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Differences in Glucosylation of Exogenous Hydroquinone by Two Morphologically Different Lines of *Catharanthus roseus* Cells

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Marked differences were found in the glucosylation of exogenous hydroquinone to arbutin by two morphologically different lines (CrA and CrB) of suspension-cultured *Catharanthus roseus* cells of the same origin. Cells of CrA were nearly spherical in shape, dense and smaller than those of CrB, while cells of CrB were cylindrical in shape with well developed vacuoles. Arbutin productivity in CrB was remarkably larger than that in CrA at every stage of growth. After hydroquinone addition, hydroquinone glucosyltransferase was induced within 6 hours and the activity reached a peak within 24 hours in both strains. However, the maximum activity in CrB was 5-fold higher than that in CrA, and this value corresponded with that of the arbutin yield. Arbutin accumulation per cell fresh weight was also 2.6-fold higher with CrB than CrA. These substantial differences in the glucosylation of hydroquinone to arbutin in the two cell lines indicate that selection of a superior cell strain is important for efficient production of compounds by biotransformation with plant cells.

Introduction

The efficiency of *de novo* synthesis can vary widely in different cell lines from the same plant species, and the selection of a superior cell line to produce useful metabolites is generally necessary[1,2]. On the other hand, although many xenobiotics, such as aromatic compounds[3,4], steroids[5], cardenolides[6], flavanones[7,8] and 2-phenylpropionic acid[9], have been glycosylated with cells in suspension, only a little is known about differences in bioconversion by different cell lines of the same plant species[10,11].

Arbutin, the monoglucoside of hydroquinone (HQ), is the main active component of *Arctostaphylos uva-ursi* Sprengel and is a potent suppresser of the synthesis of melanin in human skin[12] without having any apparent side effects[13]. We have reported that a suspension culture of *Catharanthus roseus* can efficiently convert exogenous supplied hydroquinone (HQ) into arbutin, particularly at high concentration of sugar[14], and we have described the successful production of arbutin in large amounts in a jar fermentor[15]. In the present work, we examined the glucosylation of HQ to arbutin by using two morphologically different cell lines of suspension-cultured *Catharanthus roseus* cells of the same origin, and we compared the glucosylation in these cell lines in detail.

Materials and Methods

1. *Cell culture*

Cell suspensions of *Catharanthus roseus* (L.) G. Don cell strain A (CrA) and strain B (CrB), which

had been established at Tohoku University, were subcultured weekly in Linsmaier and Skoog's liquid medium[16] supplemented with 30 g/*l* of sucrose and 2.2 mM 2, 4-dichlorophenoxyacetic acid by the transfer of 20 m*l* of the suspension to 180 m*l* of fresh medium in a 500 m*l* flask. Suspensions were cultivated in the dark on a gyratory shaker (110 rpm) at 26°C. The cultures in jar fermentors were conducted as in our previous work[15].

2. *Addition of hydroquinone (HQ)*

HQ (200 mM) was added at intervals of 24 h to give a concentration of 4 mM or 6 mM in the culture after the cells had cultivated for various times. In the case of jar fermentor culture, glucose (2.7 M aqueous solution) was added twice to a concentration of 55 mM at the first and second additions of HQ. Addition of HQ was continued until the cells no longer consumed the HQ supplied.

3. *Analysis of arbutin and HQ*

The cell suspension was disrupted by sonication and the cell debris was removed by centrifugation at 10000 rpm for 10 min. Total arbutin in the supernatant was analyzed by HPLC (TRI ROTAR-II. Japan Spectroscopic, Tokyo, Japan) on a CAPCELL PAK C_{18} column (Shiseido, Tokyo, Japan) in 5% methyl alcohol (adjusted to pH 2.5 with phosphoric acid) with monitoring at 230 nm. In some experiments, arbutin and HQ in the medium after cultivation were analyzed by the same method as described above. For determination of arbutin content within the cells, the suspension cells were filtered through filter paper (Advantec No. 2, Toyo Roshi Kaisya, Tokyo, Japan), and the collected cells were washed twice with deionized water, resuspended in deionized water, and disrupted by sonication. Arbutin was analyzed by HPLC as described above.

4. *Assay of HQ glucosyltransferase activity*

Cells (0.3 g) were collected by filtration, suspended in 0.6 m*l* of 50 mM phosphate buffer (pH 5.0) containing 10 mM mercaptoethanol, and sonicated four times for 30 s periods with 30 s intervals at 4°C. After centrifugation at 12000 rpm for 15 min, the supernatant was used as the preparation of crude enzyme.

Enzymatic activity was assayed as follows. An aliquot of the preparation of crude enzyme (100 μl) was added to 80 μl of a reaction mixture that contained 10 mM UDP [U-^{14}C] glucose (0.05 μ Ci), 10 mM HQ, 10 mM mercaptoethanol, and 50 mM phosphate buffer (pH 5.0), and the mixture was incubated at 37°C for 60 min. Incubation was stopped by addition of 0.2 m*l* of ethanol supplemented with 0.01 mmol of cold arbutin as a carrier. A 20 μl aliquot of the mixture was subjected to thin-layer chromatography on a plate of silica gel 60 F (Merck) with mobile phase of chloroform, methanol and water (30 : 10 : 1). After development, arbutin was located under UV light (254 nm), the appropriate region of gel was scraped off the plate, and the radioactivity was measured in a scintillation counter.

5. *Estimation of cell death*

The ability to reduce TTC was used as the criterion of cell viability. Fresh cells (about 0.2 g) were washed in 20 m*l* of phosphate buffer (pH 7.0) and suspended in 3 m*l* of a solution of TTC (0.6% in phosphate buffer, pH 7.0). After incubation overnight in the dark at 26°C, the cells were washed twice with water. Red pigment (reduced TTC) was extracted twice by heating in 95% ethanol at 85°C for 5 min. and the absorbance was measured at 485 nm. The time at which the ability to reduce TTC was lost coincided with the time when both HQ consumption and arbutin formation ceased.

Results and Discussion

1. *Ability to produce arbutin*

Strain B(CrB) of *Catharanthus roseus* was derived spontaneously from strain A(CrA)[17]. Both strains showed almost the same growth rate (data not shown), but the cell shapes are very different. As shown in **Fig. 1**, cells of CrA were globular in shape, dense and smaller than those of CrB, while cells of CrB were cylindrical in shape with well developed vacuoles. Both strains converted HQ, added to the medium, to arbutin.

In order to compare the arbutin productivity of the two strains, HQ was periodically added to the cultures until the cells no longer consumed the HQ supplied. **Table 1** shows that arbutin yield with CrB was 1.3-to 1.7-fold higher than that with CrA in either flask or jar fermentor cultures.

Several researchers have reported that the efficiency of biotransformation changed during culture stages[18-20]. We examined the arbutin yield at various stages of growth (**Fig. 2**). CrB formed much larger amounts of arbutin than CrA on the basis of medium volume throughout growth. On the basis of fresh weight, arbutin productivity in CrB cells diminished with advancing growth stage,

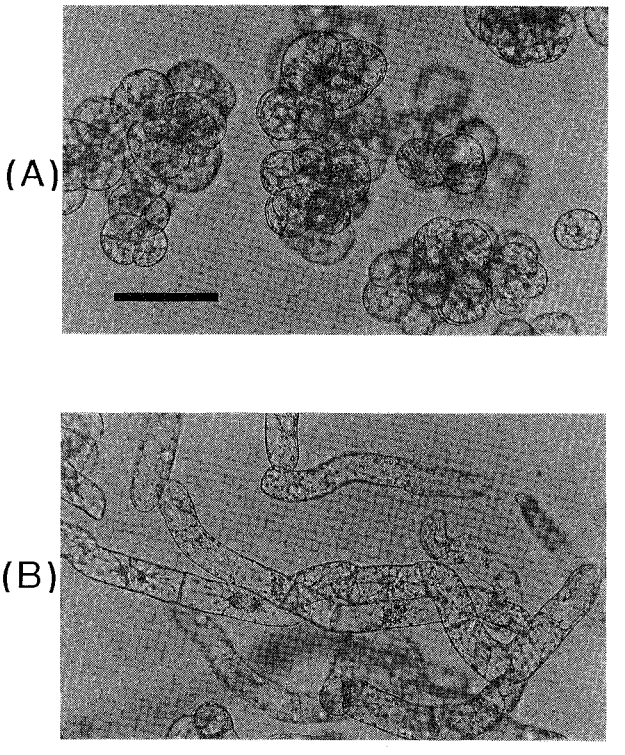

Fig. 1 *Catharanthus roseus* cells cultured for 4 days.
(A) CrA strain, (B) CrB strain. The bar indicates 100 μm.

Table 1. Arbutin yields of CrA and CrB in various cultures.

Cultures	Cell density (g fr wt/l)	Arbutin yield (g/l)	
		CrA	CrB
500 ml flask	130	0.7	1.2
5 l jar fermentor	135	1.8	2.4
5 l jar fermentor*	340 (CrA), 250 (CrB)	4.1	6.0

* High-density cultivation.

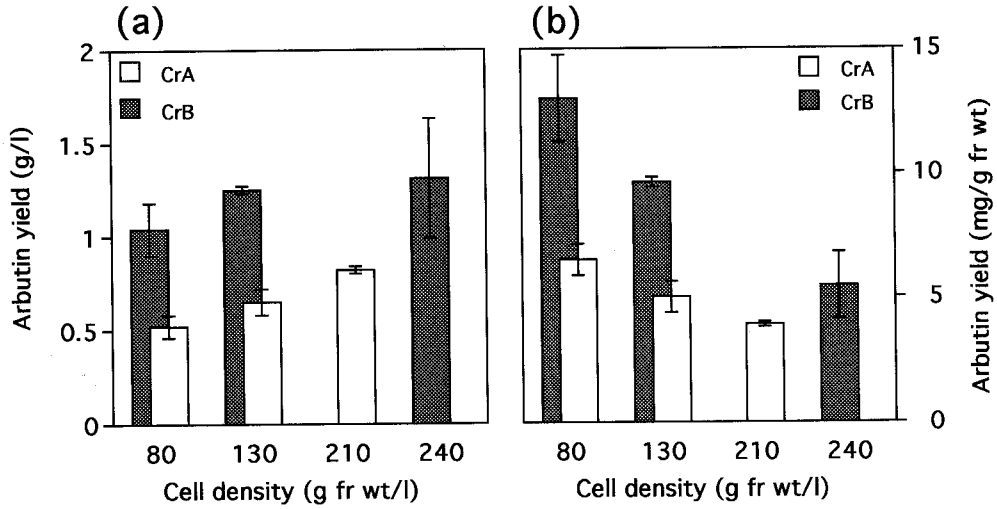

Fig. 2 Arbutin production by the two strains at different stages of flask culture.
Each flask was inoculated with 10 g cell fresh weight (fr wt)/*l*. After the cells had reached the exponential growth phase (80 g fr wt/*l*, 5-day-old cultures), the late exponential growth phase (130 g fr wt/*l*, 6-day-old cultures) or the stationary phase (210–240 g fr wt/*l*, 8-day-old cultures), HQ was repeatedly added once a day to make 4 mM in flask cultures until the cells no longer consumed the HQ supplied and arbutin formation stopped (total frequency of HQ supply, 3 or 4 times in each flask; incubation period, 3 or 4 days). Arbutin yield was expressed per liter of the suspension (a) or on the basis of cell fresh weight (b).

and was not very different from that in CrA cells when the cell density was more than 200 g fresh weight per liter.

2. *HQ glucosyltransferase activity*

We next examined changes in HQ glucosyltransferase activity of both cell strains in the exponential growth phase when HQ was administered repeatedly to the cells until they did not consume the HQ supplied. The experiments were performed in flask culture. The results are shown in **Fig. 3**. Enzyme activity was not detected in either of the cell strains before HQ was supplied. When HQ was added, the enzyme was detected within 6 hours and reached a peak within 24 hours in both strains. However, the maximum enzyme activity of CrB was 5-fold higher than that of CrA, this value corresponding well with the difference of the final arbutin yield per cell fresh weight. Arbutin formation stopped at around 50 hours in CrA, while CrB continued to generate arbutin until 70 hours with high activity. The CrB cells survived until 70 hours, but all of the CrA cells had died by 50 hours.

The difference in glucosyltransferase acitivity between the two cell strains may be the reason the arbutin productivity was significantly different in the two strains. The higher activity of the enzyme may minimize the contact time of toxic HQ with the cells by promptly converting added HQ into its glucoside, so improving the cell viability. As we reported before[15], HQ concentration in the medium strongly influenced arbutin productivity and a lower concentration of HQ was better to produce arbutin. In the case of CrB, 4 mM HQ in the medium was completely converted to arbutin within 12 hours, whereas CrA converted only 2.3 mM of HQ and 1.7 mM remained intact after 24 hours, at the next point of HQ supply.

HQ glucosyltransferase in both strains was not detected in either strain before HQ supply, but was detected in both strains within 6 hours thereafter. The enzyme was inducible in both strains.

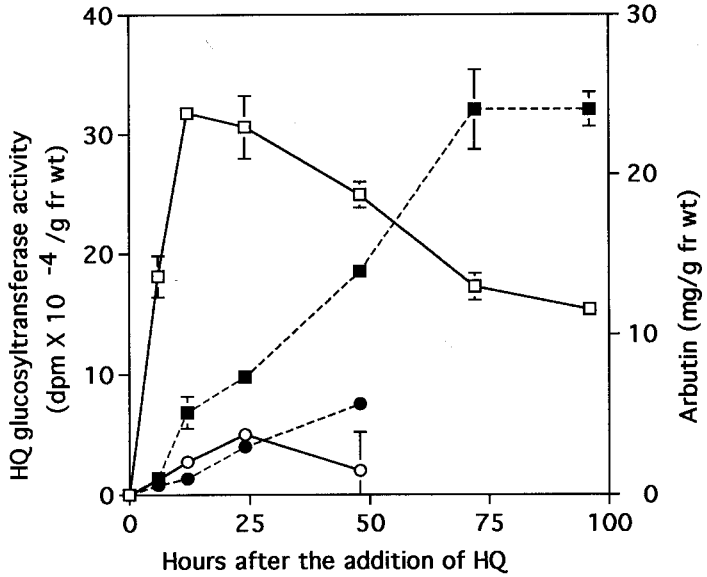

Fig. 3 Changes of HQ glucosyltransferase activity and arbutin production of the two strains after HQ addition.
HQ was periodically added to make 4 mM at intervals of 24 h until the cells did not consume the HQ supplied in flask cultures. Solid lines show the enzyme activity of CrA (○) and CrB (□). Broken lines show arbutin production per cell fresh weight of CrA (●) and CrB (■).

Both inducible and constitutive glucosylation enzymes for other exogenous phenolics have been reported. Tanaka *et al.*[21] reported that *Mallotus japonicus* cells glucosylated exogenous *o*-hydroxybenzoic acid(OHB), as well as *p*-hydroxybenzoicacid(PHB), into corresponding *o*-glucosides; PHB glucosyltransferase was a constitutive enzyme, whereas OHB glucosyltransferase was inducible.

There have been many reports on glucosyltransferase activities for other biotransformations in cell suspension culture[20-22]. For example, the activity of salicyl alcohol glucosyltransferase from *Gardenia jasminoides* cell culture was 25 pkat/mg protein[20], that of *p*-hydroxybenzoic acid glucosyltransferase from *Mallotus japonicus* cells was 14 pkat/mg protein at maximum[21], and that of scopoletin glucosyltransferase from tobacco cells was 9 pkat/mg protein[22]. These levels are similar to that of HQ glucosyltransferase in CrA, *i. e.*, 17 pkat/mg protein. On the other hand, the enzyme activity of CrB was higher at 96 pkat/mg protein. This higher activity was one factor in our previous success with high-level production of arbutin from HQ(9 g/*l*)[15].

3. *Accumulation of arbutin within the cells*

We next investigated arbutin accumulation within the cells. These experiments were done with high-density cell culture in a jar fermentor[15], which gives more stable arbutin production than flask culture. Glucose was supplied with HQ to the culture in order to enhance arbutin production by improving the cell viability after the addition of HQ[14].

The changes in the amount of arbutin within the cell or in the medium are shown in **Fig. 4**. When HQ was added to the culture, arbutin accumulated in the cells during the first 2 or 3 days, and thereafter in both the cells and the medium. Arbutin production stopped with cell death on day 5 in both strains. The rate of arbutin accumulation of CrB was 4. 9-fold higher than that of CrA during first two days. The maximum amount of arbutin accumulated within CrB cells was 19. 0 mg/g fresh weight, which was 2. 6-fold higher than that of CrA cells.

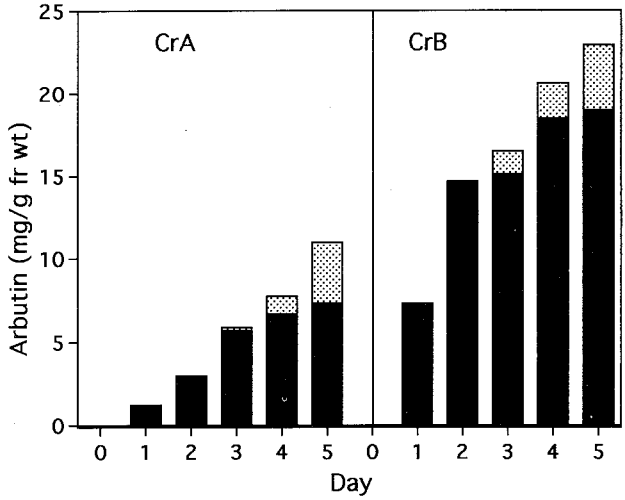

Fig. 4 Arbutin accumulation within the cell (■) or in the medium (▨) with the two strains. After the cell density had increased over 250 g fr wt/*l* during high-density cultivation in a jar fermentor, HQ was added to make 6 mM at intervals of 24 h until the cells did consume the HQ supplied. Glucose was added twice to a concentration of 55 mM, at the starting point of HQ addition and on the second day thereafter.

The difference of the maximum accumulation of arbutin in the two strains may be due to the difference of vacuolar volume. Suzuki *et al.*[17] reported that the cell size of CrB was 2- to 3-fold larger than that of CrA at every culture stage. Since most of the volume of both cells at any cell age is accounted for by vacuoles, based on microscopic observation of the stained vacuoles (data not shown), the vacuole of CrB is presumed to be also 2- to 3-fold larger than that of CrA, which value corresponds well with the difference of arbutin accumulation. Yamamoto *et al.*[23] reported that vacuoles in high-alkaloid-producing cell lines of *Coptis japonica* and *Thalictrum minus* were larger than those in low- or non-producing cell lines. They[23] suggested that development of vacuoles might be prerequisite for alkaloid synthesis, to prepare a reservoir for storage of the alkaloids. In the case of *C. roseus* cells, we observed that cells expanded about 2-fold with a corresponding expansion of the vacuoles as the accumulation of arbutin progressed (data not shown). Thus, the significantly larger vacuoles of CrB may contribute substantially to the larger productivity of arbutin in comparison with CrA.

Our data indicate that cells with high biotransformation ability may be selected by using the criterion of well-developed vacuoles. The selection of superior cell strains would be a useful means of improving the production of compounds by biotransformation in plant tissues, as has already been established for *de novo* synthesis[1,2].

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References

1) Matsumoto, T., 1984. Soshikibaiyo, **10**: 150-153.
2) Parr, A. J., 1989. J. Biotechnol., **10**: 1-26.
3) Umetani, Y., S. Tanaka, M. Tabata, 1982. In "Proc. 5th Intl. Cong. Plant Tissue & Cell Culture" (ed. by Fujiwara, A.), p. 383-384, Maruzen, Tokyo.

4) Langebartels, C., H. Harms, 1984. Z. Pflanzenphysiol., **113**: 201-211.
5) Furuya, T., 1978. In "Frontiers of Plant Tissue Culture" (ed. by Thorpe, T. A.), p. 191-200, University of Calgary Press.
6) Döller, von P. C., A. W. Alfermann, E. Reinhard, 1977. Planta Med., **31**: 1-6.
7) Lewinsohn, E., E. Berman, Y. Mazur, J. Gressel, 1986. Phytochemistry, **25**: 2531-2535.
8) Lewinsohn, E., E. Berman, Y. Mazur, J. Gressel, 1989. Plant Sci., **61**: 23-28.
9) Furuya, T., M. Ushiyama, Y. Asada, T. Yoshikawa, 1987. Phytochemistry, **26**: 2983-2989.
10) Tabata, M., Y. Umetani, M. Ooya, S. Tanaka, 1988. Phytochemistry, **27**: 809-813.
11) Courtois, D., D. Yvernel, B. Florin, V. Petiard, 1988. Phytochemistry, **27**: 3137-3142.
12) Akiu, S., Y. Suzuki, Y. Fujimura, T. Asahara, M. Fukuda, 1988. Proc. Jpn. Soc. Invest. Dermatol., **12**: 138-139.
13) Itabashi, M., H. Aihara, T. Inoue, J. Yamate, S. Sanni, M. Tajima, C. Tanaka, Y. Wakisaka, 1988. Iyakuhin Kenkyu, **19**: 282-294.
14) Yokoyama, M., S. Inomata, S. Seto, M. Yanagi, 1990. Plant Cell Physiology, **31**: 551-555.
15) Inomata, S., M. Yokoyama, S. Seto, M. Yanagi, 1991. Applied Microbiology and Biotechnology, **36**: 315-319.
16) Linsmaier, M. E., F. Skoog, 1965. Physiol. Plant, **18**: 100-127.
17) Suzuki, K., S. Amino, Y. Takeuchi, A. Komamine, 1990. Plant Cell Physiology, **31**: 7-14.
18) Umetani, Y., E. Kodakari, S. Yamamura, S. Tanaka, M. Tabata, 1990. Plant Cell Reports, **9**: 325-327.
19) Tabata, M., Y. Umetani, S. Tanaka, 1984. Plant Cell Tissue Organ Culture, **3**: 3-9.
20) Mizukami, H., T. Terao, A. Asano, H. Oshihara, 1986. Plant Cell Physiology, **27**: 645-650.
21) Tanaka, S., K. Hayakawa, Y. Umetani, M. Tabata, 1990. Phytochemistry, **29**: 1555-1558.
22) Hino, F., M. Okazaki, Y. Miura, 1982. Plant Physiology, **69**: 810-813.
23) Yamamoto, H., K. Nakagawa, H. Fukui, M. Tabata, 1986. Plant Cell Reports, **5**: 65-68.

《和文要約》

2系統のニチニチソウ懸濁培養細胞による ヒドロキノンの配糖化能力の違い

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起源が全く同じでありながら，形態的に大きく異なる2系統のニチニチソウ懸濁培養細胞(CrA株及びCrB株)の，ヒドロキノン(HQ)からアルブチンへの配糖化反応の違いを検討した．形態が円柱状で液胞がよく発達しており細胞の体積も大きなCrB株は，球状で体積がより小さいCrA株に比べて，いずれの培養齢でもアルブチン生成能力が勝っていた．HQを培養液に添加すると，両株とも6時間以内にHQ配糖化酵素が誘導され，その活性は24時間以内に最大に達した．しかしその酵素活性は両株で大きく異なり，最大活性がCrA株よりCrB株が5倍高かった．この値は両株のアルブチン生成量の違いと一致した．さらに細胞中のアルブチン蓄積量を調べた結果，細胞生重量当たりCrB株が2.6倍多かった．起源が同じ株でありながらHQの配糖化に大きな違いが示されたことは，植物組織の生物変換による有用物質生産においても，二次代謝産物の *de novo* 生産の場合と同様に細胞選抜が有効な手段となりうることを示唆している．