Original Paper Plant Tissue Culture Letters, 2 (2), 52–58 (1985)

Physiological Differences between Lactose-adapted and Non-adapted Cells of Japanese Morning-glory

Shigeru HISAJIMA,*,** Tsuneo WATANABE,* Yuji ARAI* and Trevor A. THORPE**

**Institute of Applied Biochemistry, University of Tsukuba, Ibaraki, Japan 305*
***Plant Physiology Research Group, Department of Biology, University of Calgary, Calgary, Alberta, Canada T2N 1N4*

(Received May 17, 1985)
(Accepted August 10, 1985)

To study the mechanism of lactose adaptation, several trials were carried out using original non-adapted and lactose-adapted callus cultures of Japanese morning-glory (*Pharbitis nil* L. var Violet). In both types of cells the activities of lactase, UDPgalppase, UDPGppase and UDPgal epimerase were present. Non-adapted cells thus appeared to be potentially capable of lactose metabolism. However, UDPgal epimerase activity in these cells was very low and cell wall lactase activity was about 30% of that of the lactose-adapted cells. When lactose-adapted cells were cultured on lactose medium, the glucose content remained low and galactose high throughout the culture period. Thus, it appears that these cells preferentially utilized the glucose moiety over galactose. Contents of G-6-P, G-1-P and Gal-1-P in galactose-grown original cells increased about 3 times over those in sucrose-grown original cells. UDPG and UDPgal contents increased about 1.5 times and only marginally, respectively, over those of sucrose-grown original cells. In comparison, the contents of G-6-P, G-1-P and Gal-1-P in galactose-grown lactose-adapted cells increased about two times over those of sucrose-grown original cells and decreased by about 20% of those of galactose-grown original cells. This suggests that the adapted cells have a greater capacity to metabolize lactose and galactose than the non-adapted cells. Based on these results, inhibition by lactose and galactose, and adaptation to galactose and lactose are discussed.

Although cultured cells generally grow best on sucrose or glucose as carbon/energy sources, they are capable of growing on a variety of other carbohydrates.[1–3] Plant cells can become adapted and grow on D-galactose[4] and the oligosaccharides melibiose,[5,6] lactose,[7–11] raffinose[6,12,13] and stachyose[6,14] that contain D-galactose. In the free state galactose is usually toxic to cultured tissues,[3] and normally most plant cells do not come into contact with these galactose-containing substances.[15]

A few years ago, we isolated a lactose-adapted cell line from lactose-sensitive cells of Japanese morning-glory.[11] The adapted cells could utilize lactose as the sole carbon source. The non-adapted cells grew on sucrose, but could not grow on lactose or galactose, while the adapted cells grew better on lactose than sucrose, and could also grow on galactose as sole carbon source. The mechanism of lactose metabolism in lactose-adapted cells has been proposed in

Abbreviations: UDPgal=UDP galactose, UDPG=UDP glucose, UDPgalppase=UDP galactose pyrophosphorylase, UDPGppase=UDP glucose pyrophosphorylase.

the previous paper.[16] Our present interest has been to examine further the mechanism of lactose adaptation and the mechanism of lactose and galactose toxicity.

In this communication, physiological differences between lactose-adapted and non-adapted cells are examined.

Materials and Methods

Plant material. Lactose-adapted and non-adapted cells of Japanese morning-glory (*Pharbitis nil* L. var. Violet) were cultured in a medium containing Murashige-Skoog's basal salts, yeast extract (0.3% w/v), agar 0.75% and carbohydrate (3% w/v).[16] Cultures of lactose-adapted and non-adapted cells were grown on lactose and sucrose respectively, except as indicated in different experiments. The stock cells were subcultured at 2 week intervals.

Extraction of sugars and sugar phosphates. Methods were based on the previously reported procedure[16] and that of Young and Bieleski.[17]

Assay of sugars and sugar phosphates. Glucose,[18] fructose,[19] sucrose[20] and lactose[21] were measured spectrophotometrically. G-6-P,[22] G-1-P,[23] Gal-1-P,[24] UDPG[25] and UDPgal[26] were also measured spectrophotometrically.

Enzyme extraction and assays. Cells (2 g) were homogenized with 5 ml of 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol in a cold teflon homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 15 min. The extraction was repeated thrice and supernatants were collected. The solution was dialyzed against 6 *l* of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM mercaptoethanol for 7 hr. After dialysis, the solution was centrifuged at $15,000 \times g$ for 20 min. The supernatant fraction was used as the cytoplasmic fraction.[16] The initial precipitate was washed with cold water by centrifugation and suspended in 4 ml of cold water. The suspension was used as the cell wall fraction.

The standard reaction mixture for lactase (EC 3.2.1.23) activity contained 2 μmol of lactose, 20 μmol of McIlvain (citrate-Na_2HPO_4) buffer (pH 4.5), and an appropriate volume of the enzyme preparation in a total volume of 200 μl. Reducing sugars were measured spectrophotometrically.[21] The standard reaction mixture for galactokinase (EC 2.7.1.6) contained 3 μmol of ATP, 3 μmol of D-galactose, 4 μmol of $MgCl_2$, 12 μmol of NaF, 20 μmol of Tris-HCl buffer (pH 7.5) and an appropriate volume of enzyme preparation in a total volume of 300 μl. Galactose-1-phosphate was measured spectrophotometrically.[24] The standard reaction mixture of UDPgalppase (EC 2.7.7.10) contained 2 μmol of UTP, 2 μmol of gal-1-P, 4 μmol of $MgCl_2$, 12 μmol of NaF, 12 μmol of glycine buffer (pH 9.0) and an appropriate volume of the enzyme preparation in a total volume of 200 μl. UDPgal was measured spectrophotometrically.[26] The standard reaction mixture for UDPGppase (EC 2.7.7.9) contained 2 μmol of UTP, 2 μmol of G-1-P, 2 μmol of $MgCl_2$, 12 μmol of NaF, 12 μmol of glycine buffer (pH 8.0) and an appropriate volume of enzyme preparation in a total volume of 200 μl. UDPG was measured spectrophotometrically.[25] The standard reaction mixture for UDPG epimerase activity contained 2 μmol of UDPgal, 16 μmol of glycine buffer (pH 9.0) and an appropriate volume of enzyme preparation in a total volume of 200 μl. UDPG was measured spectrophotometrically.[25] Methods used to detect galactose dehydrogenase (EC 1.1.1.48) and UDPgal uridyltransferase (EC 2.7.7.12) were based on procedures of Kurtz and Wallenfels,[27] and Zollner and Heuckenkamp[24], respectively. One enzyme unit is defined as the amount of enzyme which hydrolyzed 1 μmol of substrate or formed 1 μmol of product in 1 min in 100 μl of standard reaction mixture at 35°C.

Results and Discussion

Occurrence and cellular distribution of some enzymes involved in lactose metabolism

The occurrence and cellular distribution of some enzymes involved in lactose metabolism were compared in original and lactose-adapted cells of Japanese morning-glory. The results are given in **Table 1**. The data for the lactose-adapted cells are similar to those published previously.[16] However, at that time the cell line was 3 years old and the present data were obtained from a 4 year-old cell line, thus indicating the stability of the line in culture. Only lactase activity was present in both cell wall and cytoplasmic fractions of original and lactose-adapted cells. Galactokinase, UDPgalppase, UDPGppase and UDPgal epimerase activities were detected only in cytoplasmic fractions of both cells. However, the activity of UDPgal epimerase in the non-adapted cells varied from a trace to a very low level. Galactose dehydrogenase and uridyl transferase activities were not detected in either type of cell. Cytoplasmic lactase, galactokinase, UDPgalppase and UDPGppase activities in non-adapted cells were almost equal to those in lactose-adapted cells. However, cell wall lactase and cytoplasmic UDPgal epimerase activities of the original cells were less than 30 and 18%, respectively, of those in the lactose-adapted line.

A previous report proposed a scheme for lactose metabolism in lactose-adapted cells of Japanese morning-glory.[16] In the scheme, lactose was hydrolyzed prior to its penetration across the cell membrane by cell wall-bound lactase (β-galactosidase). In the cells, the monosaccharides were converted to sucrose. Galactose utilization involved phosphorylation by galactokinase and transformation into UDPgal and UDPG by UDPgalppase and UDPgal epimerase, respectively. The UDPgal could also be used in cell wall formation. The conversion of glucose to sucrose was the same in both the lactose-adapted and the non-adapted morning-glory cell lines. Glucose utilization involved phosphorylation by hexokinase and transformation into UDPG by UDPGppase. UDPG was used for sucrose synthesis.[28]

The present results suggest that the original cells have a system which is apparently sufficient to utilize lactose. However, it is suspected that these cells take up lactose at a slower rate that the lactose-adapted cells, probably due to the low cell wall lactase. In addition, lactose (galactose) metabolism may be limited by the epimerase reaction. These conclusions however do not indicate why galactose toxicity leading to necrosis should occur in the majority of the original cells.[11]

Table 1. Occurrence and cellular distribution of some enzymes (m unit·g^{-1}FW) of lactose metabolism in 10 day-old Japanese morning-glory callus.

	Non-adapted cells		Lactose-adapted cells	
	cytoplasm	cell wall	cytoplasm	cell wall
Lactase	31	3.5	44.2	11.7
Galactose dehydrogenase	0	0	0	0
Galactokinase	60.5	0	80.5	0
UDPgalppase	203	0	236	0
UDPGppase	302	0	269	0
UDPGal epimerase	T-13	0	73	0
Uridyl transferase	0	0	0	0

T: trace activity

Changes in carbohydrate contents during culture

Changes of sugar contents in cells and medium of the original cell culture have been reported previously.[29] In the present study changes of the sugar content in cells and medium of lactose-adapted cell culture were examined during the culture period. The results are given in **Table 2.** The growth curve of lactose-adapted cells was similar to that of non-adapted cells. However, generally speaking, changes in sugar contents of cells and medium of lactose-adapted cells were different from those of the original cells.

At the start of culture, about 15% of the lactose was hydrolyzed by autoclaving. Repeated experiments revealed that usually less than 10% of lactose was hydrolyzed by autoclaving. Less than 10% of the medium sucrose was also hydrolyzed. In culture about 20% of the lactose was hydrolyzed by day 10 (**Table 2**), while 80% of the sucrose was hydrolyzed by the same day.[29] Similarly, about 30% of the lactose remained while only 6% of the sucrose was left at day 20 in culture.[29] Therefore, the medium lactose was apparently hydrolyzed more slowly than sucrose.

In the medium, the ratio of glucose to galactose was about 1 throughout the culture period, suggesting that the monosaccharides were derived from lactose. No lactose was present in the cells at any stage of the culture period. This finding supports the lactose transport mechanism reported previously,[16] namely that uptake required prior hydrolysis.

From the medium of the non-adapted cells, preferential uptake of glucose over fructose was observed,[29] while here almost equal uptake of glucose and galactose was observed from the medium by the lactose-adapted cells (**Table 2**). Medium sucrose decreased and conversely reducing sugars increased with culture age of the non-adapted cells.[29] On the other hand, medium lactose decreased slowly, and reducing sugars did not increase at any stage of culture of the adapted cells (**Table 2**). Furthermore, early in culture reducing sugars decreased, and in the cytoplasm, glucose, fructose, galactose and sucrose were present throughout the culture period, while no lactose could be detected.

The previous report showed that around day 4, active cell division occurred and cell number increased.[29] Cells need energy for this process. In the present experiment, microscopically clusters of small cells were also observed around day 4, suggesting active cell division. Glucose content was lower than other free sugars examined throughout the culture period. This may

Table 2. Sugar contents of cytoplasm (μmol·g^{-1}FW) and medium (μmol·g^{-1}) during culture and growth (g·flask^{-1}) of lactose-adapted Japanese morning-glory callus.

	Day in culture					
	0	4	8	12	16	20
Cytoplasm						
glucose	0.83	0.49	0.83	1.1	1.1	1.0
fructose	1.5	1.0	0.8	1.8	1.8	2.4
galactose	1.7	2.6	3.9	2.5	2.3	2.4
sucrose	20.2	11.6	14.0	22.0	24.2	23.8
lactose	0	0	0	0	0	0
Medium						
glucose	14	6	11	13	10.5	10.0
galactose	15.7	7.8	12	11.4	10.4	11.1
lactose	70.2	69.1	61.0	52.0	32.0	24.5
Total sugar	85.0	76.0	72.5	64.2	42.8	35.2
Growth	1.0	1.0	1.8	3.1	6.0	8.3

suggest a more active role for glucose among free sugars in energy supply. The glucose moiety of lactose was apparently preferentially metabolized to the galactose moiety (**Table 2**). Around day 4, the glucose content dropped to its lowest level, while it remained constant at other culture stages. Fructose and sucrose also decreased at day 4 in the cultures. The decrease in the content of these three sugars may be due to the energy demand of cell division. In contrast, at day 4, the galactose content increased. This may suggest that galactose may have a different role than the other free sugars. The galactose content reached a maximum at day 8 and dropped at day 12. At around day 12, cell expansion and active cell wall synthesis are probably occurring. Thus, the decrease may indicate the need for cell wall components.

Contents of metabolic intermediates of cells grown on different kinds of sugars

The contents of sugar phosphates and nucleotide sugars involved in lactose metabolism were compared among the cells grown on different sugars. Based on the preceding experiments (**Table 2**), cells at the metabolically very active stage (day 4) were selected. The results are given in Table 3.

When non-adapted cells were grown on galactose, the contents of phosphomonosaccharides such as G-6-P, G-1-P and Gal-1-P, increased about 3 times and the contents of nucleotide sugars such as UDPG and UDPgal increased about 1.5 times and only a little, respectively, over those of sucrose grown original cells.[29] When the non-adapted cells were grown on lactose, the content of phosphomonosaccharides increased twice and the content of nucleotide sugars increased only a little over those of sucrose-grown original cells. As the original cells had the enzymes needed for lactose metabolism (**Table 1**), a major part of the metabolic intermediates were presumably derived from medium galactose or lactose.

Galactose appeared to be metabolically inactive even in lactose-adapted cells until day 8 (**Table 2**). Also, even in lactose-adapted cells, the content of each metabolic intermediate was lower in lactose-grown cells than in galactose-grown cells (**Table 3**). These results support the idea that lactose-adapted cells utilize the glucose moiety of lactose more efficiently than the galactose moiety (**Table 2**).

Low epimerase activity in galactose-sensitive original cells of sugar cane resulted in the accumulation of galactose and UDPgal on the galactose containing medium.[4] In contrast, appropriate epimerase activity of the galactose-adapted cells of sugar cane prevented the accumulation of galactose and UDPgal. UDPgal accumulation resulted in necrotic symptoms and adaptation of sugar cane cells to galactose was due apparently to increased epimerase activity.

In the present study, it appears that the low epimerase activity of the non-adapted cells did not cause a marked accumulation of UDPgal, when the cells were cultured on galactose-containing medium (**Table 1, 3**). It also appears that the appropriate epimerase activity in

Table 3. Contents (nmol·g^{-1}FW) of metabolic intermediates among the cells of Japanese morning-glory grown for 4 days on different carbohydrates.

Sugar fed	G-6-P	G-1-P	Gal-1-P	UDPG	UDPgal
Original strain					
sucrose	480	458	515	1,050	1,500
galactose	1,360	1,310	1,638	1,566	1,634
lactose	1,000	1,058	1,152	1,142	1,252
Adapted strain					
galactose	1,062	1,062	1,372	1,298	1,354
lactose	924	764	1,130	1,010	1,064

adapted cells caused only a slight decrease in contents of UDPgal, Gal-1-P and other sugar phosphates (**Tables 1, 3**). The mechanisms of inhibition of morning-glory cell growth by galactose and adaptation to galactose or lactose apparently differ from those of sugar cane.

In mammalian galactosemia, lack of uridyl transferase and galactokinase activities are thought to be due to marked accumulation of Gal-1-P and galactitol, respectively.[30] The accumulation of Gal-1-P probably results in the typical symptoms. As in the mammalian galactosemia, Gal-1-P content increased in non-adapted cells of Japanese morning-glory when the cells were cultured on galactose or lactose (**Table 3**). However, the level of Gal-1-P in galactose-grown original cells was similar to those of G-1-P and G-6-P (**Table 3**). Also, there was no difference in galactokinase activity between the adapted and non-adapted cells (**Table 1**). The mechanisms of galactose- or lactose-adaptation and inhibition in morning-glory cells therefore apparently differ from those of mammals. The contents of sugar phosphates in galactose- and lactose-grown adapted cells were lower than those in galactose- and lactose-grown original cells (**Table 3**). Thus, the adapted cells apparently metabolize lactose and galactose more efficiently than the original cells.

Enzymes to metabolize lactose were present in the original cells. However, this capacity was not realized initially, and only with continuous subculture in the presence of lactose was a population of cells capable of efficiently growing on lactose obtained.[11] Adaptation produced increased capacity of the lactose metabolizing enzymes, but the increases were less than those obtained with lactose-adapted sugar cane.[4] Thus, the adaptation of Japanese morning-glory cells to lactose appears, therefore, to involve functional activation of the lactose-metabolism enzyme system.

This work was supported by the research project fund of the University of Tsukuba, Japan, to S. H., and by NSERC of Canada Grant No. A-6467 to T. A. T. Part of this work was carried out when S. H. was a Visiting Scientist at the University of Calgary during 1983–84.

References

1) Street, H. E., 1969. In "Plant Physiology: A Treatise," Vol. VB (ed. by Steward, F. C.), p. 3–224, Academic Press, New York.
2) Maretzki, A., M. Thom, L. G. Nickell, 1974. In "Tissue Culture and Plant Science 1974," (ed. by Street, H. E.), p. 329, 361, Academic Press, London.
3) Thorpe, T. A., 1982. In "Tissue Culture in Forestry," (ed. by Bonga, J. M., D. J. Durzan), p. 325–368, Marinus Nijhoff, Netherlands.
4) Maretzki, A., M. Thom, 1978. Plant Physiol., **61**: 544–548.
5) Nickell, L. G., A. Maretzki, 1970. Plant Cell Physiol., **11**: 183–185.
6) Gross, K. C., D. M. Pharr, R. D. Locy, 1981. Plant Sci. Lett., **20**: 333–341.
7) Hess, D., G. Leipoldt, R. D. Illg, 1979. Z. Pflanzenphysiol., **94**: 45–53.
8) Urmantseva, V. V., V. P. Kholodova, V. N. Paukov, 1979. Sov. Plant Physiol., **26**: 373–380.
9) Mitchell, E. D., B. B. Johnson, T. Whittle, 1980. In Vitro, **16**: 907–912.
10) Chaubet, N., V. Petiard, A. Pareilleux, 1981. Plant Sci. Lett., **22**: 369–374.
11) Hisajima, S., T. A. Thorpe, 1981. Acta Physiol. Plant., **3**: 187–191.
12) Wright, K., D. H. Northcote, 1972. J. Cell Sci., **11**: 319–337.
13) Thorpe, T. A., E. J. Laishley, 1974. Phytochemistry, **13**: 1323–1328.
14) Verma, D. C., D. K. Dougall, 1977. Plant Physiol., **59**: 81–85.
15) Goodwin, T. W., E. I. Mercer, 1983. Introduction to Plant Biochemistry, 2nd ed., p. 252, Pergamon Press, Oxford.
16) Hisajima, S., T. A. Thorpe, 1985. J. Plant Physiol., **118**: 145–151.
17) Bieleski, R. L., R. E. Young, 1963. Anal. Biochem., **6**: 54–68.
18) Bergmeyer, H. U., E. Bernt, T. Schmidt, H. Stork, 1974. In "Methods of Enzymatic Analysis,"

Vol. 3 (ed. by Bergmeyer, H. U.), p. 1196–1201, Academic Press, New York.
19) Bernt, E., H. U. Bergmeyer, 1974. In "Methods of Enzymatic Analysis," Vol. 3 (ed. by Bergmeyer, H. U.), p. 1364–1367, Academic Press, New York.
20) Hisajima, S., T. Ito, 1981. Biol. Plant., **23**: 356–364.
21) Hisajima, S., T. Ito, 1983. Agric. Biol. Chem., **47**: 107–109.
22) Lang, G., G. Michael, 1974. In "Methods of Enzymatic Analysis," Vol. 3 (ed. by Bergmeyer, H. U.), p. 1238–1242, Academic Press, New York.
23) Bergmeyer, H. U., G. Michael, 1974. In "Methods of Enzymatic Analysis," Vol. 3 (ed. by Bergmeyer, H. U.), p. 1233–1237, Academic Press, New York.
24) Zoller, N., P. U. Heuckenkamp, 1974. In "Methods of Enzymatic Analysis," Vol. 3 (ed. by Bergmeyer, H. U.), p. 1288–1291, Academic Press, New York.
25) Keppler, D., K. Decker, 1974. In "Methods of Enzymatic Analysis," Vol. 4 (ed. by Bergmeyer, H. U.), p. 2225–2228, Academic Press, New York.
26) Keppler, D., K. Decker, 1974. In "Methods of Enzymatic Analysis," Vol. 4 (ed. by Bergmeyer, H. U.), p. 2221–2224, Academic Press, New York.
27) Kurtz, G., K. Wallenfels, 1974. In "Methods of Enzymatic Analysis," Vol. 3 (ed. by Bergmeyer, H. U.), p. 1180–1184, Academic Press, New York.
28) Hisajima, S., 1979. Mem. Tokyo Univ. Agric., **21**: 1–55.
29) Hisajima, S., Y. Arai, T. Ito, 1978. J. Jpn. Soc. Starch Sci., **25**: 223–228.
30) Mayers, J. S., L. R. Miller, F. K. Myers, 1970. Biochem. Biophys. Res. Commun., **39**: 661–665.

《和文要約》

アサガオのラクトース耐性および非耐性株における生理的差異

久島　繁*,**・渡辺恒夫*・新井勇治*・Trevor A. Thorpe**

*筑波大学応用生物化学系代謝化学研究室
**キャルガリー大学生物学科

ラクトース適応のメカニズムを検討するため，アサガオの非耐性および耐性培養株を用いて，いくつかの検討を行った．両種細胞にラクターゼ，UDP-ガラクトースピロフォスフォリラーゼ，UDP-グルコースピロフォスフォリラーゼおよびUDP-ガラクトースエピメラーゼ活性は存在した．それゆえ，非適応細胞も潜在的にはラクトース代謝能を持つものと思われた．しかし，この細胞ではUDP-ガラクトースエピメラーゼ活性は極めて弱く，細胞壁結合型ラクターゼ活性は適応細胞のそれの約30%であった．ラクトース適応細胞をラクトース培地で培養した場合，培養期間を通じて細胞内グルコース含量は低く，ガラクトース含量は高かった．それゆえ，これらの細胞はガラクトースよりグルコースをプレファレンシャルに利用するものと思われる．ガラクトースに培養した非適応細胞のG-6-P, G-1-PおよびGal-1-Pの含量はショ糖で培養したそれの約3倍であった．また，UDPGおよびUDP galの含量はショ糖で培養した場合のそれぞれ約1.5倍，僅増であった．それに比して，ガラクトース培養非適応細胞のG-6-P, G-1-P, Gal-1-Pの含量はショ糖培養のそれの約2倍となっており，ガラクトース培養適応細胞のそれより約20%低かった．この事は適応細胞が非適応細胞に比して大きなガラクトースおよびラクトース代謝能を持っている事を示唆している．これらの結果に基づきラクトースおよびガラクトースの阻害とそれに対する適応について論議した．