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Joh, Toshio

Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

Yoshimoto, Makoto

Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

Honjoh, Ken-ichi

Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University | Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

Miyamoto, Takahisa

Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

他

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Changes in Translatable RNA Population during Hardening of *Chlorella ellipsoidea* C-27

**Toshio Joh, Makoto Yoshimoto, Ken-ichi Honjoh,
Takahisa Miyamoto and Shoji Hatano**

Laboratory of Food Hygienic Chemistry, Faculty of Agriculture,
Kyushu University 46-09, Fukuoka 812, Japan

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The effects of hardening on gene expression in *Chlorella ellipsoidea* C-27 were investigated. *In vitro* translation products of translatable poly(A)⁺RNA prepared from unhardened and hardened *Chlorella* cells were separated by SDS-polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis, and visualized by fluorography. A time course study showed that changes in profile of poly(A)⁺RNA occur from the 1st hour of hardening. A comparison of *in vitro* translation products from unhardened and 24-h hardened cells indicated that 13 polypeptides newly appear and 10 polypeptides increase in abundance during hardening. The results suggest that expression of specific genes is involved in the development of frost hardiness.

INTRODUCTION

Some plant species develop freezing tolerance when exposed to low nonfreezing temperature (Graham and Patterson, 1982; Guy, 1990). It is known that during this cold acclimation many physiological and biochemical changes occur in plant cells: alterations in lipid composition, increases of sugar and soluble protein content, and changes in enzyme activities (Graham and Patterson, 1982; Uemura and Yoshida, 1984; Calderon and Pontis, 1985; Lynch and Steponkus, 1987; Guy, 1990). Weiser (1970) has first suggested that the biochemical, biophysical, and physiological changes that occur in plant cells during cold acclimation involve the altered gene expression. This suggestion is now supported by evidences that cold acclimation-specific changes in mRNA and protein profiles are identified in a wide variety of higher plants (Guy, 1990; Thomashow, 1990). However, the functions of the cold-induced proteins and the molecular mechanisms regulating their changes are still unclear.

We previously have showed that hardened cells of *Chlorella ellipsoidea* C-27 are able to survive slow freezing to -196°C (Hatano et al., 1976a), and the involvement of RNA and protein syntheses and lipid changes in the hardening process of the algal cells suggests the similarity of their hardening mechanism to that of higher plants (Hatano et al., 1976b; Hatano, 1978; Hatano et al., 1981, 1982). We also recently indicated alteration in profiles of *in vivo* soluble proteins during hardening of the strain C-27 (Yoshimoto et al., 1991). In order to further investigate the molecular genetic basis of frost hardiness, we analyzed *in vitro* translation products of poly(A)⁺RNA from unhardened and hardened cells.

This paper demonstrates that changes in gene expression at transcriptional level

are involved in the development of frost hardiness of *Chlorella ellipsoidea* C-27.

MATERIALS AND METHODS

Plant materials

Chlorella ellipsoidea IAM C-27 was grown synchronously in the MC medium of Watanabe (1960), at 25°C, under 10 klux, with 1.3% CO₂ in air, at a concentration of about 1.0×10^{10} cells per liter, under a 16-h light/B-h dark regime, as described previously (Hatano *et al.*, 1976a). Inasmuch as the cells were hardened most at the L₂ stage (an intermediate stage in the ripening phase of the cell cycle) (Hatano *et al.*, 1976a), L₂ cells were used here.

Hardening

L₂ cells synchronized at 25°C were directly hardened at 3°C. During hardening, the culture was aerated with 1.3% CO₂ in air and kept in the light (10 klux). Portions of the cell culture were withdrawn at intervals and centrifuged at 1,000 X g for 5 min at 3°C. After washing once with distilled water, cells were suspended in RNA extraction buffer (0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 10 mM EDTA, and 1% SDS) at a concentration of about 1.5×10^9 cells/ml. The cell suspension was frozen in liquid nitrogen and stored at -80°C.

Isolation of poly(A)⁺RNA

The frozen suspension was thawed at 4°C and to this an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and 0.2 volumes of 2-mercaptoethanol were added. The mixture was homogenized with glass beads of 0.5 mm in diameter in a reciprocal shaker (Vibrogen-Zellmühle; Edmund Bühler Co., Tübingen, F.R.G.), operated at 4,500 rpm at 4°C for 12 min. The homogenate was freed from the beads by passage through a sintered-glass funnel and the filtrate was centrifuged at 3,500X g for 10 min at 4°C. The upper aqueous phase containing total nucleic acids was washed twice with an equal volume of phenol/chloroform/isoamyl alcohol to remove proteins and cell debris completely. Total nucleic acids were precipitated with 0.05 volumes of 5 M NaCl and 2.5 volumes of ethanol overnight at -20°C, washed with cold 70% ethanol and then dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Total RNA was precipitated overnight at 4°C by the addition of 5 M LiCl to a final concentration of 2 M. Poly(A)⁺RNA was purified from the RNA by the use of an oligo(dT)-cellulose (Sigma, St. Louis, MO, U.S.A) as described by Maniatis *et al.* (1982).

Analysis of *in vitro* translation products

Poly(A)⁺RNA (0.5 to 1.0 μg) was translated by using a wheat germ extract *in vitro* translation kit (Boehringer Mannheim Yamanouchi, Tokyo, Japan) in the presence of 80 mM CH₃COOK, 2 mM Mg(CH₃COO)₂, and 2.2 MBq of [³⁵S]methionine (37 TBq/mmol; Hungarian Academy of Sciences, Budapest, Hungary). The translation products were separated by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli (1970) and by the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) procedure of O'Farrell (1975). Following electrophoresis, the gel

was fixed, treated with EN³HANCE (NEN, Boston, MA, U.S.A), dried and then exposed to Kodak XAR-5 film with an intensifying screen at -80°C.

RESULTS

We previously reported changes in pattern of *in vivo* proteins during hardening in *C. ellipsoidea* C-27 (Yoshimoto et al., 1991). To confirm that these changes occur at transcriptional level, *in vitro* translation products of poly(A)⁺RNA from hardened cells for various periods up to 24 h were analyzed. Figure 1 shows the results of SDS-PAGE of the *in vitro* translated proteins. Polypeptides of 51, 47, 30, 26, 23, 18, and 15 kDa apparently increased during hardening (indicated by arrowheads on the right side), while polypeptides of 28 and 16 kDa disappeared (arrowheads on the left side). Especially 47 kDa polypeptide seemed to increase in a two-step process, that is, this polypeptide once increased at the 1st hour of hardening, but decreased at and after the 2nd hour of hardening, and then increased again after the 6th hour of hardening. Changes in the profiles of the translation products occurred from the 1st hour of hardening, suggesting that response of gene expression to low temperature is relatively sensitive.

Furthermore, *in vitro* translation products from unhardened and 24-h hardened cells were analyzed in detail by 2D-PAGE. In Fig. 2, alphabetized and numbered spots

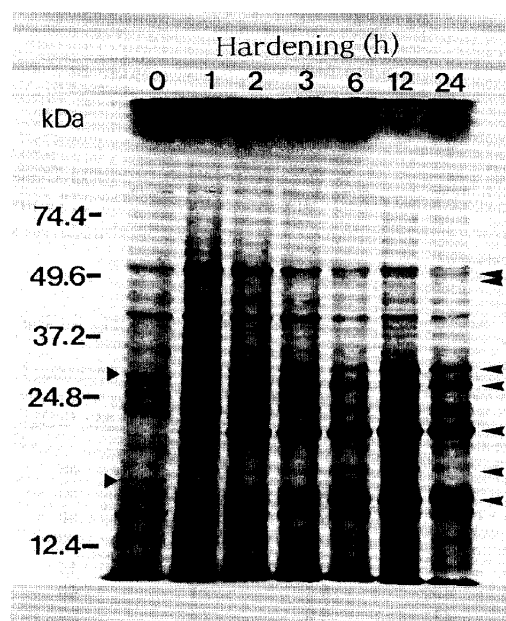


Fig. 1. Fluorograph of *in vitro* translation products from poly(A)⁺RNA of hardened cells for various periods. The [³⁵S] methionine-labeled translated proteins of 2×10^5 cpm were loaded in each lane and separated by 12.5% polyacrylamide gel containing 0.1% (w/v) SDS. Arrowheads on the right and left sides of the electrophoregraph indicate bands of increasing and decreasing translated polypeptides during hardening, respectively.

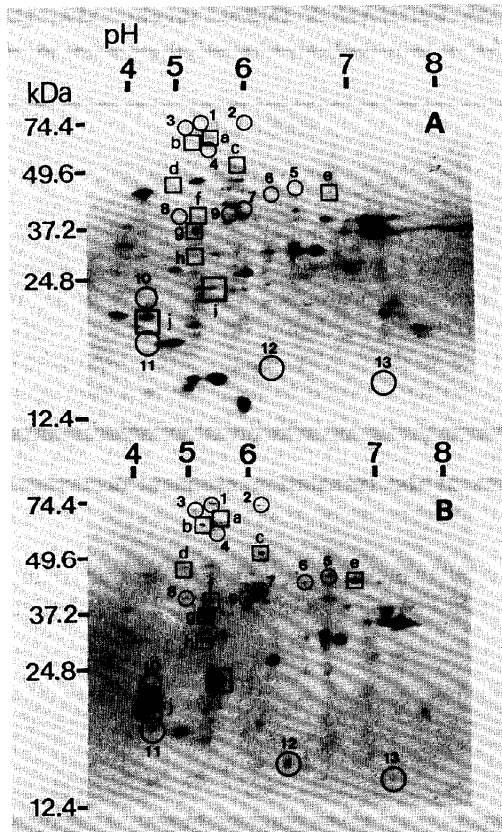


Fig. 2. 2D-PAGE patterns of *in vitro* translation products from poly(A)⁺RNA of unhardened (A) and 24-h hardened (B) cells. Open circles and squares indicate spots of polypeptides which newly appeared and increased in abundance at the 24th hour of hardening, respectively.

Table 1. Analysis of *in vitro* translation products during hardening.

Induced			Increased		
spot*	Mr (kDa)	PI	spot*	Mr (kDa)	PI
1	74	5.3	a	65	5.3
2	73	5.9	b	64	5.2
3	71	5.1	c	50	5.9
4	62	5.3	d	47	5.0
5	45	6.6	e	44	6.8
6	43	6.4	f	40	5.3
7	41	5.9	g	37	5.3
8	40	5.0	h	30	5.3
9	40	5.8	i	23	5.4
10	22	4.3	j	21	4.3
11	18	4.3			
12	15	6.2			
13	14	7.3			

*Spots are corresponding to Fig. 2.

indicate polypeptides that newly appeared and increased in abundance during hardening, respectively. The results showed that at least 13 polypeptides newly appear and 10 polypeptides increase in abundance and some polypeptides decrease or disappear during hardening. The relative molecular masses and the isoelectric points of polypeptides that newly appeared and increased by a 24-h hardening were summarized in Table 1. Of these, 4 polypeptides (spots e, i, j, and 7) increased markedly by a 24-h hardening. These results suggest that at least 23 translatable poly(A)⁺RNA species are induced at the 24th hour of hardening and the proteins encoded by them are involved in the hardening process.

DISCUSSION

It is known that expression of specific genes is involved in the development of frost hardiness in higher plants and proteins encoded by these genes play an important role in the hardening process (Guy, 1990; Thomashow, 1990). We previously reported alterations in profiles of *in vivo* soluble and membrane proteins during hardening in *C. ellipsoidea* C-27 (Yoshimoto et al., 1991). In order to confirm that these alterations in the *Chlorella* are involved in gene expression in the same way as higher plants, poly(A)⁺RNA were prepared from hardened cells for various periods up to 24 h and the *in vitro* translation products were analyzed. As expected, changes in profiles of translation products during hardening were observed; 23 polypeptides newly appeared or increased in abundance at the 24th hour of hardening (Figure 2 and Table 1), suggesting that the development of frost hardiness in the *Chlorella* is also accompanied by expression of specific genes.

As compared the number of *in vivo* newly appeared or increased polypeptides during hardening with that of *in vitro*, there were 19 polypeptides *in vivo* as against 23 *in vitro* (Yoshimoto et al., 1991). As the molecular mass of the changed polypeptides ranged between 74 and 14 kDa *in vitro* as against between 141 and 27 kDa *in vivo* (Yoshimoto et al., 1991), *in vivo* changed polypeptides are larger than them of *in vitro*. Normally, in *in vitro* translation experiments proteins of high molecular mass seem to be troublesome to be synthesized and in other reports about *in vitro* translation proteins of high molecular mass can not be detected abundantly, either (Danyluk and Sarhan, 1990; Koga-Ban et al., 1991; Lee et al., 1991). Consequently, the difference in range of the molecular mass of the changed polypeptides between *in vitro* and *in vivo* may come from the problem of *in vitro* translation system itself. Also, in comparison of the molecular mass and the isoelectric point of the changed polypeptides between both, any polypeptide does not agree except one polypeptide (spot h in Figure 2 and Table 1). Inside cells, some polypeptides translated from poly(A)⁺RNA are processed to become mature proteins. Therefore these disagreements are presumed to be attributable to post-translational modification of protein that occurs only *in vivo*.

Although proteins of high molecular mass could not be synthesized abundantly, 23 polypeptides newly appeared or increased in abundance during hardening in the *Chlorella*, showing that its development of frost hardiness involves expression of specific genes in the same as higher plants. Guy (1990) and Thomashow (1990) summarized many reports about changes in synthesis and accumulation of proteins

during cold acclimation in plants. According to them, 16 polypeptides reported in tomato are the most numerous in the number of increased proteins during cold acclimation in one plant. As compared with that, the number of newly appeared or increased proteins in the *Chlorella*, 23 polypeptides, seems to be very abundant. Although, of course, all of these polypeptides would not be involved in the development of frost hardiness, the fact that in spite of unicellular plant (but maybe because of unicellular plant) such many changes in protein synthesis occur during hardening shows that a mechanism of the development of frost hardiness in the *Chlorella* may be somewhat different from that in higher plants.

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