Note

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# Detection of Glycoproteins in Carrot Somatic Embryos Induced by 2,4-D or Stress Treatment.

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Although artificial seeds can be utilized as a useful propagation system for many plants, their practical application in agriculture poses some technical problems. One such problem is that the conversion rate of synthetic seeds to normal seedlings is low. In alfalfa, the conversion rate of somatic embryos induced at a lower concentration (10$\mu$M) of 2,4-dichlorophenoxyacetic acid (2,4-D) is higher than that induced at a higher 2,4-D concentration (50$\mu$M), due to a deficiency of essential storage proteins in somatic embryos at higher 2,4-D concentrations [1]. In carrots, a previous study has shown that somatic embryogenesis can also be induced by stress treatment (osmotic stress or heavy metal stress), with a higher conversion rate of somatic embryos than that induced by 2,4-D treatment [2]. In that study, the patterns of glycoprotein binding to Con-canavalin A (Con A) in carrot somatic embryos induced by 2,4-D or stress treatment did not differ and were quite similar to those in zygotic embryos [2]. On the other hand, the results of another study have suggested that the carbohydrate chains of glycoproteins play an important role in carrot somatic embryogenesis [3]. Embryogenesis-specific glycoproteins have also been detected in rice embryogenic cells [4], suggesting that glycoproteins are essential in embryogenesis. However, in carrot somatic embryos, different glycoprotein-binding patterns have not been detected with Con A. Thus, we decided to investigate the patterns of glycoprotein binding to lectins other than Con A in carrot somatic embryos induced by 2,4-D and stress treatment.

In the present study, we examined two factors that might affect the conversion rate of somatic embryos to normal seedlings. First, we compared the patterns of glycoprotein-binding to Con A, lentil seed lectin (LCA) and wheat germ lectin (WGA) in carrot somatic embryos induced by 2,4-D and stress treatment to those in zygotic embryos. In addition, because 2,4-D that had been incorporated in embryogenic cells induced by 2,4-D treatment, may have inhibited the conversion rate of somatic embryos to normal seedlings when the embryogenic cells were transferred to the 2,4-D free medium for induction of somatic embryos, we examined the amount of incorporated 2,4-D in embryogenic cells and the effect of 2,4-D on the development of somatic embryos.

Ten-day-old *Daucus carota* L. cv. US-Harumakigosun seedlings grown on vermiculite were surface-sterilized with a sodium hypochlorite solution (chlorine concentration of 1%) for 15 min and then rinsed five times with sterile distilled water. Segments 10 mm long were cut from the hypocotyls and each segment was placed in semi-solidified (0.2% Gelrite) Murashige and Skoog's medium (MS medium) [5] containing $4.5\times10^{-6}$M 2,4-D and cultured at 25°C under light conditions (16h light/8h dark, 6000lux). Embryogenic cells formed after 1 month of culture with 2,4-D were suspended in 300m$l$ Erlenmeyer flasks with 100m$l$ of liquid MS medium containing $4.5\times10^{-6}$M 2,4-D. These suspension cultures were gently shaken on a gyratory shaker (100rpm) at 25°C in darkness and transferred every two weeks to fresh MS medium with 2,4-D.

In order to obtain relatively uniform cell clusters, 14-day-old cultures were successively filtered through stainless steel meshes with pore sizes of 1000, 63 and 37$\mu$m, respectively, and rinsed with an excess amount of fresh MS medium without 2,4-D. Cell clusters remaining on the 37$\mu$m mesh were collected and rinsed again five times with fresh MS medium without 2,4-D. The remaining cell clusters were suspended in fresh MS medium without 2,4-D, at a final density of 0.2m$l$ packed cell volume (at 100×g) per liter of medium and cultured in 300m$l$ Erlenmeyer flasks containing 100m$l$ of the suspension at 25°C in darkness.

Apical tip segments of 1-week-old seedlings prepared by the same method described above were put on semi-solidified (0.2% Gelrite) MS medium containing 0.5mM $CdCl_2$, 0.3M NaCl or 0.7M sucrose and cultured at 25°C under light conditions (16h light/8h dark, 6000lux). For $CdCl_2$ or sucrose stress, the explants were transferred to an MS stress free medium after 2 weeks and cultured at 25°C under light condi-

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tions. For NaCl stress, they were transferred to an MS stress-free medium after 3 weeks and cultured under the same conditions. After about 4 weeks, somatic embryos formed according to the normal developmental process.

## 1. Analysis of glycoproteins in carrot somatic embryos

About 4 weeks after transfer to the 2,4-D free or stress free medium, the length of each somatic embryo was measured and classified into three categories (0.5-1.0, 1.0-2.0 or 2.0-3.0 mm). Each embryo was homogenized with sample buffer (pH 6.8) that contained 125 mM Tris-HCl, 10% (w/v) SDS, 0.004% (w/v) bromophenol blue, 20% (w/v) glycerol and 100 mM DTT. The homogenate was centrifuged at 100,000×g for 30 min at 4°C and the supernatants then analyzed by SDS-PAGE on 12% acrylamide gels according to the method of Laemmli [6]. Proteins in the gels were stained with Coomasie brilliant blue R-250 (CBB) dissolved in 10% (v/v) acetic acid and 50% (v/v) 2-propanol. The standards for determining protein molecular masses were phosphorylase B (Mr 97,400), bovine serum albumin (Mr 66,200), ovalbumin (hen egg: Mr 45,000), carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500) and lysozyme (hen egg: Mr 14,400) (Japan Bio-rad, Tokyo, Japan). Staining with peroxidase-conjugated Con A, LCA and WGA (Seikagaku kougyou, Tokyo, Japan) was performed using a modification of the method of Ochiai *et al.* [7] as described previously [8].

Protein patterns on CBB stained SDS-gels were the same among 2,4-D induced embryos, stress induced embryos and dry seed (data not shown). Glycoprotein patterns stained with peroxidase-Con A (**Fig. 1-A**) and peroxidase-LCA (**Fig. 1-B**), were also very similar and no glycoprotein was stained with peroxidase-WGA (data not shown) in any of the four somatic embryos or dry seed samples.

These data differ from the results obtained by Stuart *et al.* [1]. They demonstrated that storage proteins formed during embryogenesis accumulate in dry seeds and somatic embryos induced at a lower concentration of 2,4-D (10 $\mu$M), but not in somatic embryos induced at a higher concentration of 2,4-D (50 $\mu$M). They also suggested that these storage proteins are essential for development of embryos. However, the protein patterns detected in carrot somatic embryos in the present study were quite similar among the somatic embryos. These results indicate that in carrots, detectable proteins stained with CBB or lectins are not essential to the development of somatic embryos.

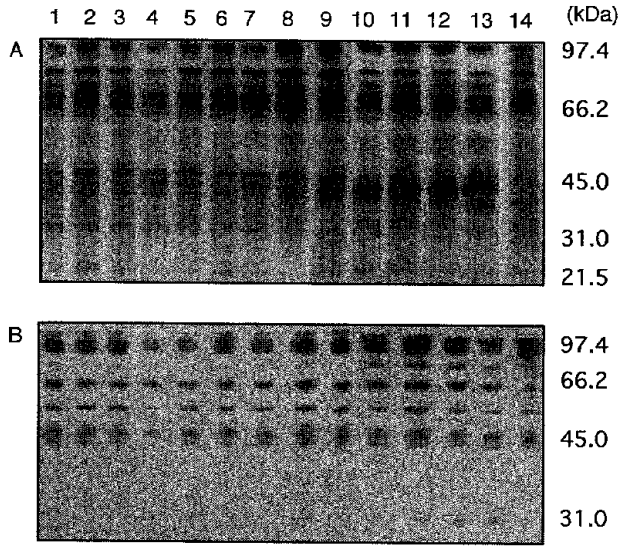


**Fig. 1** Variations in protein patterns stained with lectin in somatic and zygotic embryos.
Extracts of somatic embryos induced by 2, 4-D (lane 1, 2 and 3), NaCl (lane 4, 5 and 6), $CdCl_2$ (lane 7, 8 and 9) and sucrose (lane 10, 11 and 12), dry seeds (lane 13) and zygotic embryos 4-days after germination (lane 14), were analyzed by SDS-PAGE. Glycoproteins were visualized either by staining with peroxidase-Con A (A) or peroxidase-LCA (B). 20$\mu$g of protein was loaded into each lane. Numbers on the left refer to the positions of molecular mass markers. The length of embryos in lanes 1, 4, 7 and 10 was 0.5-1.0 mm, that in lanes 2, 5, 8 and 11 was 1.0-2.0 mm and that in lanes 3, 6, 9 and 12 was 2.0-3.0 mm.

## 2. Effects of incorporated 2,4-D on somatic embryogenesis

The 2,4-D incorporated in embryogenic cells was analyzed using radiolabeled 2,4-D. Embryogenic cells maintained in MS medium containing 2,4-D were transferred to MS medium containing 1.5 M Bq/*l* of $^{14}$C-2,4-D (equivalent to $4.5\times10^{-6}$M). After 14 days, cell clusters of 37-63 $\mu$m in diameter obtained as previously described were rinsed five times with fresh MS medium. The clusters were extracted with 80% (v/v) acetone. The extracts were filtered and dried *in vacuo*. The dried sample was dissolved in scintillator (ACS II, Amersham Japan, Tokyo, Japan) and radioactivity was determined using liquid scintillation counting (LS 5000 TA; Beckman Instruments Inc.). The 2,4-D incorporated in embryogenic cells was estimated as $1\times10^{-9}$M by dividing the amount of the incorporated 2,4-D by the fresh weight of cell mass.

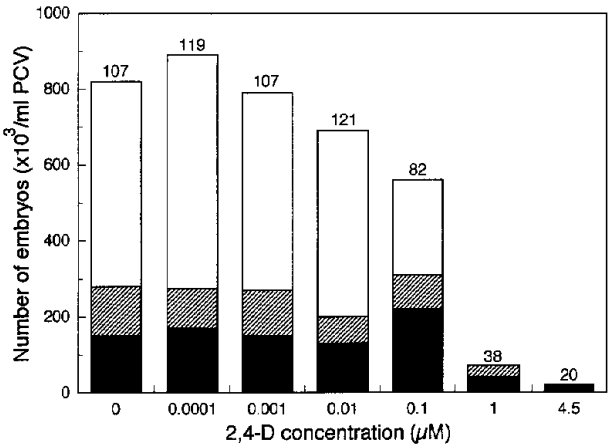


**Fig. 2** Influence of 2,4-D on the development of embryos.
The number of somatic embryos (open box: torpedo shaped embryos, hatched box: heart shaped embryos, closed box: globular embryos) was counted 14 days after transfer to MS media containing different concentrations of radiolabeled 2,4-D. Numbers on each bar indicate fresh weight per m*l* packed cell volume (100×g).

For the induction of somatic embryos, embryogenic cells were transferred to MS without 2,4-D, and thus the concentration of 2,4-D in embryogenic cells was most likely lower than $1\times10^{-9}$M.

Embryogenic cells maintained in MS medium containing $4.5\times10^{-6}$M 2,4-D, were transferred to MS media containing different concentrations (0, $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ and $4.5\times10^{-6}$M) of 2,4-D. The number of embryos formed after 2 weeks was then counted. As shown in **Fig. 2**, $1\times10^{-10}$ to $1\times10^{-8}$ M of 2,4-D had no effect on the development of somatic embryos, $1\times10^{-7}$M of 2,4-D weakly inhibited it and $1\times10^{-6}$ or $4.5\times10^{-6}$ M of 2,4-D almost completely inhibited it. All experiments were repeated three times and the same tendency was observed in all experiments. These data suggest that the incorporated 2,4-D ($1\times10^{-9}$M) was not the main factor inhibiting the development of somatic embryos induced by 2,4-D.

The low conversion rate of synthetic seeds to normal seedlings in carrot somatic embryos induced by 2,4-D could not be explained by the change in proteins stained with CBB or lectin and/or incorporated 2,4-D. This suggests that undetectable minor changes in somatic embryos induced by 2,4-D may affect their development.

## ACKNOWLEDGEMENTS

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