Note

Further Characterization of C₆-Aldehyde-forming System in Alfalfa Cells Cultured *in vitro*Jiro SEKIYA, Koji MUNESHIKA
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Volatile C₆-aldehydes such as hexanal, *cis*-3-hexenal and *trans*-2-hexenal, along with the corresponding alcohols, characterize flavors of plant tissues and are found in a variety of plants.¹⁾ Lipoxygenase and hydroperoxide lyase (HPO lyase) are responsible for the enzymatic formation of these C₆-aldehydes from linoleic and linolenic acids originating from lipids.²⁾ In a previous paper we reported that cultured green cells of alfalfa had the ability to produce the C₆-aldehydes from linoleic and linolenic acids.³⁾ The present paper describes further characterization of the C₆-aldehyde-forming enzyme system of the cultured alfalfa cells.

Cultured alfalfa cells (*Medicago sativa*) were induced in May 1976 from germinating seeds and cultured on Linsmaier-Skoog agar medium containing 2,4-dichlorophenoxyacetic acid (2,4-D, 1 μ M) or 1-naphthaleneacetic acid (NAA, 10 μ M) under illumination with fluorescent lamps (about 3000 lux).³⁾ The cells were subcultured every 7 to 8 weeks. Cells cultured for about 40 days after transfer to fresh medium were harvested and 0.5 g (fresh weight) of the cells was homogenized with 10 ml of McIlvaine's buffer, pH 6.3. The homogenate was used as the enzyme

solution without centrifugation. The homogenate (10 ml) was incubated with or without a substrate (6 μ mol) for 10 min at 35°C in a sealed 50 ml-flask. HPO lyase activity was determined with 13-hydroperoxylinoleic acid as a substrate, and the overall C₆-aldehyde-forming activity with linoleic acid as a substrate, both by the headspace method.⁴⁾ Lipoxygenase activity was determined with an oxygen electrode, as described previously.⁴⁾ Protein was determined by the method of Lowry *et al.*⁵⁾ and chlorophyll by the method of Mackinney.⁶⁾

When the homogenate of alfalfa green cells cultured *in vitro* was incubated with the C₁₈-fatty acids, hexanal and hexenals (*cis*-3-hexenal and *trans*-2-hexenal) were formed from linoleic and linolenic acids, respectively, as reported previously.³⁾ The homogenate of the green cells consumed oxygen with linoleic acid (Table I) and HPLC analysis of the hexane extract of the reaction mixture confirmed the formation of 9- and 13-hydroperoxides of linoleic acid (data not shown). The homogenate treated with heat at 100°C showed neither oxygen consumption nor the hydroperoxide formation. This indicates that the green cells contained lipoxygenase. The green cell homogenate also catalyzed the formation of hexanal from 13-hydroperoxylinoleic acid (Table I). This means that the HPO lyase was involved in the green cells. Thus, lipoxygenase and HPO lyase are responsible for the enzymatic C₆-aldehyde formation in the green cells. The overall C₆-aldehyde-forming activity of the green cells increased with increasing extent of greening during the successive subcultures for more than 4 years. In contrast, white cells cultured with 2,4-D did not show the C₆-aldehyde-forming activity (Table I). Therefore, the C₆-aldehyde-forming enzyme system may be related to the greening of alfalfa cells.

Figure 1 shows the effects of illumination on the enzyme activities of cultured green cells. When the green cells were transferred to the dark from the light conditions, lipoxygenase and the C₆-aldehyde-forming activity decreased to almost zero, and the low levels of the activities were maintained during the following dark period. In contrast, HPO lyase activity decreased temporally to 50% of the

TABLE I. ENZYME ACTIVITIES CONCERNED IN VOLATILE C₆-ALDEHYDE FORMATION OF CULTURED ALFALFA CELLS

The cultured cells used were about 40 days-old after the transfer to fresh medium. Values are per g fresh weight.

Tissues	Chlorophyll (μ g)	Protein (mg)	Lipoxygenase (μ mol/min)	HPO Lyase (μ mol/10 min)	C ₆ -Aldehyde-forming activity (μ mol/10 min)
Cells cultured with NAA					
54 months-old	160	28	0.80	2.01	0.62
Cells cultured with 2,4-D					
54 months-old	4	25	trace	0.10	trace
Green leaves					
July, 1981	1442	77	1.06	6.14	3.32

FIG. 1. Effect of Illumination on the Enzyme Activities Concerned in C_6 -Aldehyde Formation in Cultured Alfalfa Cells.

Alfalfa green cells cultured for 40 days in the light were transferred to the dark conditions at time zero. The cells in the dark were again transferred to the light 72 h after the first transfer. The chlorophyll content (A) and the enzyme activities (B) were determined at the times indicated. Symbol (Δ) in (B) indicates lipoxxygenase activity: ($\bullet$), HPO lyase activity; ($\circ$), hexanal-forming activity from linoleic acid.

original level after dark incubation for 24 h, but returned to the original level later even in the dark (Fig. 1). When the cells were transferred to the light again, lipoxxygenase and the C_6 -aldehyde-forming activity were restored to the original levels. Therefore, lipoxxygenase activity of the cultured green cells of alfalfa is light-dependent, but HPO lyase is only slightly light-dependent. Consequently, the level of the C_6 -aldehyde-forming activity of the cultured

green cells is dependent on the illumination. Light-dependency of the lipoxxygenase activity is also the case with the mustard seedling.⁷⁾ Thus, the green alfalfa cells cultured *in vitro* are advantageous materials for investigating the environmental factors which affect the level of the C_6 -aldehyde-forming activity.

When the C_6 -aldehyde-forming activity of green tissues is affected by environmental factors such as temperature,⁴⁾ light illumination as presented here, the developmental stage⁸⁾ and plant species,⁹⁾ it can be attributed mainly to the change in the level of lipoxxygenase rather than HPO lyase.

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