Original Paper Plant Biotechnology, **15**(1), 17-22(1998)

Changes in Protein Pattern during Stress-induction of Carrot (*Daucus carota* L.) Somatic Embryogenesis

Yoshinobu TACHIKAWA, Tsutomu SAITOU, Hiroshi KAMADA and Hiroshi HARADA

Gene Experiment Center, Institute of Biological Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki 305-8572, Japan

Received 12 June 1997; accepted 7 October 1997

Abstract

To clarify the molecular biological aspects occurring during the induction of somatic embryogenesis, the stress-induced changes in protein pattern and the proteins associated with somatic embryogenesis induced by stress in carrot (*Daucus carota* L.) were investigated. In two dimensional polyacrylamide gel electrophoresis (2D-PAGE), 6 spots with molecular weight of 63-65 kilo Dalton (KDa) showing pI ranging from 6.0-6.5, and 2 spots of molecular weight 45 KDa with a pI of 5.0 were detected in the extracts of apical tip segments treated with cadmium chloride, sucrose and sodium chloride. Three spots out of the 6 spots of 63-65 KDa molecular weight and the two 45 KDa spots were also detected in the extracts of 2,4-dichlorophenoxy acetic acid (2,4-D)-induced embryogenic cells, but not in those of non-embryogenic cells. Furthermore, these 5 spots were also detected in the extracts of hypocotyl segments cultured with 2,4-D for more than 4 weeks. Judging from the relationship between accumulation of these proteins and acquisition of embryogenic competence, these proteins may be related to acquisition and/or maintenance of embryogenic competence.

1. Introduction

Since the independent discoveries by Reinert (1958) [1] and Steward *et al.* (1958) [2] that carrot somatic cells cultured *in vitro* were able to form somatic embryos, somatic embryogenesis has been extensively investigated as a model system to understand the mechanism of zygotic embryogenesis, because morphological changes of somatic embryos are similar to those of zygotic ones. Especially, in carrot somatic embryogenesis which is the most established experimental system, a lot of molecular biological analyses have been conducted on the developmental processes of somatic embryos (Wurtele *et al.*, 1993, Sato *et al.*, 1995, Lin *et al*, 1996) [3-5]. It is well known that carrot somatic embryogenesis can be readily induced by transferring somatic tissues cultured on 2,4-dichlorophenoxyacetic acid (2,4-D)-containing medium to 2,4-D-free medium. However, to use only one system with 2,4-D is unsuitable for analysis of biochemical changes prerequisite for induction of somatic embryogenesis, because 2,4-D caused many other physiological phenomena including somatic embryogenesis (Lovell *et al.*, 1987, Morre *et al.*, 1988) [6, 7].

On the other hand, it has been reported that carrot somatic embryogenesis is induced by the treatments of apical tip segments with high concentrations of sucrose (Kamada *et al.*, 1989 and 1993) [8,9], NaCl (Kiyosue *et al.*, 1989) [10] or heavy metal ions such as Cd^{2+} (Kiyosue *et al.*, 1990) [11] in phytohormone-free medium. Futhermore, heat stress also induces somatic embryogenesis in phytohormone-free medium (Kamada *et al.*, 1994) [12]. In these methods, after the segments were transferred to phytohormone-free medium without these substances or high temperature, somatic embryos formed directly on the surface of the explants without visible callus formation. In addition to the induction system with 2,4-D, those systems with other chemicals or by heat provide us with useful experimental systems to detect biochemical changes strongly related to the induction of somatic embryogenesis. Because high concentrations of these chemicals or heavy metals or high temperature induce many physiological changes in explants, differences between protein patterns before and after these treatments include many changes which are not directly concerned with somatic embryogenesis. So it is important to detect common changes in all induction systems treated with different chemicals.

In addition to the previous reports indicating that 2,4-D is a suitable substance for induction of somatic embryogenesis, 2,4-D is thought to be a stress agent, because 2,4-D is known as a strong herbicide. It is possible to say that appropriate physiological stresses including 2,4-D might trigger the induction of somatic embryogenesis and important changes for acquisition

of embryogenic competence might occur during the stress treatments. Therefore, the common changes in protein pattern induced by some stresses were investigated.

In this report, several proteins related to induction of somatic embryogenesis commonly detected in different induction systems are reported and their possible role(s) on acquisition and/or maintenance of embryogenic competence are discussed.

2. Materials and Methods

2.1 Plant material

Seven- to ten-day-old seedlings of *Daucus carota* L. cv. US-Harumakigosun grown on vermiculite under a 16 h light (6000 lux)/8 h dark condition at 25°C were used as a plant material.

2.2 Induction of somatic embryogenesis by stress treatments

The carrot seedlings grown on vermiculite were surface-sterilized with 20 % (v/v) sodium hypochlorite solution (a final concentration of available chlorine was 1%) for 15 min, then rinsed three times with sterilized distilled water. One-cm-long segments of apical tips were excised from the seedlings. These explants were cultured in plastic Petri-dishes (9 cm in diameter) containing 30 m*l* of Murashige and Skoog's agar (0.8%) medium (Murashige and Skoog, 1962) [13] (hereafter referred to as MS medium) in which 2, 4-D, sodium chloride, sucrose or cadmium chloride was added. The most effective treatments for induction of somatic embryogenesis were as follows ; 2,4-D (1 mg/*l*) for more than 2 weeks, sodium chloride (0.3 M) for 3 weeks (Kiyosue *et al.*, 1989) [10], sucrose (0.7 M) for 2 weeks (Kamada *et al.*, 1989 and 1993) [8,9] or cadmium chloride (0.5 mM) for 2 weeks (Kiyosue *et al.*, 1990) [11]. Culture conditions were the same as described above.

2.3 Culture of embryogenic and non-embryogenic cells

Yellowish nodular callus was formed on hypocotyl segments cultured on MS agar medium supplemented with 2,4-D (1 mg/*l*) for 1 month. When the callus was transferred to 2,4-D-free MS medium, somatic embryos were formed. The callus was designated as embryogenic cells. Embryogenic cells were suspended in 300 m*l* flasks with 100 m*l* of liquid MS medium containing 1 mg/*l* of 2,4-D and cultured on a gyratory shaker (100 rpm) at 25°C in the darkness. Embryogenic callus was subcultured at 2 week intervals by transferring them to fresh MS medium with 2,4-D.

Small cell clusters obtained by passing the embryogenic suspension cultures through a stainless-steel mesh (1 mm in pore size) were subcultured in MS liquid medium containing 2,4-D (1 mg/*l*) and after more than 6 months of the culture, an actively proliferating suspension composed of small cell clusters was obtained. The suspension culture did not form somatic embryos even after transfer to 2,4-D-free MS medium. Thus, the cells were designated as non-embryogenic cells. The non-embryogenic cells were subcultured by the same method as for embryogenic cells.

2.4 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The two dimensional equilibrium pH gradient system of O'Farrell (1975) [14] was employed with some modifications. Embryogenic and non-embryogenic cells, and explants (*ca.* 100 mg fresh weight) cultured on MS medium with 2,4-D, NaCl, sucrose or $CdCl_2$ were put in Eppendorf tubes and homogenized by a glass pestle in 100 μl of lysis buffer composed of 8.5 M urea, 2% (v/v) ampholytes, 5% (v/v) glycerol and 10 mM dithiothreitol(DTT). The homogenate was centrifuged at 16000 × g for 15 min and the protein content of the supernatant was measured by a Bio-Rad Protein Assay Kit. An aliquot of the supernatant (equivalent to 100 μg of proteins) was subjected to two-dimensional gel electrophoresis. Samples were loaded at the basic end of the focusing gels. The upper (anode) buffer was 20 mM NaOH and the lower (cathode) buffer was 10 mM H_3PO_4. Isoelectric focusing was conducted for 12 h at 400 V plus 1 h at 800 V. The focused gels were equilibrated for 20 min in 62.5 mM Tris-HCl (pH 6.8) containing 2.3% (w/v) sodium dodecyl sulfate(SDS), 10% (v/v) glycerol and 0.05% (w/v) DTT, and run on a second dimension gel. The second dimension SDS gel was 1 mm thick and consisted of a 14 cm separation gel of 11% acrylamide overlaid with a 1 cm stacking gel of 3% acrylamide. After electrophoresis, the proteins were detected by silver staining.

3. Results

3.1 Analysis of the changes in protein pattern induced by various stresses

Total proteins were extracted from explants cultured on MS medium supplemented with sodium chloride (0.3 M), sucrose (0.7 M) or cadmium chloride (0.5 mM) as a stress substance and applied to 2D-PAGE. **Fig. 1** shows the 2D-PAGE patterns of proteins extracted from apical tip segments cultured on MS medium supplemented with or without each stress substance. Over 400 polypeptide spots were detected in the explants treated with each stress substance

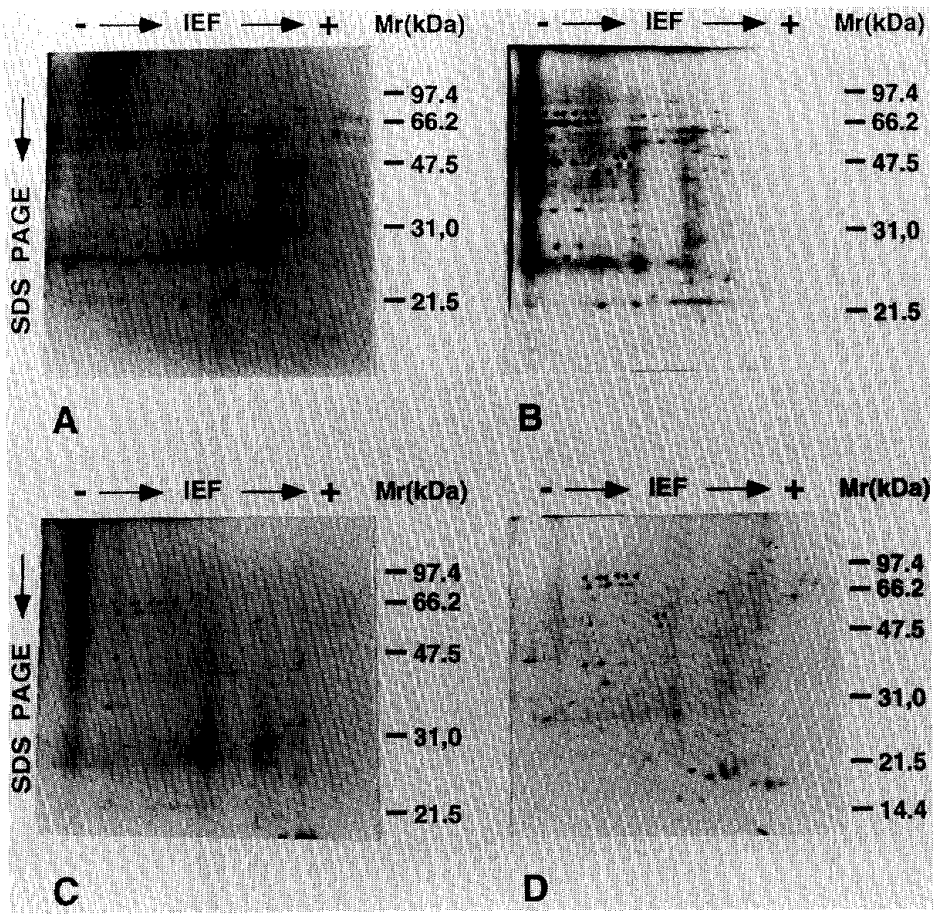

Fig. 1 2D-PAGE analysis of total proteins of apical tip segments treated with various kinds of stress.
Proteins were extracted from apical tip segments cultured on MS medium without any addition for 3 weeks (A, control), and from the segments cultured on MS medium with cadmium chloride (0.5 mM) for 2 weeks (B), sucrose (0.7 M) for 2 weeks (C) and sodium chloride (0.3 M) for 3 weeks (D). Supernatant containing 100 μg protein was applied on each gel. Arrow heads indicated spots which were commonly detected in apical tip segments treated with all of the kinds of stress, but not in those without stresses.

(**Fig. 1-B, 1-C, 1-D**). In the explants cultured on a medium without any stress substances, only about 200 polypeptide spots were detected both after 3 weeks (**Fig. 1-A**) and 2 weeks (data not shown) of culture and were not different among them. By comparing these patterns, 8 common stress-induced spots indicated with arrow heads in **Fig. 1** were detected in all stress treatments, that did not occur in the treatments without any stress substances. Based on the molecular weights, these proteins were divided into two groups. One (6 spots) had 63-65 KDa molecular weight with estimated pI ranging from 6.0-6.5, and another (2 spots) had 45 KDa molecular weight with an estimated pI of 5.5. Based on their molecular weights, the proteins were designated as ECP (Embryogenic Cell Protein) 63-1, 63-2, 65-a, 65, 65-b, 65-c, 45-1 and 45-2, respectively.

3.2 Comparison of the stress-induced protein pattern to those of embryogenic and non-embryogenic cells

Total proteins extracted from embryogenic and non-embryogenic cells were applied to 2D-PAGE (**Fig. 2-A, 2-B**). The protein patterns were very similar to each other. However, 3 spots (ECP 63-1, 63-2 and 65) among the 6 spots of 63-65 KDa molecular weight and both two 45 KDa spots (ECP45-1 and 45-2), which were observed in stress treatments, were also detected in the extract of embryogenic cells but not in that of non-embryogenic cells (**Fig. 2-A, 2-B** and **2-C**).

3.3 Changes in protein pattern of hypocotyl segments cultured with 2,4-D

Callus was formed on hypocotyl segments treated with 2,4-D and these callus include embryogenic cells. Callus formation on hypocotyl segments was observed about 4 weeks after the culture with 2,4-D. So, we tried to research when the spots, which were observed in the extracts of apical tips induced by stress and

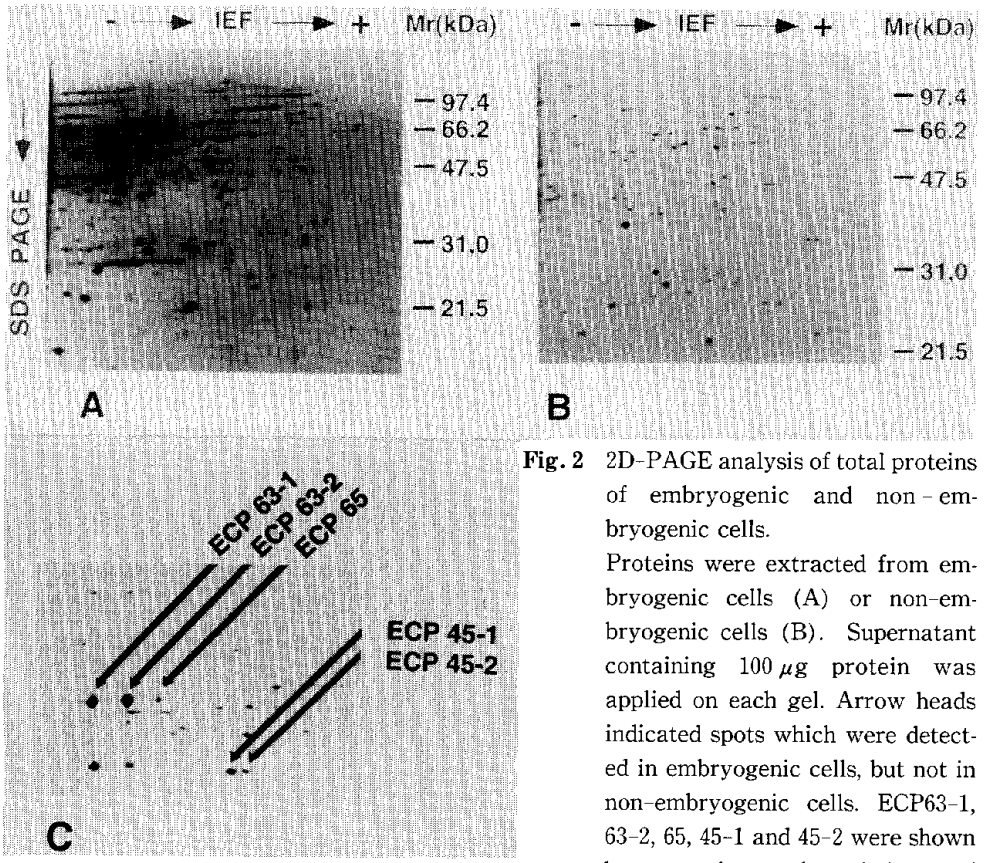

Fig. 2 2D-PAGE analysis of total proteins of embryogenic and non-embryogenic cells.
Proteins were extracted from embryogenic cells (A) or non-embryogenic cells (B). Supernatant containing 100 μg protein was applied on each gel. Arrow heads indicated spots which were detected in embryogenic cells, but not in non-embryogenic cells. ECP63-1, 63-2, 65, 45-1 and 45-2 were shown by arrows in an enlarged picture of **Fig. 2-A** (C).

embryogenic cells, were detected on hypocotyl segments treated with 2,4-D.

Total proteins extracted from hypocotyl segments cultured on MS medium supplemented with 2,4-D (1 mg/l) for various periods were applied to 2D-PAGE (**Fig. 3**). Five spots (ECP63-1, 63-2, 65, 45-1 and 45-2) out of 8 spots, which were observed in apical segments induced by stress, were detected in the extracts of hypocotyl segments treated with 2,4-D for 4 and 6 weeks (**Fig. 3-B, 3-C, 3-D**), but did not occur in those of explants cultured for 2 weeks (**Fig. 3-A**). These spots were the same as those shown in the extracts of embryogenic cells (**Fig. 2-A, 2-C**).

4. Discussion

Change in the relative abundance of mRNA and protein species during morphological changes in both zygotic and somatic embryogenesis have been reported in some plant species. However, most of the genes and proteins isolated were related to storage protein synthesis or late embryogenesis (Due *et al*, 1989, Baker *et al.*, 1988) [15,16] and few were related to early embryogenesis (Wurtele *et al.*, 1993, Sato *et al.*, 1995, Lin *et al*, 1996) [3-5]. So we analyzed the biochemical changes prerequisite for induction of somatic embryogenesis by comparing some stress-induced somatic embryogenesis in carrot.

In carrot somatic embryogenesis induced by some kinds of stress, change of the physiological conditions of somatic cells and induction of the synthesis and/or accumulation of many proteins was observed (**Fig. 1**). Some kinds of stress, including heat shock, salts and heavy metal stresses, affect variously physiological conditions of plants and cultured tissues. In general, these stresses result in serious damage or denaturation of intracellular proteins and trigger changes of metabolism and synthesis of proteins, some of which are related to tolerance to those stresses. The 8 protein spots (ECP63-1, 63-2, 65-a, 65, 65-b, 65-c, 45-1 and 45-2) reported here, which could be detected in apical tips by all stress treatments, might be related to tolerance to these stresses. However, among the 8 spots, 5 proteins, ECP63-1, 63-2, 65, 45-1 and 45-2, were also detected in embryogenic cells but not in non-embryogenic cells (**Fig. 2**). Furthermore, these proteins were observed in hypocotyl segments 4 weeks after the culture with 2, 4-D and increased corresponding to the length of culture period on MS medium with 2,4-D (**Fig. 3-B** and **3-C**), but not in extracts after 2 weeks of culture with 2,4-D (**Fig. 3-A**). Embryogenic cells appeared on the surface of hypocotyl segments while they were cultured on MS medium with 2,4-D. About 4weeks after the start of culture with 2,4-D, calli were formed on hypocotyl segments and increased according to the length of culture period on MS medium with 2,4-D. So, the larger amount of calli were formed on hypocotyl segments treated with

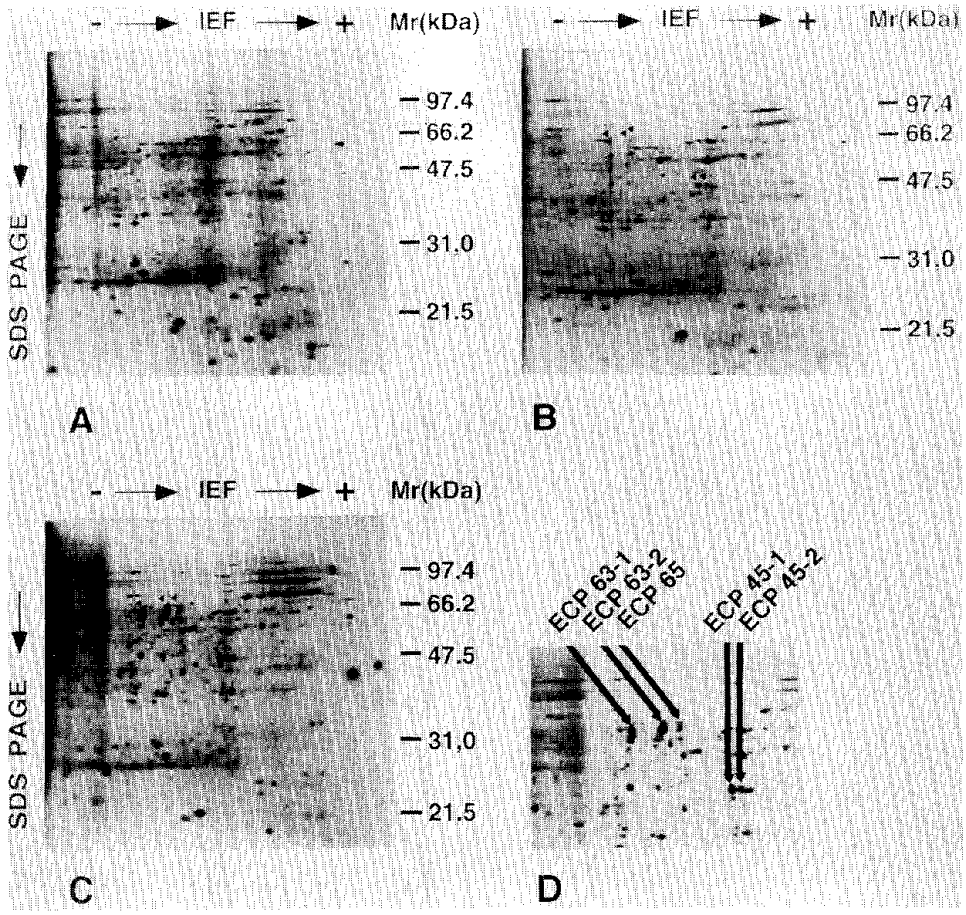

Fig. 3 2D-PAGE analysis of total proteins of hypocotyl segments treated with 2,4-D for various periods.

Proteins were extracted from hypocotyl segments treated with 2,4-D for 2 weeks (A), 4 weeks (B) and 6 weeks (C). Supernatant containing 100 μg protein was applied on each gel. Arrow heads indicated spots which were commonly detected in both apical tip segments treated with all kinds of stresses and hypocotyl segments treated with 2,4-D, but not apical tip segments without stresses. ECP63-1, 63-2, 65, 45-1 and 45-2 were shown by arrows in an enlarged picture of **Fig. 3-C** (D).

2,4-D, the more abundant the proteins (ECP63-1, 63-2, 65, 45-1 and 45-2) were in hypocotyl segments. And these proteins existed most abundantly in embryogenic cells. Furthermore, the degree of somatic embryogenesis was highest in embryogenic cells between apical tips induced by stresses and embryogenic cells (Kamada *et al.*, 1993, Kiyosue *et al.*, 1989. Kiyosue *et al.*, 1990, Kamada, *et al.*, 1994, Hogwash *et al.*, 1996) [9-12, 17]. These results indicate that these proteins may be directly related to induction of somatic embryogenic competence and/or early events of somatic embryo development prior to morphological differentiation of embryos rather than stress tolerance.

ECP63-1, 63-2, 65, 45-1 and 45-2 were also detected in mature somatic embryos and zygotic embryos in seeds (data not shown). In addition to that both somatic and zygotic embryos of carrot can produce embryogenic callus readily and produce somatic embryos directly in the absence of phytohormone (Smith *et al.*, 1989) [18], it was also reported that excised zygotic embryos of holly (*Ilex aquifolium*) (Hu *et al.*, 1978) [19] and *Brassica campestris* have been observed to produce somatic embryos without exogenous application of phytohormone. These show that both zygotic and somatic embryos have higher embryogenic tendency. And embryogenic cells which had ECP63-1, 63-2, 65, 45-1 and 45-2 had lost them after they had lost embryogenic competence to become non-embryogenic cells. Considering these results, ECP63-1, 63-2, 65, 45-1 and 45-2 may play important roles not only in induction of embryogenic competence, but also in maintenance of that competence.

In this report, it has been shown that ECP63-1, 63-2, 65, 45-1 and 45-2 were identified as embryogenic cell proteins, which were directly related to induction of somatic embryogenesis by stress treatments. The possibility that ECP63-1, 63-2, 65, 45-1 and 45-2 are related to acquisition and/or maintenance of em-

bryogenic competence is raised. However, the physiological functions of these proteins (genes) in the induction of somatic embryogenesis remains to be clarified. The relationship between accumulation of these proteins and acquisition of embryogenic competence will be clarified by further molecular analysis, such as cloning of the cDNAs and genomic DNA, analysis of 3′ flanking and promoter regions, and determination of subcellular localization of these proteins by immunohistochemistry.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and we wish to thank Dr. D. Macer, Institute of Biological Sciences, University of Tsukuba, for critical reading of the manuscript.

References

[1] Reinert, J., 1958. Naturwissenschaften, **45**: 344-345.

[2] Steward, F. C., Mapes, M. O., Smith, J., 1958. Am. J. Bot., **45**: 707-708.

[3] Wurtele, E. S., Wang, H., Durgerian, S., Nikolau B. J., Ulrich T. H., 1993. Plant Physiol., **102**: 303-312.

[4] Sato, S., Toya, T., Kawahara, R., Whittier, R. F., Fukuda, H., Komamine, A., 1995. Plant Mol. Biol., **28**: 39-46.

[5] Lin, X., Hwang, G.-J. H., Zimmerman, J. L., 1996. Plant Physiol., **112**: 1365-1374.

[6] Lovell, P. H., Lovell, P. J., Nichols, R., 1987. Ann. Bot., **60**: 41-47.

[7] Morre, D. J., Crane, F. L., Barr, R., Penel, C., Wu, L. Y., 1988. Physiol. Plant., **72**: 236-240.

[8] Kamada, H., Kobayashi, K., Kiyosue, T., Harada, H., 1989. In Vitro Cell. Develop. Biol., **25**: 1163-1166.

[9] Kamada, H., Ishikawa, K., Saga, H., Harada, H., 1993. Plant Tissue Culture Letters, **10**: 33-37.

[10] Kiyosue, T., Kamada, H., Harada, H., 1989. Plant Tissue Culture Letters, **6**: 162-164.

[11] Kiyosue, T., Takano, K., Kamada, H., Harada, H., 1990. Can. J. Bot., **68**: 2301-2303.

[12] Kamada, H., Tachikawa, Y., Saitou, T., Harada, H., 1994. Plant Tissue Culture Letters, **11**: 229-232.

[13] Murashige, T., Skoog, F., 1962. Physiol. Plant., **15**: 473-497.

[14] O'Farrell, P. H., 1975. J. Biol. Chem., **250**: 4407-4021.

[15] Due L. III, Crouch, M., Harada, J., Ho. T,-H. D., Mundy, J., Quatrano, R., Thomas, T., Sung, Z. R., 1989. Plant Mol. Biol., **12**: 475-486.

[16] Baker, J. C., Steele, C., Dure, I. I. I., 1988. Plant Mol. Biol., **11**: 227-291.

[17] Higashi, K., Kamada, H., Harada, H., 1996. Plant Cell Tissue and Organ Culture, **45**: 109-114.

[18] Smith, D. L., Krikorian, A.D., 1989. Amer. J. Bot., **76**: 1832-1843.

[19] Hu, C. Y., Ochs, J. D., Mancini, F.M., 1978. Z. Pflanzenphysiol., **89**: 29-40.