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# Esterase isozyme patterns in developing plant regeneration from calli of *Citrus junos* Sieb.

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## ABSTRACT

**The callus from the hypocotyl region of immature embryo of *Citrus junos* Sieb. was efficiently induced in the ½ MS medium containing 45μM BA after 8 weeks culture. The callus was developed into the two callus type, embryogenic callus and nonembryogenic callus, which can be distinguished by visual examination depending on color and appearence. *In vitro* regeneration of callus established efficiently in the hormone-free MS medium from the embryogenic callus. In order to investigate the physiological changes depending on the developmental stage of embryo, the embryo was formed in the MS medium. The embryogenic and nonembryogenic callus, and the various stages of the somatic embryo were examimed the changes of esterase activity, and their isozyme patterns as well. The protein content and esterase activities was gradually increased on the developmental stages of embryo. Total protein pattern were different by the SDS-PAGE and were appeared strong band of 23 KD in the torpedo stage. The pattern of the esterase isozyme was exhibited a difference between embryogenic callus and nonembryogenic callus. It was appered pI 6.0, 8.0, 8.2 in the embryogenic callus. Also the new band of pI 4.75 was appeared in the cotyledon. These results suggest that the changes of esterase activities and isozyme patterns are importent factor in the differentiation and development of citrus.**

***Key words :*** *Citrus junos* Sieb., embryogenic callus, nonembyogenic callus, total protein contant, esterase, isozyme pattern.

## INTRODUCTION

In plant tissue culture, somatic embryogenesis and plant regeneration process was observed both plant physiology, biochemical regulator and genetic expression process. Plant regeneration from callus tissue is essential for the utilization of in vitro culture techniques in morden plant breeding. As an approach to plant regeneration, somatic embryogenesis has several stages. The stages was globular, heart-shaped, torpedo and cotyledon embryos. Also, it was inducted through less high concentration of auxin.

Many plants undergo changes in plant regulator during growth and development and isozyme expression (Dewitte and Coppens, 1990). Changes in activity and isozyme pattern are correlated with growth and development and plant growth regulation. Isozymes analysis

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at different stages of culture widely used tools in physiological, genetic and biochemical studies underlying the process of regeneration and differentiation (Coppens and Gillis, 1986).

Isozyme or protein patterns are widely used distingluish closely related plant species, varieties or cultivars. The esterase isozyme refers to a group of enzymes that includes a host of ester hydrolase(Kidambi, 1990). Refinement of isozyme analysis by isoelectric focusing and combination of isozyme provide a reliable marker system for organigenesis and embryogenesis of plants in callus tissues. Everett and and coworkers(1985) used the developmentally regulated isozyme system of esterase to distingulish between embryogenic and shoot-forming maize tissue cultures. Isozyme system of esterase is known as a biochemical maker between embryogenic and shoot forming maize tissue culture (Everett *et al*, 1985 ; Coppens and Gillis, 1986).

In *Citrus*, plant regeneration from shoot tip, immature ovules, cotyledon, hypocotyl and leaf of Trivita orange, Naval orange(*C. sinensis*), sweet orange(*C. siinensis*), Trifoliate orange(*Poncirus trifoliate*), *Citrus junos* (L.) Osbeck., *Citrus junos* Sieb., *Citrus junos* Blanco. has been reported(Kobayashi *et al* , 1984; Kobayashi *et al*, 1990, Shinozaki *et al*, 1992; Hiramatsu *et al*, 1987; Ikeda and Yoshida, 1993; Sim *et al*, 1988; Song et al, 1991). In addition, our laboratory was achieved plant regeneration from hypocortyl of immature seed of *Citrus junos* Sieb.(Park *et al*, 1995). Also, isozyme analysis is very useful for both classification and genetics. Hirai and his co-workers has carried out a series of experiments using this technique (Hirai *et al*., 1986; Hirai and Kajiura, 1987, Kensuke, 1993).

We intended to extend the number of Citrus species and cultivars in which callus could be readily isolated from immature embryo and subsequently cultured *in vitro* and regenerated into functional plant.

The objectives of this study were to determine the different esterase isozyme and protein banding patterns with the each stage of the plant tissue culture and to established an efficient system for somatic embryogenesis and plant regeneration in *Citrus junos* Sieb. by embryogenic callus from the hypocotyl region of immature embryo. It was also our aim to proffer the fundamental information of genetic analysis about a system phylogeny.

## METERIALS AND METHODS

### Plant meterials

Immature fruit of *Citrus junos* Sieb. were obtained from immature seed in age from 4 month after flowering be used for the experiment. Seeds was surface sterilzed in 70%(V/V) ethanol for 10min and 5%(W/V) NaOCl for 15 min and rinsed 3 times in sterilled water. Immature emrbryo was seperated from the seed and it was excised from surface-sterilizd seeds. It was transplanted onto the callus induction media.

### Callus induction and plant regeneration

For callus induction, the culture medium($\frac{1}{2}$ MS) consisted of Murashige and Skoogs (1962), inorganic salts supplemented with 3% sucrose, 0.8% Difco-Bacto agar and 45$\mu$M BA. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were incubated in a room thermostatically controlled to maintain a temperature of 25$\pm$2℃ under white flourescent light (2000 Lux. 16h photoperiod). After callus induction, the embryogenic callus was transplanted onto MS medium without any growth regulators.

### Extraction and electrophoresis of protein

Protein was determined by the Lowry(1951) method using Bovine Serum Albumin as a standard.

About 1g of young fresh material was extracted with 50mM Tris-HCl buffer(pH 7.2), at 4℃. The homogenated was centrifuged for 30min at 13.000 rev./min and supernatant was used for protein analysis.

Protein molecular weight were determinred by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed by the method of Laemmli (1970) with 12% (W/V) polyacrylamide gel. Electrophoresis was performed for 4 h at 150V at 4℃.

Standard markers Bio-Rad was phosphorylase b (97.4 KD), bovineserum albumin (66 KD), egg albumin (45 KD), carbonic anhydrase (31 KD), trypsin inhibitor (21.5 KD), lysozyme (14.5 KD).

**Esterase assay**

Esterase assay were prepared as described by Coppens and Gillis (1987).

About 1g of young fresh material was extracted with 100mM Tris-HCI buffer (pH 7.2), at 4℃. The homogenate was centrifuged for 5min at 15.000 rev./min and supernatant was used for isozyme analysis.

Esterase was tested by measuring the absorbance at 290 nm after 15 minutes incubation at 30℃. Each value was the mean of three separated experiments.

**Isoelectric focusing(IEF) and staining for the enzyme activiy.**

IEF were prepared as described by Stegman and Park (1979). IEF was performed at 4℃, 5% polyacrylamide gel containing 0.1% carrier ampholytes (sigma) in the pH range 3～10 for esterase.

Electrode strips were saturated with 0.01 M $H_3PO_4$ as an anolyte and 0.2 M NaOH as a catolyte. IEF standard proteins was Cytochrom C (pI 9.6), Lentil lectin (3 band, pI 7.8, 8.0, 8.2), Human hemoglobin C (pI 7.5), human hemoglobin A (pI 7.1), equine myoglobin (pI 7.0), Human carbonic anhydrase (pI 6.5), bovin carbomin anhydrase (pI 6.0), $\beta$-Lactoglobulin b (pI 5.1), Phycocyanin (pI 4.75)

After electrophoresis, each gel was stained for enzyme activity. The staining procedures were were stained by the method of Kidambi *et al* (1990) and Wetter and Dyck (1983). 20mg of $\alpha$-naphtyl acetate was dissolved in 0.5*ml* of acetone and the volume was made up to 100mM Tris-HCl buffer (pH 7.0). Than 40mg of Fast Blue RR Salt was dissolved by vigorous stirring. Gel were incubated 37℃ for 15 min. When the staining is judged to be complete, the solution discarded and the gel is rinsed and stored in 7% acetic acid.

## RESULT AND DISCUSSION

**Callus induction and plant regeneration**

The highest production rate of somatic embryos was influenced by age of the explants and a part of the plant. Generally, they use immature embryos, young leaf, young anthotaxy, hypocotyl tissue, leaf lobes, root to induction of callus. Among the rest, the immature embryos showed high embryogenic but a reduced embryogenic capacity mature embryos due to loss of embryogenic potential(Lee *et al*, 1993).

Hidaka and Omura (1989) studied the efficient control of embryogenesis using friable callus originating from the hypocotyles of premature embryos in Citrus. Kobayashi *et al* (1984) effectively induced nucellar callus in ovules of Trovita orange cultured on Murashige and Tucker(MT) medium supplemented with 10mg BA/liter. In this experiment, we used the hypocotyl reglon of immature seed in age from 4 month after flowering for callus induction (Figure 1A). Callus induced on MS medium containing 45$\mu$M BA. After 6～8 week of culture, callus was consisted two distinguished types. The first one was recognized as nonembryogenic callus, the callus was easily distinguished by bright yellowbrown color and humidity from other ones. Whereas the other as embryogenic callus, the callus appeard to have a more compact surface structure and yellow-green color than the surounding tissues (Firgure 1B).

Nabors *et al* (1983) found that, The calli were developed

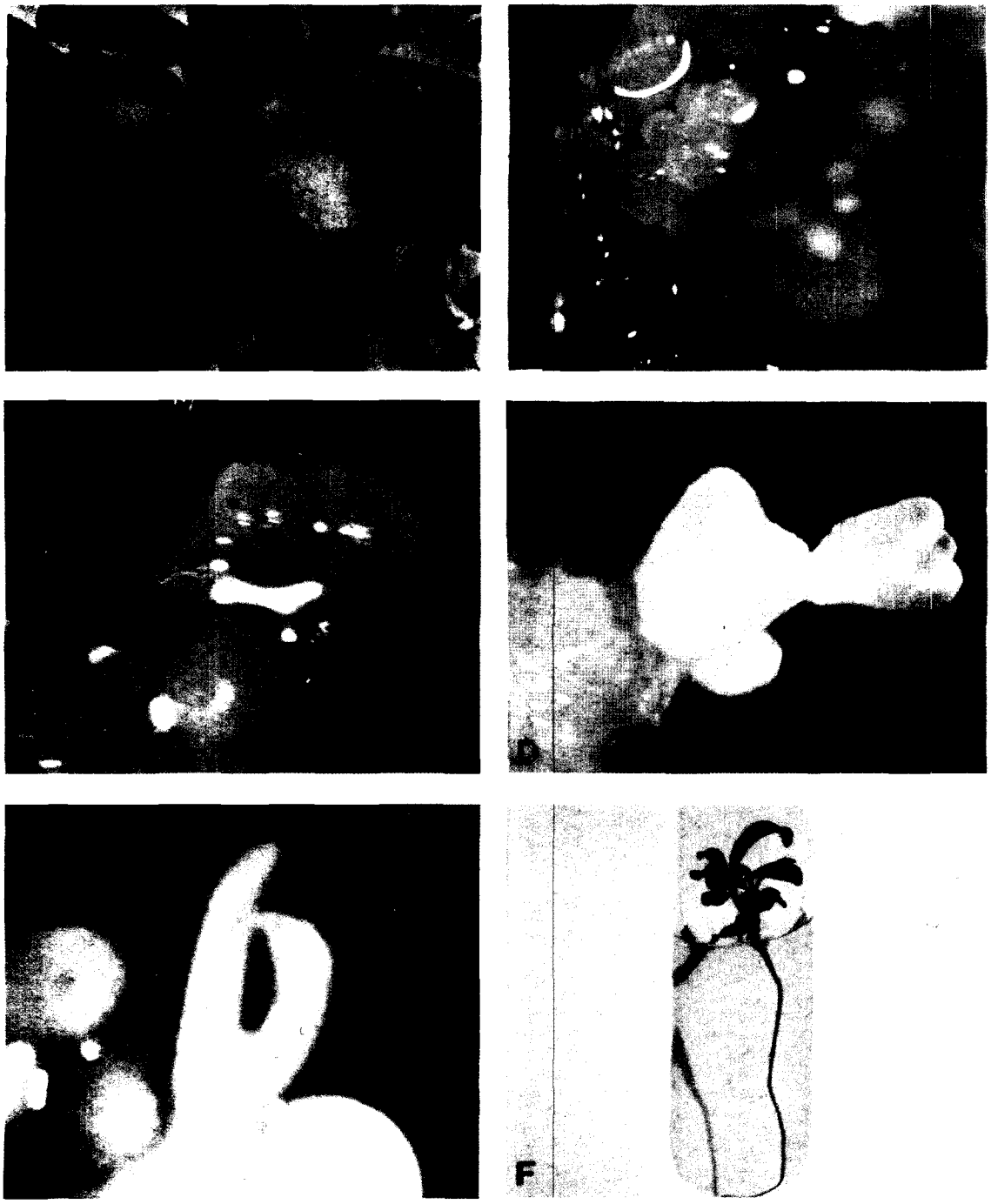


**Fig 1.** Callus induction and plant regeneration from embryogenic callus in the hormone free MS medium.
A, Immature seed in age from 4 month after flowering for callus induction.
B, Proliferation of embryogenic callus and nonembryogenic callus on $\frac{1}{2}$ MS medium with 50 $\mu$M BA.
C, The globular emborys formed from embryogenic callus after 2 weeks culture.
D, The heart-shaped embryos formed from embryogenic calli after 3 weeks culture.
E, The cotyledon after 8 weeks culture.
F, The pantlet formation from embryogenic calli after 15weeks culture.

into two callus type, embryogenic and nonembryogenic callus, which can be distinguished by viual examination depending on color and appearance (Chen and Luthe, 1987, Jung *et al.*, 1993)

Plant regeneration occurred from cultured embryogenic callus on medium without supplemented growth regulators. After 2 weeks culture, the embryogenic callus became green in color by degrees. This embryogenic callus showed greenly nodular structures and grew slowly. Directly the majority of embryogenic callus developed globular embryos without an intervening a callus multiplication. It could be separated easily from the other parts (Figure 1C). After 3 week cultures, as globular embryo developed into heart-shaped stages. This heart-shaped embryos appeared bipolar structures, and indicated root and cotyledon initials (Figure 1D). It was developed rapidly torpedo-shaped embryo. Distict tissue differentiation was in 8-week cultured cotyledonary stage embryo (Figure 1E). ultimately, the plantlet was formed after 15week cultured (Figure 1F).

The total period between initiation of embryoculture and transfer to the greenhouse varied from 4 to 5 months.

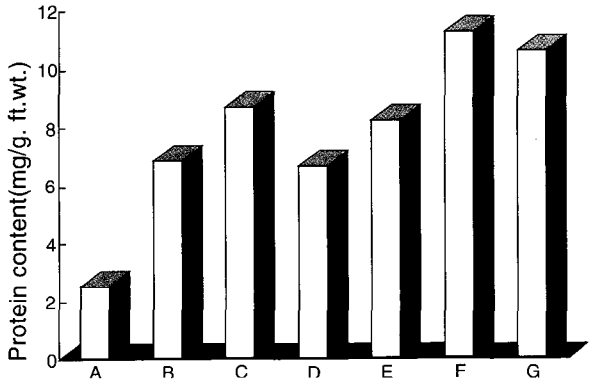


**Fig 2.** Comparison of soluble protein content of the nonembryogenic and embryogenic callus and the various stages of *Citrus* somatic embryo.
A, nonembryogenic callus B, embryogenic callus C, immatured somatic embryo D, globular embryo E, heart-shaped embryo F, torpedo embory G, cotyledonary embryo

**Extraction and electrophoresis of protein**

The results presented in Figure 3 clearly show that the total pretein of embryogenic callus, nonembryogenic callus and each stages have different mobilities in SDS-PAGE. And a further increased amount of protein was observed in the following stages of embryogenesis (Figure 2).

In comparison of the protein patterns among the callus in a nonembryogenic and embryogenic state, and

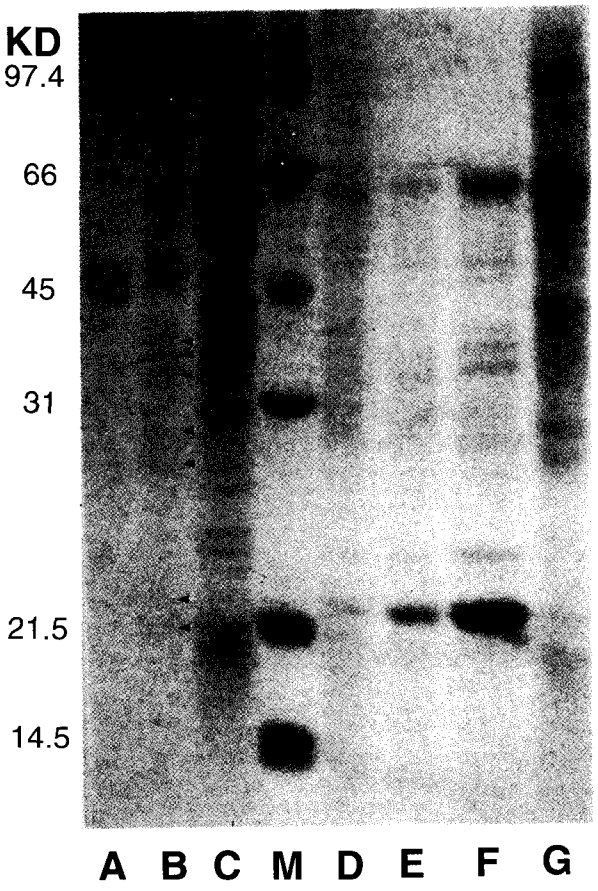


**Fig 3.** The protein patterns of nonembryogenic and embryogenic callus and the various stages of *Citrus* somatic embryo.
A, nonembryogenic callus B, embryogenic callus C, immatured somatic embryo D, globular embryo E, heart-shaped embryo F, torpedo embory G, cotyledonary embryo

somatic embryos. We are found three characteristic protein bands of embryogenic callus. These protein bands had molecular weight of 28KD, 25KD and 21KD. these were common protein (47KD) in both callus.

Three bands (63KD, 39KD, 37KD) of embryogenic callus reappeared in globular stage embryos. 32KD protein band appeard newly globular stage. Two protein bands (63KD, 23KD) appeared a specific band of torpedo stage. Five protein bands (31KD, 39KD, 54KD, 55KD, 63KD) appeared from globular stage and remained until cotyledonary stage.

Changes of protein patterns during embryogenesis from topedo stage to cotyledonary stage were more striking than during globular stage to torpedo stage. This result was with anatomical analysis that tissue differentiation was significantly correlated to morphological differentiation.

Change of morphologic and development from callus induction to plant regeneration was due to a special gene combination (Kim et al, 1992). Also, the developmental program of a cell in volves the appearance of disappearance of apecific proteins at a particular stage which reflects that the differentiation is regulated by differential gene expression (Rao et al, 1990).

In this study we compared changes of protein patterns to morphological differntiation during the embryogenesis, that was expressed differented protein patterns each other.

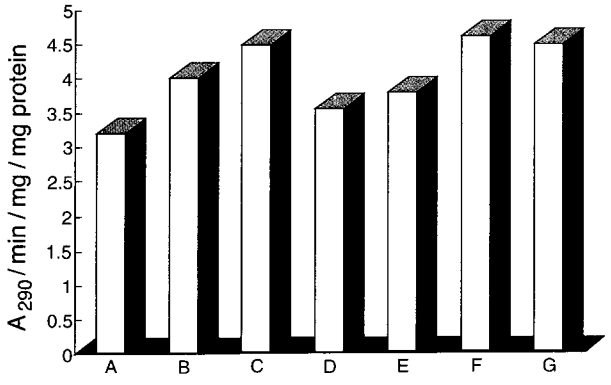


**Fig 4.** The esterase activity in nonembryogenic callus and embryogenic callus and the various stages of *Citrus* somatic embryo.
A, nonembryogenic callus B, embryogenic callus
C, immatured somatic embryo D, globular embryo
E, heart-shaped embryo F, torpedo embory
G, cotyledonary embryo

**Esterase assay**

A charicteristic change associated with the development of callus was a significant increase in the esterase activity. (Figure 4)

During the progress of differentiation and growthing, the callus was getting incerase in the activity. In all types of callus, the globular embryo are charicteried by a very low activity for the esterase. Eapecially torpedo embryo stage is very strong.

Mangolion et al(1994) report the analytical conditions used to obtain electrophoretic pattern for esterase isozymes to be used as markers of genetic uniformity of callus tissues from long-term cultures and of the plants regenerated from callus of C. peruvianus.

From the work reported here it is clear the a rise in total esterase is necessarily related to growth and development. It appears that it is the relative level of isoenzymes rather than the level of tatal esterase that is related to plant growth. The selective response of the plant tissue to growth substances in the development of specifics esterase isozymes.

**IEF and staining for the enzyme activity**

The associated of growth with the development of the plant regenerations as affected by esterase is evident from the data presented in Figure 5.

Esterase isozyme patterns showed unclear, differences between embryogenic and nonembryogenic callus. Nonembryogenic callus exhibited more number(5) of bands compared to embryogenic callus(4).

Two bands were common, band pI 6.8, 6.4 in both the types. In embryogenic callus, we obtained isozyme

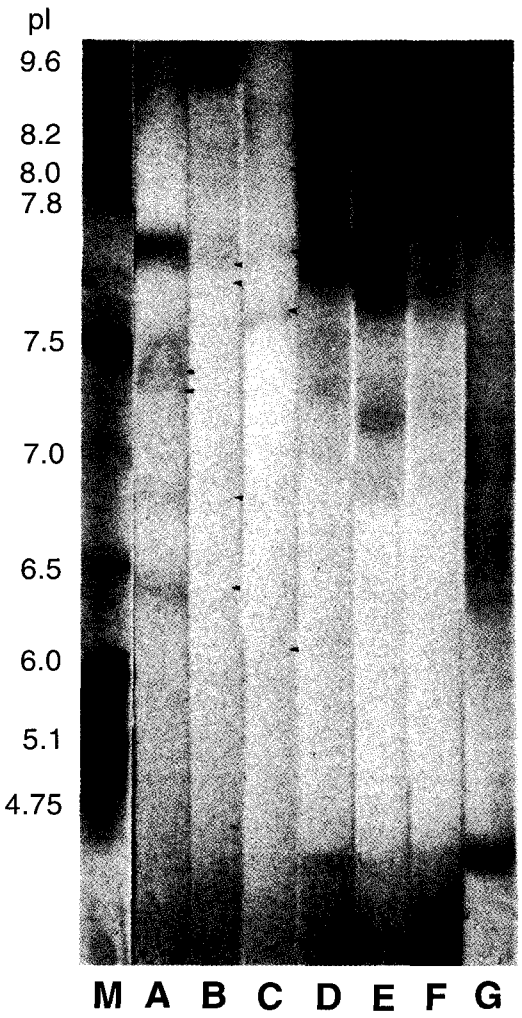


**Fig 5.** The change of esterase pattern in nonembryogenic and embryogenic callus and the various stages of *Citrus* somatic embryo.
A, nonembryogenic callus B, embryogenic callus
C, immatured somatic embryo D, globular embryo
E, heart-shaped embryo F, torpedo embory
G, cotyledonary embryo

patterns of pI 7.6, 7.75. Whereas, nonembryogenic callus showed pI 7.4, 7.3, 7.7 of esterase patterns.

Specifis esteras isozymes(pI 7.6, 7.75) were found in embryogenic callus not detectable in nonembryogenic callus. These results suggest that the isozyme profile may be used as markers in distinguishing embryogenic and nonembryogenic callus.

In the region pI 7,5~9.6, the isozymes are absent or very weak in embryogenic callus, but strogly active in developing embryos. Three specific bands (pI 6.0, 8.0, 8.2) appeard from globular stage, and globular stage are characterized by a very high activity for he isozyme patter pI 8.2, 7.75. The strong activity we observed in esterase bands prevails in differentiated leaf tissue. That is, this situation consider that those specific isozyme patterns were detected along morphological changes during embryogenesis.

Embryogenesis and leaf morphogenesis can be recognized by examination of mainly esterae patterns(Dewitte *et al*, 1990) The isozyme analysis at different stages of culture may unrevel. The physiological or biochemical changes underlying the process of differentiation, and is important in tissue specific biochemical characteristics which are expresed and maintained in culture (Sandalios, 1974; Coppens and Gills, 1987; Rao et al, 1990).

In this paper, we observed that refinement of isozymes analysis by isoelectric focusing and combination of isozymes profiles provided a reliable marker system for regeneration of plant from barley callus tissues. these result suggest that the esterase isozyme profile may be used as markers in distinguishing embryogenic, nonembryogenic callus and embryogenesis.

## ACKNOWLEDGMENT

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