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벼 callus로부터 재분화 과정에서 생성되는 특수한 단백질의 동정

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Indentification of Specific Proteins synthesized During Somatic Embryogenesis of Rice

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Summary

This study was conducted to investigate the physiological and biochemical elements relating embryogenisis. Also we examined the differences of protein in embryogenic callus and nonembryogenic callus by SDS-PAGE and two-dimensional gel electrophoresis. In this study, we have focused attention on the process of total proteins during sometic embryogenesis of rice. When compared the proteins of embryogenic callus and nonembryogenic callus, some different proteins were observed and serval proteins were increased in embryogenic callus. Some proteins were decreased or disappeared in embryogenic callus. Near 43kD protein band was only observed in embryogenic callus. The other bands were similar to each lines. The result of two-dimensional gel electrophoresis, E-callus specific proteins were observed. This Results may indicate that these proteins were associated with somatic embryogenesis.

Introduction

Developments in plant tissue culture techniques offer possibilities of introducing into plants variability that could be utilized for crop improvement. One of the primary objectives of today's plant breeders is to use this variability to develop high yielding varieties with the ability to resist or tolerate adverse environmental conditions. The effors to do so require the new technique as well as the traditional breeding methods.

Tissue cultures of rice have been started from orgen culture of excised roots and young hybrid plants (4). Regeneration of plants was done from the calli of embryo, anther, root and various explants of rice(9.18). Extensive experiments using rice tissue culture have been performed to apply the results of these studies to the areas of genetics and practical breeing.

The greatest advances in increasing regeneration frequency have come from the realization

that rice tissue cultures produce different types of calli which may differ in their regenerative potentials(8,13,15,16). At least two types of rice callus are present: One is "embryogenic"(E) callus that is smooth, white, knobby appearance and is composed of small isodiametric cells which average 31㎛ in diameter. The orther is "Non embryogenic"(NE) callus that is yellow to translucent. wet rough to crystalline in appearance and is composed of larger, elongated cells which average 52㎛ in diameter and 355㎛ in length(11.19). Embryogenic callus is produced on a small fraction of individual callus(frequently less than 10 %) and comprises a small fraction of a given callus.

The E-callus was frequently developed plants by somatic embryogenesis whereas NE-callus was occationally developed shoots or roots by organogenesis. Vasil and Vasil(20) described that embryogenic callus of the Gramineae are generally white to pale-white in color, compact and organized in nature, and contain large numbers of samll, riching cytoplasmic, starch-containing meristematic cells. The terms E. and NE. are used freely in the literature to describe callus even though the existence of true embryoids is not usally histologically demonstrated. Verification of physiological and biochemical change in the cells having high regeneration ability was very usefulness to efficient regeneration. The callus having high regeneration ability was identified with eyes in 5-7 days after the transfer of callus on to regeneration medium and after a week, fresh weight and dryweight were increased 2.5 times and 3.8 times respectively.

In recent year, many rescarchers have been studied the differences between E-callus and NE-callus. But these studies were poor and not clear in molecular level. Therefore, E-callus and NE-callus were isolated from the rice mature seed culture. From isolated callus we want to examine the physiological and biochemical element relating embryogenesis. Also we want to increase the regeneration ability from rice callus. This study was performed to know the difference of protein in E-callus and NE-callus by SDS-PAGE and two dimonsional gel electrophoresis.

Materials and Methods

Eight varieties including two subspecies, *japanica and indica-japonica* hybrids(Tongil type) were used in this experiment(Table 1). Mature seeds were selected and dehusked as experimental materials. Seeds were surface sterilized in 70% ethanol for 1 min. and 1% sodium hypochloite solution for 40 min followed by rinse three times with sterlie distilled water.

Table 1. Rice varieties used for this study.

Japonica type	Tongil type
Palgongbyeo	Chilseongbyeo
Nagdongbyeo	
Tamjinbyeo	Hangangchalbyeo
Chucheongbyeo	
Yeongdeogbyeo	Yongmunbyeo

The sterilized seeds were placed on solid N6 medium with 2,4-D(1㎎/1), sucrose(30 g/1) and gelrite(2 g/1). The media were adjusted to a pH 5.8 before autoclaving. The cultures were incubated at 27℃ in the dark and were subcultured every 2 weeks in the same medium. E-callus and NE-callus were subcultured every 2 weeks in the same medium. E-callus and NE-callus were divided and maintained by working under the microscope and other methods, at the same time initiated callus were subcultured 7-8 times. Isolated E-callus were transfered to solid N6 shooting medium with

NAA(1㎎/1), kinetin(5㎎/1), sucrose(30 g / 1), and gelrite(2 g /1).

Rice callus were routinely propagated in N_6 liquid medium with 1㎎/1 2,4,E-D. Nonorganized cells were subcultured every two weeks on fresh medium. Embryogenic cultures were initiated at filteration a 170㎛ nylon mesh. After 6 weeks, somatic embryos were harvested and used in this study.

Protein was extracted from callus by grinding with a motar and pestle in 12.5mM Tris-Cl, pH 6.8, 4%SDS, 20% glycerol, 10% 2-mercaptoethanol(30㎎of callus/100㎕ of extraction buffer) in 1.5-㎖ Eppendorf tubes. And other extracted proteins were needed for IEF Gel, 9.5M urea, 2% Np-40, 2% ampholytes, 5% 2-mercaptoethanol. In this extraction procedure, the extraction buffer was differ from SDS PAGE buffer. The homogenates were centrifuged at 12,000 rpm for 30min.

The supernatants were removed in a fresh tube and stored at −20℃. SDS-PAGE was carried out according to the procedure of Leammli(1970)(12) with minor modification of the slab gel(Gradiet polyacrylamide 4%, 15% 1.5㎜ thick) containing 0.1% SDS 5㎕ of the extract was applied to each lane. Standard proteins (Pharmacia LMW Calibration Kits) for identifying moleculer weight were also used. The electrophoresis was performed at room temperature with a constant voltage of 100V.

Two-dimensional gel electrophoresis was performed as described previously(3).

After electrophoresis the gels were soaked in stain solution(1% coommassie bule R−250) and gently shaked for 2 to 3 hours. The gels were removed and put them in destaining solution I(50% methanol, 10% acetic acid) and shaked for 1 hour. The gels were soaked in destaining solution Ⅱ(7% acetic acid, 5% methanol) and destained until the background is clear.

Silver staining was performed by following procedure. The polyacrylamide gels, first must be fixed in fixing solution(50% ethanol, 5% acetic acid) and washed in tray on shaker to remove the fixation reagents and SDS. After fixation and washing, the gel was soaked in silver reagent. The silver regent was replaced by reducing reagents. At the ended of the recommended time, spots begin to appear through the gel. The final soakings was with the stabilizer reagents three times(14).

Results and Discussion

The frequency of regeneration on solid medium varied with the varieties of rice examined (table 1). The varieties "Yeongdeogbyeo" showed higher frequencies of regeneration than these of other varieties. The result was correspond to other data(17).

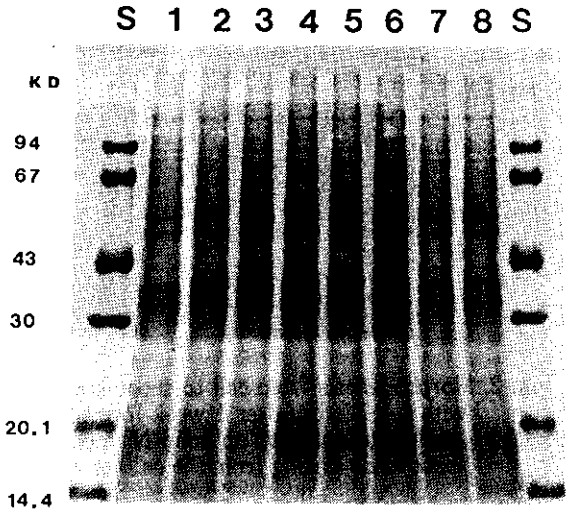

Fig. 1. SDS-PAGE of protein patterns from 8 rice cultivars.
s:protein standard lane 1:Tamjinbyeo 2:Chilseongbyeo, 3:Palgongbyeo, 4:Yeongdeogbyeo, 5:Chucheongbyeo, 6:Hangangchalbyeo 7:Yongmunbyeo, 8:Nagdongbyeo.

So we used Yeongdeogbyeo which was most effective in our study. And we also assayed total proteins extracted from 8 varieties. To do this, proteins were extracted from 30 ㎎ of callus respectively and assayed by SDS gel electrophoresis(Fig. 1) No marked difference was observed among protein patterns of the callus on SDS-PAGE from each eight varieties.

It is important for physiological and biochemical investigations, as well as for mass propagation, to suspension cultures of embryogenic cells which can maintain high embryogenic potential and to induce somatic embryos. We obtained somatic embryoid which was the potential for regeneration by suspension culture. Suspension cultures are more suitable for physiological and biochemical investigation of differentiation than callus cultures grown on solid media. Because the former obtain much homogeneous cell line than that of the latter. In our experiments, N^6 medium with 1㎎/1 2,4, -D was suitable for suspension culture(Fig. 2) In earlier report we found many differences of the callus having high and law regeneration ability. Callus cultures from the mature embryo of rice variety gave rise to two kinds of callus, embryogenic and nonembryogenic calluses.

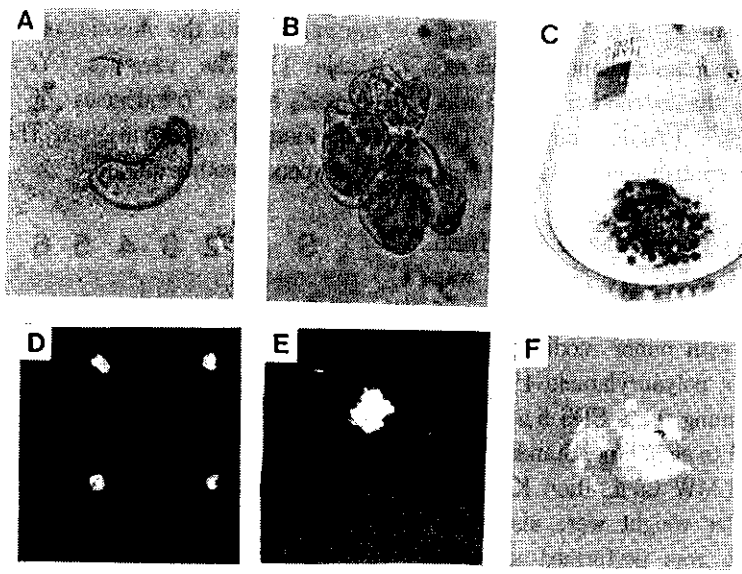

Fig. 2. Morphogenesis from cell to shoot-buds A:rice suspension culture(N_6 medium supplemented with 2,4-D). B: after 1 week formed cell cluster C: uniformed embryogenic callus. D,E: E -callus, transferred on solid N^6 media with 5㎎/1 kinetin, 1㎎/1 NAA, F: after 1 week shoot-buds arising from the calli on the shooting media

The former callus was characterized by white, compact and organized structure in appearance which came to be embryoids, the latter callus was characterized by yellow th brownish, wet and soft structures n appearance.

Protein pattern was markedly different between callus of the non-selected on callus and shoot induction meadia.(Fig. 3) In the non-selected callus on callus induction media, strong bands were present whereas in the non-selected callus on shoot induction media, weak bands were present.

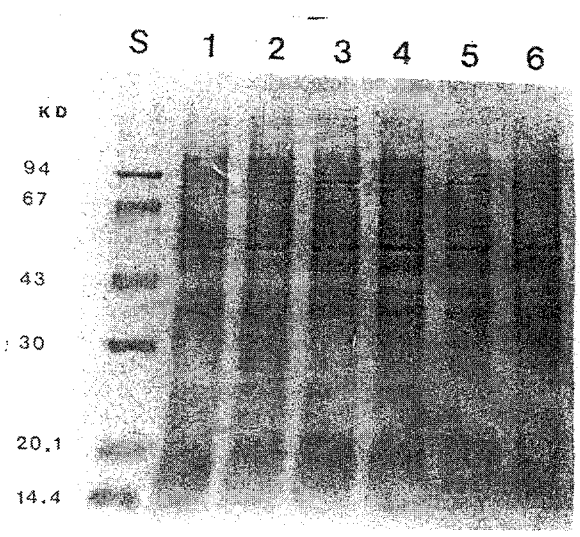

Fig. 3. SDS-PAGE pattern of the callus lines cultured on N_6 medium. S: protein standard, lane 1,2,3: non-selected callus on callus induction media(1:1 week 2:2 weeks, 3:3 weeks), lane 4, 5, 6: non-selected callus on shooting media (4:1 week, 5:2 weeks, 6:3 weeks). Arrow indicate decreased protein band.

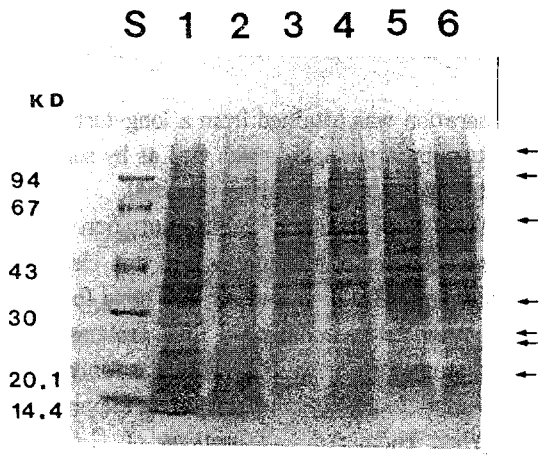

Fig. 4. SDS-PAGE pattern of seed embryo(1), 2-days after germinated embryo(2), E-callus(3,4,5). Arrow of the right indicate the change of band pattern.

When compared SDS-PAGE pattern of every stage, some proteins were decreased and other protein bands were similar to all stages (Fig. 4).

Difference of the protein band after SDS-PAGE separation between E-callus and NE-callus were observed at the third subculture of each lines. When compared to two callus lines, some bands were decreased in the E-callus line. Other bands were similar between E-callus and NE-callus(Fig. 5)

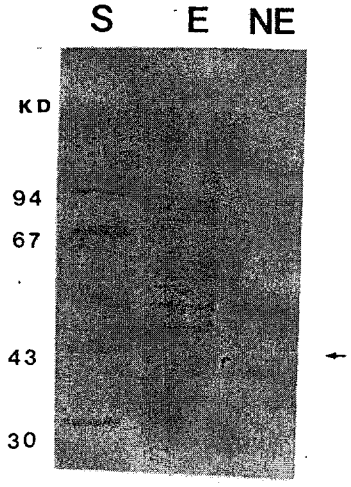

Fig. 5. SDS-PAGE pattern of the two callus lines cultured on N_6 medium at third subculture E: embryogenic callus line NE: non-embryogenic callus line s: protein standard. Arrow of the right indicate specific protein in E-callus.

Two-dimensional gel electrophoresis of proteins is a standard technique for the analysis of complex protein mixtures. The high resolution that can be obtained with this technique makes it well studied for comparative studies, e.g. of cells at different developmental stages. The separation obtained by the O'Farrell technique for two-dimensional electrophoresis of proteins(14) which applies isoelectric fo-

cusing in the first dimension and SDS gel electrophoresis in the second, is not achievable by one dimensional techniques. Combined with sensitive detection methods such as silver staining or radiolabeling, nanogram quantities of protein can be detected.

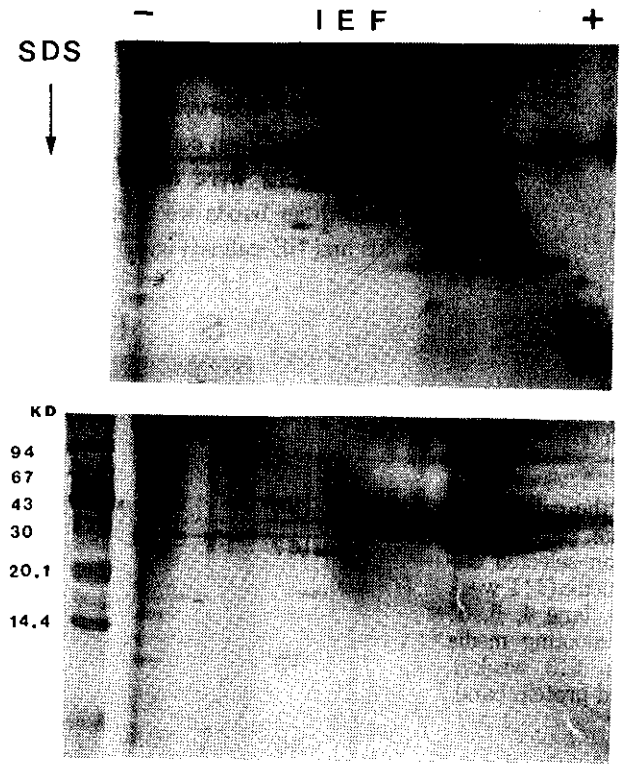

Fig. 6. Two-dimensional gel electrophoresis of E-callus and NE-callus protein was extracted from 30㎎ of E-callus and NE-callus. After centrifugation, 5 μl of the extract were applied to the first dimension.

In this experiment, we also used two-dimensional gel electrophoresis and silver staining methods. As a result of two-dimensional gel electrophoresis and silver staining, E-callus specific protein spots were observed(Fig. 6) The ability of plant regeneration and the type of differentiation (embryogenesis and organogenesis) considerably varied with genotypes used in rice cultures(1). In the experiment where the aim was to obtain efficient plant regeneration from rice callus cultures, embryogenic calli were initiated from mature seed and immature embryo(7) and root section (2,6). The results also show that efficient plant regeneration was attained from a long-term subculture by organogenesis as well as by somatic embryogenesis in an earlier report(2).

The mechanism of differentiation and organogenesis of rice callus are not clear in many respects. In this study, we have focosed attention on the behavior of total proteins. For this study, we used SDS-PAGE rutinly and two-dimensional gel electrophoresis mainly.

When compared the proteins of E-Callus and NE-callus, some different proteins were observed and serval proteins were increased. Some proteins were decreased or disapperared in E-callus. This result may indicate that

decrated or disappeared proteins were associated with somatic embryogenesis. So the other aspects should also be studied in detail.

Nevertheless, the protins of E-callus, some different proteins were observed and several proteins were increased. Some proteins were decreased or disappeared in E-callus. This result may indicate that decrated or disappeared proteins were associated with somatic embryogenesis. So the other aspects should also be studied in detail.

Nevertheless, the proteins and genes specifically associated with somatic embryogenesis have been difficult to isolate. In our experiment. two-dimensional protein gel comperisions have shown that the vast majority proteins appeared constant between E-callus and NE-callus.

Futher studies in progress will take advantage of the cDNA and antibody probes to clarify the specifc functions and the developmental roles of these genes and their proteins. This investigations will provide insights into mechanisms of embryogenic gene regulation and the molecular bases of developmental process.

摘　　要

본 硏究는 植物의 發生과 分化에 關한 生化學的 分子生物學的 究明을 위해 組織 發生 段階의 특이적인 遺傳子의 발현조절 기작과 役割을 硏究하기 위한 基礎 지식을 얻고자 遂行되었다. 벼를 實驗材料로 하여 현미로부터 callus를 誘道하고 이들 callus에서 여러가지 方法을 通해 embryogenic callus와 nonembryogenic callus를 分離해 somatic embryogenesis에 관련된 특수한 蛋白質을 찾고자 하였고 그 結果를 要約하면 다음과 같다.

지금까지의 報告에 의하면 재분화가 잘되는 callus와 그렇지 않은 callus간에는 여러가지 면에서 차이가 난다고 알려져 있다. 재분화가 잘되는 callus는 표면이 거칠고 흰색에 가까우며 組織이 단단하다. 반면에 재분화가 되지않는 callus는 表面에 윤기가 나며 단단하지 않다. 이러한 報告를 基礎로 하여 N_6 media에 1㎎/1의 2,4-D가 添加된 培地에서 2주 정도 callus를 유기시키고 유기된 callus는 다시 培養하였다. 7-8차 계대배양하면서 embryogenic callus와 nonembryogenic callus를 分離한 다음 이를 試料로 하여 protein을 抽出하여 SDS-PAGE 상에서 비교하여 보았다. 그 結果 두 callus간에 차이가 있음을 알 수 있었다. 7-8 차 계대배양한 두 種類의 callus로 부터 protein을 분리하여 two-dimensional gel 전기영동에 의해 비교하여 본 結果 몇 개의 蛋白質이 서로 다름을 알 수 있었다.

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