Pergamon

PII: S0031-9422(97)00784-X

Phytochemistry, Vol. 47, No. 8, pp. 1483–1487, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

EFFECTS OF GROWTH TEMPERATURE ON LIPIDS OF ADZUKI BEAN CELLS

MICHIYUKI KOJIMA,* HIROSHI SUZUKI,† MASAO OHNISHI and SEISUKE ITO

Department of Bioresource Chemistry, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan; † Agriculture Research Institute, Tokachi Federation of Agricultural Cooperatives, Obihiro, Hokkaido 080-24, Japan

(*Received in revised form* 9 *June* 1997)

Key Word Index—*Vigna angularis*; Leguminosae; Adzuki bean; suspension-cultured cells; glycerolipid; sphingolipids; sterols; molecular species.

Abstract—Suspension cultures of cells of Adzuki bean (*Vigna angularis*), a chilling-sensitive species, that had been maintained at 3° for 2 days exhibited the same sensitivity to freezing-damage as similar cultures maintained at 24°. In general, in chilling-resistant plants, acclimation increases the relative amounts of phospholipids and decreases those of sterols in membranes. Moreover, the phospholipids of acclimated cells have a higher percentage of unsaturated fatty acyl chains than those of control cells. In Adzuki bean cells grown at low temperature the concentrations of highly unsaturated glyceroglycolipids were slightly elevated but the relative amounts of stigmasterol was conspicuously elevated in all classes of sterols. No significant changes in cerebroside species were detected. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Exposure of plants to low, non-freezing temperatures frequently causes irreversible or reversible cellular injury, the extent of which depends on the temperature and the duration of exposure. However, many plants can survive freezing after a period of acclimation at low temperature [1–4]. Various changes in the composition of cells have been observed during acclimation [5–9]. Yoshida reported that, when etiolated mung bean seedlings were exposed to chilling temperatures below 5°, there was a remarkable decline in the physiological activity of cells, which was reversible or irreversible depending on the duration of chilling [3]. After chilling for less than 24 h, tissues suffered no permanent injury, but upon further prolonged chilling, cellular activities declined irreversibly [3, 4]. It has also been reported that plant cells in suspension culture can be acclimated to withstand freezing [10–13].

The use of tissue culture for physiological studies on plants allows us to take advantage of the fact that, in these simple systems, the confounding effects of cell differentiation and organ specificity, observed in whole plant materials, are reduced or eliminated. In addition, cells can be exposed to uniform environmental conditions for test treatments. In spite of these advantages, little information is available about the changes in cellular properties that accompany cold-hardening plant cells in culture.

It was reported recently that the enzyme most sensitive to chilling in plant cells is the H^+-ATPase on the tonoplast and reversible inactivation of the enzyme was detected during short-term and reversible chilling, namely 24 h at 0° [3, 4]. Kasamo reported that the H^+-ATPase activity appeared to be stimulated by phospholipids and that activation was dependent on the degree of saturation or unsaturation of fatty acyl chains and their length [14, 15]. It has been reported that the phospholipids of acclimated chilling-resistant cells have a higher percentage of unsaturated fatty acyl chains than those of non-acclimated cells and, moreover, cerebroside (CMH) was found in the tonoplast and plasma membrane as a major lipid constituent [16, 17]. In addition, the molecular species of cerebroside (CMH) in plants were shown to have different phase-transition temperatures (18–64°) [18]. However, the critical changes in lipid composition that confer tolerance to low temperatures of the early phase of chilling have yet to be determined in chilling-sensitive plants.

The present report describes the changes in the molecular species of phospholipids, namely, phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI), and of glycolipids, namely, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), as well as chan-

---

* Author to whom correspondence should be addressed.

ges in CMH and in the composition of sterol lipids, such as acyl sterol (AS), free sterol (FS), acylsteryl glycoside (ASG) and sterylglycoside (SG), when Adzuki bean cells in suspension were subjected to a low temperature (3°) for several days.

RESULTS AND DISCUSSION

During a 10-day incubation at 3°, cells in cultures derived from roots did not increase in fresh weight. In contrast, cells in cultures grown at 24° tripled in fresh weight during the same period. The ability to reduce 2,3,5-tryphenyltetrazolium chloride (TTC) of Adzuki cells in suspension that has been grown at a low temperature (3°) for 9 days decreased to 77% of the control value, although the value was almost the same as the control after only 2 days.

It is known that some plants of temperate origin can be acclimated to withstand freezing temperatures without injury by growth at low (non-freezing) temperatures, but the suspension-cultured cells derived from Adzuki beans did not appear to acclimate to low temperatures during maintenance at 3°. In the Adzuki cells, the total lipid content of cells grown at 3° was 78% of that of cells grown at 24°. When Adzuki cells in suspension were grown at a low temperature, phosphatidic acid (PA) and diacylglycerol (DG) derived from glycerolipids were not found in the lipid fractions. Growth at a low temperature had a major effect on the weight and composition of alfalfa cells [19], but hardening of Adzuki cells at 3° for 10 days did not lead to changes in the relative amounts of unsaturated and saturated fatty acids. Although the phospholipid content of alfalfa cells increased ca 10-fold during acclimation, it did not do so in Adzuki cells. When the various lipid classes in suspension-cultured cells were compared at different growth temperatures, the ratios of CMH to phospholipid (w/w) in Adzuki cells were found to be 0.03 at 25° and 0.04 at 3°.

Gas-liquid chromatography revealed that the sterols of Adzuki cells contained at least 24-methylsterol, stigmasterol and sitosterol (Table 1). The major desmethylsterols of four types of sterol lipids were stigmasterol and sitosterol, although the proportions were slightly different for each type of sterol lipid. Guye reported that, prior to and during chilling at 5° for 24 h, the molar ratio of sitosterol to stigmasterol was directly related to chilling tolerance in *Phaseolus* species [20, 21]. Hardening of cells of Adzuki bean (a chilling-sensitive plant) at 3° led to an increase in this ratio when compared with that of control cells (Table 1). However, Guye reported a decrease in this ratio in the most chilling-sensitive genotype of *Phaseolus* and species with a range of intermediate chilling-sensitivities showed no changes during a chill-warm cycle [21]. The ratio of sitosterol to stigmasterol also varied markedly into plant age, showing an increase during senescence [22].

We found that the fatty acid profiles of phospholipid classes in Adzuki cells in suspension had higher concentrations of 18:3 than those of the mother organ (roots). At least ten molecular species of PE, PC and PI were observed. The major species in the suspension-cultured cells were palmitoyl linolenin (16:0–18:3) and palmitoyl linolein (16:0–18:2), as in the case of soybean [23] (Table 2). The diacylglycerol residues in PI were less unsaturated than those in PE and PC; this difference might be attributable to differences in metabolic pathways. At least nine molecular species of MGDG and DGDG were observed. The major molecular species in suspension-cultured cells were dilinolenin (18:3–18:3), 16:0–18:3, 16:0–18:2 and stearoyl linolein (18:0–18:2). The major molecular species in the DGDG from the cells were the same as those in MGDG, although the amount of 18:3–18:3 was low and that of 18:0–18:2 species was high as compared with that in MGDG. When we compared the species of phospholipids (PE, PC and PI) and glycolipids (MGDG and DGDG) in cells grown at 24° and 3°, we found that the fatty acid combination 18:3–18:3 in *sn*-1,2-diacylglycerol residues in MGDG and DGDG increased with the decrease in temperature, while contents of the other species decreased (Table 2).

When the sphingoid composition of CMH was analyzed by GC of the fatty aldehydes obtained by acid degradation, the principal constituents were found to be *trans*-4, *trans*-8-sphingadienine, 4-hydroxy-*trans*-8-sphingenine and 4-hydroxy-*cis*-8-sphingenine. The configuration of the double bond at C-8 of CMH in suspension-cultured Adzuki cells was mostly of the *trans*-type, although the amount of the *cis*-type of 4-

Table 1. Major desmethylsterols of four types of sterol lipids from Adzuki suspension cells grown at 24° and 3° (mol%)

	FS		AS		SG		ASG	
Major desmethylsterols	24°	3°	24°	3°	24°	3°	24°	3°
24-Methylensterol	12	12	13	8	8	10	7	5
Stigmasterol	71	63	54	45	61	55	60	54
Sitosterol	18	25	33	47	31	35	33	41
Sitosterol/Stigmasterol	0.25	0.40	0.61	1.04	0.51	0.64	0.55	0.76

Table 2. Molecular species compositions of glycerolipids from Adzuki suspension cells grown at 24° and 3° (mol%)

	PE		PC		PI		MGDG		DGDG	
DAG species	24°	3°	24°	3°	24°	3°	24°	3°	24°	3°
18:3–18:3	8	5	10	10	2	3	56	78	30	53
18:3–18:2	12	12	13	9	1	7	8	4	5	3
18:3–18:1	2	2	3	4	1	2	<1	<1	<1	<1
18:3–18:0	9	7	13	8	7	8	<1	<1	<1	<1
18:2–18:2	6	11	6	16	1	5	<1	<1	<1	<1
18:2–18:1	1	2	2	5	<1	<1	5	2	7	2
18:3–16:0	35	24	30	26	62	38	15	10	15	14
18:2–16:0	22	31	16	17	22	30	11	3	11	3
18:2–18:0	5	7	7	5	4	7	3	2	25	22
18:1–16:0	<1	<1	<1	<1	<1	<1	2	1	7	3

hydroxy-8-sphingenine from the CMH in Adzuki cells was higher than that of *trans*-type [24]. The fatty acids of CMH were normal and 2-hydroxy acids, and the latter was usually predominant (97%). The principal components, of the hydroxy acids of CMH were commonly saturated at C^{16}, C^{24} and C^{22}. Reverse-phase HPLC separated CMH into more than ten peaks and the major species was identified as 1-*O*-glucosyl-*N*-2-hydroxypalmitoyl-4-*trans*, 8-*trans*-sphingadienine (d18:$2^{4t,8t}$–16h:0-Glc; 38±1%); its concentration did not change appreciably (37±1%) after cold treatment.

The molecular species composition of CMH in suspension cells was similar to in that of roots [24], except for the amounts of 1-*O*-glucosyl-*N*-2-hydroxylignoceroyl-4-hydroxy-8-*cis*-sphingenine (t18:1^{8c}–24h:0-Glc; 11%) and 1-*O*-glucosyl-*N*-2-hydroxylignoceroyl-4-hydroxy-8-*trans*-sphingenine (t18:1^{8t}–24h:0-Glc; 17%). It was reported that the activity of the tonoplast H^+-ATPase and the ATP-proton transport in suspension-cultured cells of mung bean (a chilling-sensitive plant) that has been incubated at 2° declined rapidly and reached a minimum level after 10 h [4]. Moreover, mung bean seedlings suffered irreversible injury after exposure to 0° in darkness for more than 48 h, whereas no permanent injury occurred within the first 24 h of chilling [3]. In cultures of *Catharanthus roseus*, *Glycine max* and *Nicotiana tabacum* cells grown at 15°, the concentrations of C_{18} unsaturated fatty acids increased [39]. In general, a low ambient temperature increases contents of unsaturated fatty acids in many intact plants [40] and in plant cells [39, 41]. Chilling injury to mung bean seedlings proceeds via two distinct processes: an early reversible process and a later irreversible process. In this study, we monitored the changes in lipid composition of Adzuki cells in suspension after growth at 3°. It appears that slight changes in glycerophospholipids and CMH, unsaturation of glyceroglycolipids and an increase in the relative proportion of stigmasterol might participate in the early chilling responses (during 2 days at 3°) in Adzuki bean cells.

EXPERIMENTAL

Cell cultures

Adzuki beans (*Vigna angularis* cv. Hatune) were soaked overnight and then cultured grown on 2% agar for 1 week under a constant photoperiod of 3000 lux lamp at 24°. Roots of seedlings were cut into small pieces and incubated on a solid B5 medium [25] that contained 0.1 mg l^{-1} kinetin and 1 mg l^{-1} 2,4-D, in order to generate callus cells. Suspension cultures were derived from callus cells by growth on a rotary shaker at 88 rev min^{-1} in 100-ml conical flasks under fluorescent light (3000 lux) at 24°. Suspension-cultured cells at the exponential phase of growth were subcultured every 10 days. For acclimation, cultures were transferred to fr. medium and maintained in darkness at 3° on a rotary shaker (88 rev min^{-1}). Cells were harvested by centrifugation at the times indicated and used for analysis.

Reduction of TTC

Cellular activity was determined by measuring the ability of cells to reduce TTC, essentially as described in Ref. [26] but with some modifications. Cells (100 mg, fr. wt) were incubated with 2,3,5-TTC in darkness for 2 h at room temp. and the formazan produced was extracted with 2 ml of EtOH and quantified by measuring A at 490 nm.

Extraction and separation of lipids

Total lipids were extracted from callus and suspension-cultured cells after lipases has been denatured by hot *iso*PrOH [27]. The total lipid extract was fractionated into neutral lipids, glycolipids, neutral and acidic phospholipids by CC on silicic acid and DEAE-Toyopearl 650M (Toso, Japan) [28]. For complete separation of the polar lipids, 2-D TLC [29, 30] was performed on silica gel with $CHCl_3$–MeOH–28% NH_4OH (65 : 25 : 4) for the first dimension and $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (40 : 10 : 2 : 2 : 1) for the second.

Analysis of fatty acids, sphingoids, sugars and sterols

For the determination of the components of glycerolipids, sphingolipids and sterol lipids, individual classes were degraded and analyzed [31–34].

Analyses of molecular species

For the determination of the fatty acid combination, purified MGDG and DGDG were separated [35] into the molecular species based on their *sn*-1,2-diacylglycerol moieties by reverse-phase HPLC (Shimazu Co., 6A type). An ERC-ODS column (6 × 250 mm, Erma Co.) was used for the separation with MeOH–H_2O (19 : 1). Elution was monitored by a RI detector. For the identification of molecular species of phospholipids, PE, PC and PI were degraded by phospholipase C from *Bacillus cereus* (Sigma, type III) to yield diacylglycerol moieties [36, 37]. These were converted to dinitrobenzoyl (DNB) derivatives, which were then chromatographed by HPLC (ERC-ODS-1282 column, 6 × 250 mm) using MeCN–*iso*-PrOH (4 : 1) as mobile phase. The DNB-diacylglycerol derivatives were monitored at 254 nm and the peaks detected were identified by measuring the component fatty acids after fractionation followed by methanolysis. The molecular species of CMH were analyzed directly by HPLC (Inertsil ODS-2 column, 4.6 × 250 mm) to separate the geometric isomers [38]. MeOH–H_2O (25 : 1) was used as eluent at 1 ml min^{-1} with detection at 220 nm at 40°.

REFERENCES

1. Levitt, J., *Responses of Plants to Environmental Stress*, 2 edn, Vol. 1. Academic Press, New York, 1980.
2. Li, P. H., *Hort. Rev.*, 1984, **6**, 373.
3. Etani, S. and Yoshida, S., *Plant Cell Physiol.*, 1987, **28**, 83.
4. Yoshida, S., *Plant Physiol.*, 1991, **95**, 456.
5. Lyons, J. M. and Raison, J. K., *Plant Physiol*, 1973, **24**, 445.
6. Steponkus, P. L., Uemura, M., Balsamo, R. A., Arvinte and Lynch, D. V., *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 9026.
7. Meza-B. L., Alberdi, M., Raynal, M., Cardinanos, M. L. F. and Delseny, M., *Plant Physiol.*, 1986, **82**, 733.
8. Guy, C. L. and Haskell, D., *Plant Physiol.*, 1987, **84**, 872.
9. Perras, M. and Sarham, F., *Plant Physiol.*, 1989, **89**, 577.
10. Chen, T. H. H. and Gusta, L. V., *Plant Physiol.*, 1983, **73**, 71.
11. Orr, W., Singh, J. and Brown, D. C. W., *Plant Cell Rep.*, 1985, **4**, 15.
12. Keith, C. N. and Mckersie, B. D., *Plant Physiol.*, 1986, **80**, 766.
13. Chen, P. M. and Gusta, L. V., *Can. J. Bot.*, 1982, **60**, 1207.
14. Kasamo, K. and Nouchi, I., *Plant Physiol.*, 1987, **83**, 323.
15. Yamanishi, H. and Kasamo, K., *Plant Cell Physiol.*, 1993, **34**, 411.
16. Yoshida, S. and Uemura, M., *Plant Physiol.*, 1986, **82**, 807.
17. Rochester, C. P., Kjellbom, P., Andersson, B. and Larsson, C., *Arch. Biochem. Biophys.*, 1987, **255**, 385.
18. Ohnishi, M., Imai, H., Kojima, M., Yoshide, S., Murata, N., Fujino, Y. and Ito, S., *Proc. ISF-JOCS World Congress*, 1988, pp. 930–935.
19. Borochov, A., Walker, A. and Pauls, K. P., *J. Plant Physiol.*, 1989, **133**, 671.
20. Guye, M. G., *J. Exp. Bot.*, 1988, **39**, 1091.
21. Guye, M. G., *J. Exp. Bot.*, 1989, **40**, 369.
22. Guye, M. G., *Plant Science*, 1987, **53**, 209.
23. Nishihara, M. and Kito, M., *Biochim. Biophys. Acta*, 1978, **531**, 25.
24. Kojima, M., Katagiri, Y., Furukawa, N., Mouri, H., Ohnishi, M. and Ito, S., *Res. Bull. Obihiro Univ.*, 1990, **I**, 17, 235.
25. Keith, C. N. and Mckersie, B. D., *Plant Physiol.*, 1986, **80**, 766.
26. Gamborg, O. L., Miller, R. A. and Ojima, K., *Exp. Cell Res.*, 1968, **50**, 151.
27. Kates, M. and Eberhardt, F. M., *Can. J. Botany*, 1957, **35**, 895.
28. Rouser, G., Kritchevsky, G., Simon, G. and Nelson, G. T., *Lipids*, 1967, **2**, 37.
29. Kojima, M., Ohnishi, M., Mano, Y., Sasaki, S., Ito, S. and Fujino, Y., *J. Jpn Oil Chem. Soc.* (*Yukagaku*), 1989, **38**, 619.
30. Wilson, A. C. and Kates, M., *Lipids*, 1978, **13**, 504.
31. Fujino, Y., Ohnishi, M. and Ito, S., *Agric. Biol. Chem.*, 1985, **49**, 2753.
32. Fujino, Y., Ohnishi, M. and Ito, S., *Lipids*, 1985, **20**, 337.
33. Kojima, M., Ohnishi, M. and Ito, S., *J. Agric. Food Chem.*, 1991, **39**, 1709.
34. Kojima, M., Ohnishi, M., Ito, S. and Fujino, Y., *Lipids*, 1989, **24**, 849.
35. Kojima, M., Seki, K., Ohnishi, M. and Ito, S., *Biochem. Cell Biol.*, 1990, **68**, 59.
36. Kojima, M., Sasaki, S., Ohnishi, M., Mano, Y. and Ito, S., *Phytochemistry*, 1990, **29**, 2091.
37. Mano, Y., Nishiyama, S., Kojima, M., Ohnishi, M. and Ito, S., *Cereal Chem.*, 1991, **68**, 280.

38. Kojima, M., Ohnishi, M. and Ito, S., *J. Agric. Food Chem.*, 1991, **39**, 1709.
39. MacCarthy, J. J. and Stumpf, P. K., *Planta*, 1980, **147**, 389.
40. Slack, C. R. and Roughan, P. G., *Biochem. J.*, 1978, **170**, 437.
41. Leathers, R. R. and Scragg, A. H., *Plant Science*, 1989, **62**, 217.