transgenic cell lines; TL: transgenic tobacco leaves. Arrows and arrowheads indicate a POD isoenzyme encoding a sweet potato acidic POD cDNA (swpa1) and tobacco cell line-specific POD isoenzymes, respectively.

cDNA (swpa1) [13]. To examine the expression of the sweet potato POD gene in the transgenic cell lines on the protein level, native gel analysis was performed and POD isoenzymes were stained with benzidine and hydrogen peroxide. For better separation of an isoenzyme encoding the swpa1 cDNA with a low electrophoretic mobility, two different electrophoretic times (3 h, Fig. 1A: 5 h Fig. 1B) were used. A novel protein band corresponding to the isoenzyme encoded by the swpa1 cDNA was detected on the native-PAGE in all transgenic tobacco cell lines (TC; T5, T6 and T8), which was not found in nontransgenic cell lines (NC) (Fig. 1B). These results confirmed that the sweet potato POD gene is stably incorporated into the genomic DNA in cell lines via transgenic tobacco leaves (TL; T5 and T8). From the native gel assay of POD transformants, it appears that the isoenzyme encoded by the swpa1 cDNA might be equivalent to one of the major isoenzymes in suspension cultures of sweet potato which have a low electrophoretic mobility (arrow in Fig. 1). The POD isoenzyme patterns showed a significant difference between *in vitro* cultured cells and intact tobacco leaf tissues as well as *in vitro* cultured cells of sweet potato. Dominant isoenzymes in tobacco cell lines (arrowhead in Fig. 1) might be *in vitro* tobacco cell-specific isoenzymes which are closely associated with culture stresses. The POD isoenzymes in TL with a high electrophoretic mobility are considered as tobacco leaf-specific isoenzymes compared with TC (Fig. 1).

There are many reports on plant regeneration systems from transgenic calli to transgenic plants, which are very important for molecular breeding in the field of plant biotechnology. Conversely, direct transfer of the foreign gene into *in vitro* transgenic cell lines via transgenic plants as shown in this study might be a meaningful method for the development of cell lines. The interchangeable system of hairy root and cell suspension cultures of *Catharanthus roseus* for indole alkaloid production has been proposed for the large scale production of hairy root cultures [14].

Activities of antioxidant enzymes in transgenic plants and cell lines

Fully expanded leaves of transgenic plants and the calli of the stationary growth stage were used for the determination of the activity of the four antioxidant enzymes, POD, SOD, CAT and GR. The activity of the four antioxidant enzymes showed a different level in seven transgenic tobacco plant leaves and cell lines (Table 1). Transgenic plants (T1, T3, T5, and T8) that highly express the anionic POD (swpa1) showed from 3.2 to 7.0 times higher POD specific activity (units/mg protein) than control plants, whereas transgenic cell lines (T2, T5 and T8) showed from 1.4 to 1.7 times higher POD activity. T8 transgenic tobacco plant showing the greatest POD activity marked the highest POD level in the transgenic cell lines, which is ca 1.7 times greater than in control cell lines. Average POD activity in transgenic cell lines was ca 1.3 times higher than that of control cell lines, whereas that in transgenic plants was ca 3.1 times higher than that of control plants. The POD specific activity in each transgenic cell line had a good correlation with that of leaves in each original transgenic plant ($r = 0.63$). In

Table 1. Comparison of antioxidant enzyme activity (units/mg protein) between leaf and callus derived from anionic (swpa1) transgenic tobacco. Similar results were obtained in two other independent experiments. Data are an average of three measurements in each sample

	POD		SOD		CAT		GR	
Plant	leaf	callus	leaf	callus	leaf	callus	leaf	callus
	units/mg protein (fold to control)							
CON*	2.1	7.3	33.2	55.0	0.05	0.003	0.35	0.31
T1	8.8 (4.3)	—	19.0 (0.6)	—	0.07 (1.4)	—	0.24 (0.7)	—
T2	1.8 (0.9)	10.8 (1.5)	14.9 (0.4)	58.6 (1.1)	0.05 (1.0)	0.003 (0.7)	0.26 (0.7)	0.20 (0.6)
T3	6.8 (3.2)	7.0 (1.0)	18.9 (0.6)	44.8 (0.8)	0.06 (1.2)	0.001 (0.3)	0.38 (1.1)	0.34 (1.1)
T4	3.7 (1.8)	8.0 (1.1)	11.5 (0.3)	57.8 (1.1)	0.04 (0.8)	0.002 (0.7)	0.14 (0.4)	0.14 (0.5)
T5	9.7 (4.6)	10.5 (1.4)	36.3 (1.1)	57.2 (1.0)	0.03 (0.6)	0.003 (1.0)	0.39 (1.1)	0.30 (1.0)
T6	4.4 (2.1)	9.0 (1.2)	22.5 (0.7)	52.1 (0.9)	0.06 (1.2)	0.001 (0.3)	0.21 (0.6)	0.18 (0.6)
T7	2.5 (1.2)	7.4 (1.0)	11.0 (0.3)	42.9 (0.8)	0.03 (0.6)	0.003 (1.0)	0.14 (0.4)	0.18 (0.6)
T8	14.9 (7.0)	12.1 (1.7)	31.7 (1.0)	52.0 (0.9)	0.09 (1.8)	0.001 (0.3)	0.53 (1.5)	0.40 (1.3)
Mean	6.5 (3.1)	9.3 (1.3)	20.7 (0.6)	52.2 (0.9)	0.05 (1.0)	0.002 (0.7)	0.28 (0.8)	0.24 (0.8)

*CON: nontransformed control plant or callus.
T1–T8: Bel W3 plants transformed by anionic POD (swpa1).
—: not determined owing to no callus induction.
The number in parentheses indicates the value (fold) of control plant or callus.

addition to POD, transgenic tobacco plants and cell lines in the other three antioxidant enzymes showed a similar level to that of control or slightly lower than control (Table 1). The antioxidant enzyme activity dramatically fluctuated during the cell growth [11, 15]. For exact comparison between *in vitro* cell lines and intact plants, the enzyme activity should be investigated during cell growth and plant development.

Comparison of specific enzyme activity in plant leaves and cultured cells

The average levels of the four antioxidant enzymes in tobacco cell lines were compared with those of intact plants. Interestingly, the ratio of *in vitro* cell lines to plants in specific activities of antioxidant enzymes showed a considerable difference: 2.5 in POD activity, 2.1 in SOD activity, 0.05 in CAT activity and 0.9 in GR activity. It is very interesting that the ratio in POD and CAT activity showed reverse results, even though they are enzymes using hydrogen peroxide as substrate.

In our previous results, the CAT specific activity in 97 cultured cell lines from various plant resources had no correlation with that of POD and SOD, whereas POD activity showed a slight correlation with SOD activity ($r = 0.37$) [16]. Salicylic acid (SA) inhibits the CAT activity in plants and induced PR gene expression resulting in enhanced disease resistance [17]. However, SA markedly increased the CAT activity in suspension cultures of sweet potato, but decreased the POD, SOD and GR [18]. The change in POD activity during cell growth differed from that in CAT: POD activity showed the lowest level at exponential growth stage and the highest level at the end of cell cultures [11], whereas CAT activity marked the highest at exponential growth stage, but the lowest level at the end of cell growth stages [unpublished data]. Our results suggest that the antioxidative mechanism between *in vitro* cultured cells and plants may be regulated differently.

EXPERIMENTAL

Plant material and cell cultures

Transgenic tobacco (*Nicotiana tabacum* cv Bel W3) plants carrying a sweet potato anionic cDNA (swpa1) were used for induction of *in vitro* cell lines [13]. Transgenic cell lines were induced on Petri dishes with 20 ml of MS (Murashige and Skoog) [19] medium containing 1 mg l^{-1} 2,4-D, 30 g^{-1} sucrose and 0.4% Gelrite from the leaf tissues of transgenic plants at 25° in the dark. Every month 1 g fr. wt of calli were subcultured under the same culture conditions. The calli subcultured over 5 times were used for analysis.

Enzyme activity. The calli or leaves (1 g fr. wt) were homogenized on ice with a mortar in 50 mM KPi buffer (pH 7.0, 1:2.5, w/v). The homogenate was centrifuged at 12,000 *g* for 15 min at 4°. The supernatant was used immediately for enzyme assay. The POD activity was assayed according to the method of Ref. [11] using pyrogallol as a substrate. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 20 s at pH 6.0. The SOD activity was measured by its ability to inhibit the reduction of ferricytochrome c by superoxide radicals generated by xanthine/xanthine oxidase according to the method of Ref. [20]. One unit of SOD activity is defined as that 50% inhibiting xanthine oxidase activity at *A* 550 nm. CAT activity was determined by measuring the linear rate of decrease at *A* 240 nm in

a soln of 12.5 mM H_2O_2 [21]. One unit of CAT activity is defined as that catalysing the decomposition of 1 μM H_2O_2 min^{-1}. GR activity was determined from the rate of NADPH oxidation as measured by the decrease in A at 340 nm according to the method of Ref. [22]. Protein was determined according to the method of Ref. [23] using Bio-Rad protein assay reagents.

POD gel assay. The POD native-polyacrylamide gel electrophoresis (PAGE) was conducted for 3 h or 5 h at 20 mA using 9.8% gel. After native-PAGE, POD was stained with 1% benzidine and 1.5% H_2O_2.

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