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Tissue Culture Note

Biotransformation of estragole by the plant cultured cells of *Caragana chamlagu*

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Abstract Estragole (EG) is biosynthesized in herbs including anise, basil, bay, tarragon, fennel and marjoram, and is thought to be a useful biomass for the food and health industries. Moreover, the metabolites from estragole are useful intermediates in organic synthetic chemistry. However, estragole has been transformed only by chemical methods, and no biocatalysts have been reported. In this paper, we report the biotransformation of estragole using the plant cells of *Caragana chamlagu* gave 4-methoxycinnamaldehyde (MCAL), 4-methoxycinnamyl alcohol (MCA) and 4-methoxybenzaldehyde (MBAL). In addition, we propose a reaction mechanism in the biotransformation of estragole using *Caragana chamlagu*. Since estragole generates malignant liver tumors in the rat, it is necessary to reduce exposure. The present study reveals the transformation of harmful estragole. Furthermore, we succeeded in biotransforming estragole as biomass using plant cells into useful compounds.

Key words: Biotransformation, *Caragana chamlagu*, estragole, plant cultured cells.

Estragole (EG) was biosynthesized in herbs including anise, basil, bay, tarragon, fennel and marjoram (McDonald 1999) and is thought to be a useful biomass for the food and health industries. EG is used as flavoring for foods and fragrances for cosmetics, perfumes, soaps and detergents. In 1998, it was reported that the production of EG in the U.S. exceeded one million pounds per year (McDonald 1999).

The metabolites from EG are useful intermediates in organic synthetic chemistry. For example, 4-methoxycinnamaldehyde (MCAL), which is a metabolite of estragole, was converted into (*S*)-goniothalamin analogue, and shows cytotoxic activities against human cancer cells (Fatima et al. 2006), and (−)-oudemansin shows strong antifungal activities (Umezawa et al. 1985). Furthermore, 4-methoxycinnamyl alcohol (MCA), a metabolite of EG was converted into (±)-cytoxazone as a cytokine modulator of microbial origin (Asano et al. 2005).

Plant cultured cells have the ability of oxidation, reduction, hydroxylation, hydrolysis or glycosyl conjugation to some natural and unnatural products (Ishihara et al. 2003). Recently, we studied biotransformation using plant cultured cells (Itoh et al. 2005; Sakamaki et al. 2005), and reported that olefins were oxidized by the plant cells of *Caragana chamlagu* into an epoxide or hydroxyl group (Sakamaki et al. 2004; Sakamaki et al. 2008). EG has been transformed only by chemical methods (Kishore and Kannan 2006; Sharma et al. 2005), and no biocatalysts have been applied for transformation. Therefore, we attempted the biotansformation of EG using various kinds of plant cells. In this work, we report that the reaction of EG using the plant cells of *C. chamlagu* gave MCAL, MCA and 4-methoxybenzaldehyde (MBAL). Furthermore, we describe the reaction mechanism in the biotransformation of estragole using *C. chamlagu*, and propose a novel method of utilizing estragole.

First, biocatalysts that can react with EG were screened (Figure 1). The plant cultured cells used here were *C. chamlagu*: Chinese peashrub, *Ocimum basilicum*: Basil, *O. basilicum* cv. *Purpurscens*: Dark opal basil, *Corchorus olitorius*: Jute, *Perilla frutescens* var. *crispa*: Ao shiso and *Lavandula angustifolia*:

Abbreviations: EG, estragole; MBAL, 4-methoxybenzaldehyde; MCA, 4-methoxycinnamyl alcohol; MCAc, 4-methoxycinnamic acid; MCAL, 4-methoxycinnamaldehyde

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Tasmanian lavender, and these cultures were grown in MS solid medium (Murashige and Skoog 1962) containing 3 g l^{-1} sucrose and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) at 25°C for about 21 days in the dark. In this reaction, the callus tissues (5 g wet weight) were transferred to an MS liquid medium (10 ml) containing 30 g l^{-1} sucrose and 1 mg l^{-1} 2,4-D, and were grown with shaking at 120 rpm at 25°C for 24 h in the dark. EG (15 mg) in ethanol (1 ml) was added to the suspension. The mixture was shaken at 120 rpm at 25°C in the dark, and the callus tissues (0.2 g dry weight) were filtered to separate and washed by EtOAc (2×10 ml). The filtrate was dissolved in EtOAc (2×15 ml), and washed with sat. aq. NaCl (2×5 ml). The combined organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The results of the conversions from the gas chromatographic analyses, which were performed using a Shimadzu GC-2014 equipped with a GC-column (DB-1, 25 m), are summarized in Table 1. The chemical yields were determined by GC analysis using *n*-dodecane as the internal standard, and the GC conditions and retention times of the products and *n*-dodecane are as follows: GC conditions; 1) 100°C to 150°C (25°C min^{-1}), 2) 150°C (5 min) and 3) 150 to 250°C (4°C min^{-1}), t_R/min; estragole: 4.4, *n*-dodecane: 4.6, 4-methoxybenzaldehyde: 4.9, 4-methoxycinnamaldehyde: 10.7, 4-methoxycinnamyl alcohol: 10.9. All products were identified by ^{1}H-NMR, ^{13}C-NMR, or MS. GC-MS (EI) analyses were performed using a Shimadzu GCMS-QP5000 equipped with GC-column (DB-1, 60 m) and an ionizing energy of 70 eV. HRMS analysis was performed on an Agilent G1969LC/MDS TOF. ^{1}H and ^{13}C NMR spectra were recorded using a JOEL JNM-GX400 spectrometer with tetramethylsilane as the internal standard. MCAL was identified by NMR and GC as compared with the reference (Su and Takaishi 1999) and the purchased standard sample: ^{1}H-NMR ($CDCl_3$) δ=9.66 (d, *J*=7.8 Hz, 1H), 7.53 (d, *J*=9.2 Hz, 2H), 7.43 (d, *J*=15.6 Hz, 1H), 6.95 (d, *J*=8.7 Hz, 2H), 6.62 (dd, *J*=16.0, 7.8 Hz, 1H), 3.87 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ=193.8, 162.2, 152.8, 130.3, 126.8, 126.5, 114.5, 55.4; EI-MS *m*/*z* 162 $[M]^+$ (100), 161 (60), 147 (20), 121 (38), 119 (34), 108 (37), 91 (45), 77 (35). And MCA: ^{1}H-NMR ($CDCl_3$) δ=7.33 (d, *J*=8.7 Hz, 2H), 6.86 (d, *J*=8.7 Hz, 2H), 6.56 (d, *J*=16.0 Hz, 1H), 6.20–6.27 (m, 1H), 4.30 (d, *J*=6.0 Hz, 2H), 3.80 (s, 3H), 1.57 (brs, 1H); ^{13}C-NMR ($CDCl_3$) δ=159.1, 131.1, 129.4, 127.8, 126.3, 114.1, 64.1, 55.4; EI-MS *m*/*z* 164 $[M]^+$ (44), 162 (16), 147 (9), 131 (22), 121 (100), 108 (39), 91 (30), 77 (25); HRMS (TOF-Cl) calcd for $C_{10}H_{13}O_2$ (MH^+): 165.0915 (Found: 165.0919). MBAL was identified by NMR and GC as compared with the purchased standard: ^{1}H-NMR ($CDCl_3$) δ=9.89 (s, 1H), 7.85 (d, *J*=9.0 Hz, 2H), 7.01 (d, *J*=8.5 Hz, 2H), 3.90 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ=190.8, 164.6, 132.0, 129.9, 114.3, 55.6; EI-MS *m*/*z* 136 $[M]^+$ (78), 135 (100), 107 (13), 92 (13), 77 (21). Surprisingly, all the biocatalysts used here can easily convert estragole. However, in many cases, only a few products were detected, perhaps because of the facile biodegradation of the products. Among them, *C. chamlagu* gave considerable amounts of the products. When EG (15 mg) was added to the suspension of *C. chamlagu* (5 g wet weight) and shaken at 120 rpm at 25°C in the dark for 2 days, MCAL, MCA and MBAL were obtained in 19, 5 and 2% yields, respectively.

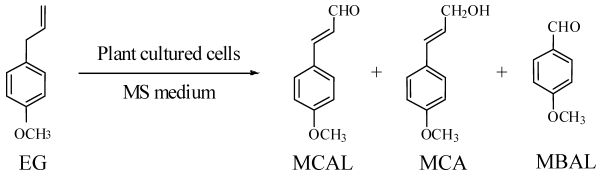

Figure 1. Biotransformation of estragole (EG) by some plant cultured cells.

The time course for the reaction using *C. chamlagu* is shown in Figure 2. The results show that more than 60% of EG was rapidly consumed in the first 2 days and then the decrease of EG was stopped for the following 4 days and then EG was decreased gradually. Corresponding to the decrease of EG, the yield of MCAL, the oxidation product, was obtained slowly up to 10% in 20 days. The other products, MCA and MBAL were increased gradually up to 7–10% in 14–20 days.

In order to elucidate the mechanism of this reaction

Table 1. Biotransformation of estragole by some plant cultured cells

Runa	Plant cultured cells	Product (%)			
		EG	MCAL	MCA	MBAL
1	*Caragana chamlagu*	36	19	5	2
2	*Ocimum basilicum*	22	1	—	—
3	*O. basilicum* cv. *Purpurscens*	22	1	—	—
4	*Corchorus olitorius*	24	2	4	—
5	*Perilla frutescens* var. *crispa*	21	1	1	—
6	*Lavandula angustifolia*	16	1	—	—

[a] Reaction time: 2 days.

EG, estragole; MCAL, 4-methoxycinnamaldehyde; MCA, 4-methoxycinnamyl alcohol; MBAL, 4-methoxybenzaldehyde

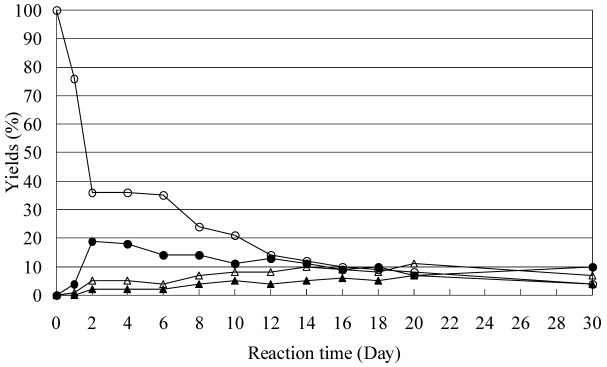

Figure 2. Time course for biotransformation of estragole (EG) by *C. chamlagu*. Reaction time: 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 30 days. Substrate and products: ○ Estragole (EG), ● 4-Methoxycinnamaldehyde (MCAL), △ 4-Methoxycinnamyl alcohol (MCA), ▲ 4-Methoxybenzaldehyde (MBAL).

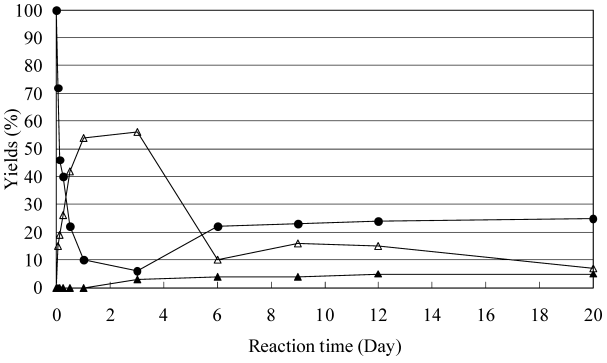

Figure 3. Time course for biotransformation of 4-methoxycinnamaldehyde (MCAL) by *C. chamlagu*. Reaction time: 1 h, 3 h, 6 h, 12 h, 1 day, 3 days, 6 days, 9 days, 12 days and 20 days. Substrate and products: ● 4-Methoxycinnamaldehyde (MCAL), △ 4-Methoxycinnamyl alcohol (MCA), ▲ 4-Methoxybenzaldehyde (MBAL).

pathway, MCAL was reacted with *C. chamlagu* and the results are shown in Figure 3. Then, MCAL (7.5 mg) was added to the suspension of *C. chamlagu* (3 g wet weight) and the resulting mixture was shaken at 120 rpm at 25°C in the dark. The starting material MCAL was decreased rapidly in the first 24 h and corresponding to the decrease of MCAL, the allyl alcohol, MCA was obtained (nearly 50–60% yield) and then the yield of MCA was decreased after 3 days and the yield of the starting material, unsaturated aldehyde, MCAL was increased again. From the results, MCAL was firstly reduced into MCA by *C. chamlagu* (nearly 60% yield in 3 days), and the product MCA was converted to give a mixture of MCAL, MCA and the degradative product, MBAL.

In addition, MCA was reacted with *C. chamlagu* and the results are shown in Figure 4. MCA (7.5 mg) was added to the suspension of *C. chamlagu* (3 g wet weight) and the resulting mixture was shaken at 120 rpm at 25°C in the dark. MCA was decreased rapidly in the first 12 h and corresponding to the decrease of MCA, MCAL was

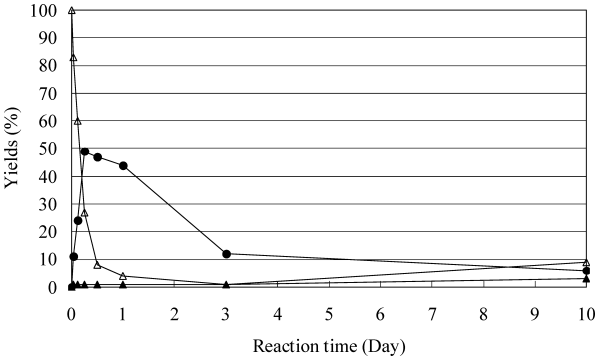

Figure 4. Time course for biotransformation of 4-methoxycinnamyl alcohol (MCA) by *C. chamlagu*. Reaction time: 1 h, 3 h, 6 h, 12 h, 1 day, 3 days, and 10 days. Substrate and products: ● 4-Methoxycinnamaldehyde (MCAL), △ 4-Methoxycinnamyl alcohol (MCA), ▲ 4-Methoxybenzaldehyde (MBAL).

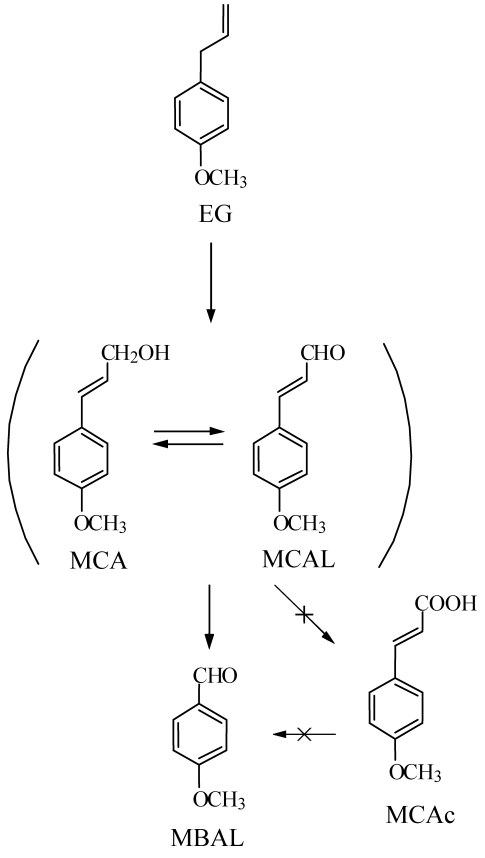

Figure 5. Reaction pathway.

obtained (about 50%). Then the yield of MCAL was decreased after 12 h and MCA was slowly and slightly increased (about 10% in 10 days). From the results, the starting material MCA was oxidized into MCAL by *C. chamlagu*, and the product MCAL was afforded to a mixture of MCAL, MCA and MBAL (6, 9 and 3% yields, respectively).

From these results, the reaction pathway of the biotransformation of EG by *C. chamlagu* is explained as shown in Figure 5. At first, EG was oxidized into MCAL

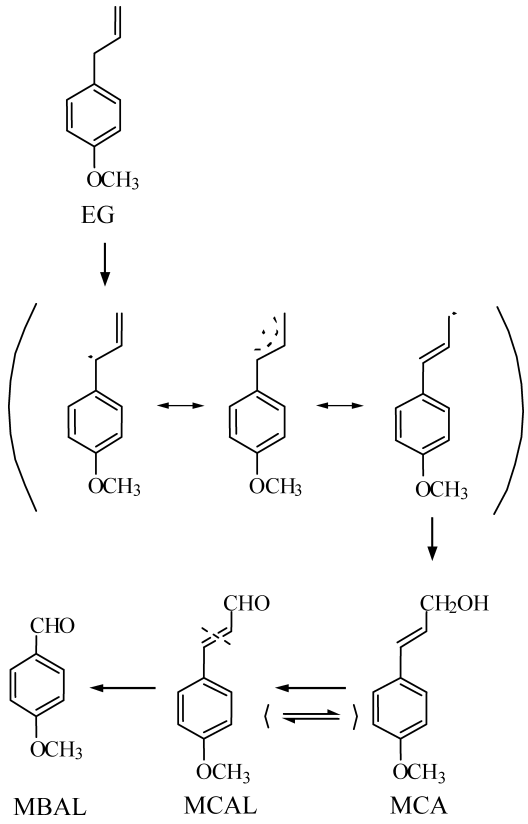

Figure 6. Reaction mechanism.

and (or) MCA by *C. chamlagu*. Next, the degradative product, 4-methoxybenzaldehyde, MBAL was obtained from MCAL or MCA. MCAL was reduced into MCA, and MCA was oxidized into MCAL by *C. chamlagu*. Since these oxidative and reductive reactions were attained to equilibrium between MCAL and MCA, the yields of MCAL and MCA were afforded to constant. The other pathway, the formation of MBAL via 4-methoxycinnamic acid (MCAc) was excluded. (In this reaction, MCAc and 4-methoxybenzoic acid as the products were not yielded and MCAc was not reacted in the reaction system.) Total yield (the yields of EG, MCAL, MCA and MBAL) was gradually reduced; it seems that MBAL was further degraded.

Tsai and co-workers reported the metabolic pathway of EG into MCA in animals (Tsai et al. 1994). Metabolic oxidation of the allyl side chain of EG may proceed into benzylic and terminal hydroxylation via radical intermediates. Moreover, Hatanaka and co-workers reported that trans-2, cis-6-nonadienal and trans-2, cis-6-nonadienol in the biooxidation of linolenic acid by plants are achieved to equilibrium state (Hatanaka et al. 1975). Therefore, we proposed the reaction mechanism in the formation of MCAL and MCA from EG (Figure 6). EG was immediately converted into MCAL and MCA via radical intermediates by *C. chamlagu* and redox equilibrium between MCAL and MCA was achieved. The production of MBAL was explained by the biooxidation of MCAL or MCA by a similar mechanism to that Ishikawa and co-workers reported in the biooxidation of coniferaldehyde and (or) ferulic acid by fungi where vanillin was obtained (Ishikawa et al. 1963). Therefore, it seems that MBAL was formed by the biooxidation of MCAL by *C. chamlagu*.

Since EG generates malignant liver tumors in the rat, it is necessary to restrict and to reduce its exposure (European Commission 2001; Rietjens et al. 2005; Smith et al. 2002). The present study reveals the transformation of harmful EG. Furthermore, it was shown that MCAL, which can easily be purchased, was transferred into MCA as an available compound by *C. chamlagu* in satisfactory yield. As limonene has been used as biomass for transformation into useful materials using biocatalysis (Chatterjee and Bhattacharyya 2001; Hamada et al. 2003; Mars et al. 2001), we succeeded in the biotransformation of EG as biomass using plant cells into useful compounds. We have further continued to investigate the biotransformation of excess producing natural products using biocatalysis.

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