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Change in Levels of Endogenous Hormone and Detection of Adventitious Bud-Related Protein during Culture of Hybrid Poplar Explants

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Addition of plant growth hormones [0.01 mg/L NAA and 0.2 mg/L benzyladenine (BA)] to a woody plant medium stimulated the adventitious bud formation of poplar explants during culture. Endogenous IAA content increased rapidly at the initial culture stage and then decreased, being followed by rapid increment again at the late culture. But the content of trans-zeatin riboside (*t*-ZR) increased continuously during the culture. Cytoplasmic soluble proteins were analyzed by one- and two-dimensional SDS-PAGE. Increased amount of 40 kD band was detected by one-dimensional electrophoresis using Coomassie Blue staining during the culture and two distinctive proteins whose mol wt is 40,000 were detected by two-dimensional electrophoresis using autoradiography and these proteins were synthesized continuously prior to the adventitious bud formation. When the midvein segments were transferred to the actinomycin D-containing medium, the spots of adventitious bud-related proteins (ABRPs) did not disappeared but weakened in intensity. So, it is concluded that genes coding for the ABRPs are regulated to some degree at the transcriptional level. Also, they were not observed in BA-free medium, suggesting that these proteins be regulated by cytokinin, which made them possible to form the adventitious bud.

Keywords : poplar explant, adventitious bud, *in vivo* labeling, adventitious bud-related protein

Adventitious bud formation *in vitro* from various explant sources had been practiced for the purpose of micropropagation and recently as a means for the selection of somaclonal variants. Poplar species also have a relatively high capacity to form adventitious buds *in vitro* from excised leaves, stems, callus and roots (Winton, 1970; Venverloo, 1973; Chalupa, 1974; Ahuja, 1983). Adventitious buds have been induced *in vitro* on callus derived from leaves and stems of hybrid Poplar clones and the number of buds formed varied markedly from callus to callus within a clone and also between clones (Ostry, 1988).

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When the explants of poplar were cultured on the woody plant medium (WPM; Russel and McCown, 1986) supplemented with a relatively higher concentration of cytokinin than that of auxin, adventitious buds formed (Aartrijk and Blom-Barnhoorn, 1984; Douglas, 1985). It has been well known that auxin is necessary to initiate and maintain callus formation and the removal of auxin from the medium is required for subsequent regeneration in many plant species including woody plant (Reynolds, 1984; Cheema, 1989). Also it has been proposed that auxin maintains callus proliferation by repressing genes necessary for organogenesis and must be removed before the genes can be expressed for the shoot regeneration (Raghavan and Nagmani, 1983; Reynolds, 1984). Smigocki and Owens (1988) enhanced shoot organogenesis and zeatin level in transformed tobacco by increasing the synthesis of isopentenyl trans-

ferase, key enzyme in cytokinin biosynthesis. And the effect of cytokinin on the shoot regeneration was also demonstrated in poplar (Colemann and Ernst, 1989).

Since the first demonstration in poplar that the initiation of adventitious bud formation in culture was accomplished by interaction of two plant growth regulators, auxin and cytokinin, the basic mechanisms of these control of developmental processes must be understood. Although the primary functions of auxin and cytokinin at the gene level have not yet been clearly demonstrated, sufficient evidences have been accumulated to suggest that the hormonal responses are mediated through RNA and protein synthesis (Yasuda *et al.*, 1980; Zurfluh and Guilfoyle, 1982; Chen and Leisner, 1985; Theologis *et al.*, 1985; Crowell *et al.*, 1990).

Therefore, it would be necessary to detect and identify the proteins related to the adventitious bud organogenesis for the understanding of the mechanism of differentiation. Probably, the adventitious bud formation which seemed to be induced by cytokinin would be a result of the synthesis of the proteins indispensible to adventitious bud formation (Hasegawa *et al.*, 1979).

In this study, the contents of endogenous IAA and trans-zeatin riboside (*t*-ZR) and the pattern of main soluble proteins from poplar explants were investigated during *in vitro* culture.

MATERIALS AND METHODS

Plant material and growth conditions

Populus nigra var. *betulifolia*×*Populus trichocarpa* was grown in a greenhouse at 27± 3℃ under 16 h photoperiod. For the explants culture, midveins were excised, surface sterilized in 0.8% sodium hypochlorite for 3 min and washed several times in sterile distilled water. After the midveins were excised 0.5 cm long, their segments were cultured in the WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA at 28± 2℃ under 16 h photoperiod.

Enzyme immunoassay for IAA

The content of IAA was estimated according to the method described by Weiler *et al.* (1981). To determine the contents of extractable IAA, 0.5 g of midvein segments was ground in 1 mL 80% acetone with 10 mg butyl hydroxytoluene (BHT)/L, and the homogenate was centrifuged for 20 min at 10,000 *g*. The pellet was reextracted twice, and the supernatant was gathered together, concentrated to the aqueous phase, and the pH adjusted to 2.5. After extraction with equal volumes of ether three times, the ether phase was dried, and treated with an excess of etheral diazomethane. The methylated material was dissolved in 1 mL tris-bufferred saline (TBS; 25 mM Tris, 1 mM $MgCl_2$, 0.01 M NaCl, pH 7.5). For enzyme immunoassay, a 100 μL-aliquot of the sample was added to an antibody-coated microwell purchased at Idetek with 100 μL IAA-alkaline phosphatase tracer. After 3 h incubation at 4℃, the solution was decanted, and the wells were washed three times with washing buffer. The surface-bound enzyme activity was assayed by adding the substrate (*p*-nitrophenyl phosphate) followed by incubation at 37℃ for 1 h. The reaction was stopped with 5 M KOH, and the amount of auxin was determined by an ELISA reader at 405 nm.

Enzyme immunoassay for *t*-ZR

The content of *t*-ZR was estimated according to the method described by Weiler (1980). The principle of immunoassay of *t*-ZR was the same above mentioned except the coated well with *t*-ZR antibody which was also purchased at Idetek.

Extraction of soluble proteins

Midvein segments of poplar were collected and transferred to 3 vol of ice-cold extraction buffer [0.2 M Tris-HCl, pH 8.5, 1 M sucrose, 56 mM 2-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride (PMSF)]. The segments were homogenized on ice using a glass tissue grinder. The homogenate was centrifuged for 10 min at 11,000 *g* to obtain a supernatant expected to contain predominantly soluble proteins. The protin concentration of the supernatant was determined using BSA as a standard.

In vivo labeling of proteins

Each midvein segments were sampled at indicated

intervals and incubated in liquid medium containing 100 μCi/mL of L-[^{35}S] methionine (spec. act. 1000 Ci/mmol; Amersham) on a rotary shaker for 3 h. At the end of labeling period, proteins were extracted as decribed above. Proteins were precipitated from the supernatant fluid by addition of an equal vol of 20% (w/v) trichloroacetic acid (TCA) for 30 min in ice. The precipitate was then washed 3 times with ethanol:ether (1 : 1) to remove TCA. Then, the homogenization buffer mentioned above was added to the pellet. An aliquot (2 μL) of the protein extract was precipitated by 10% TCA on Whatman GF/C filter. The filter was washed with 5% TCA and then with acetone. After the filter drying, its radioactivity was determined by liquid scintillation spectrometry.

Gel electrophoresis of proteins

One-dimensional SDS-PAGE was performed according to Laemmli (1970). Eighty μg of protein was subjected to each lane of the 5 to 20% gradient gels in the case of Coomassie Blue staining and 10 to 15% gradient gels for autoradiography. Standard mol wt markers were as follows: BSA (66,000), ovalbumin (45,000), trypsinogen (24,000), β-lactoglobulin (18,400) and lysozyme (14,300) to determine the mol wt of proteins. Two-dimensional SDS-PAGE with isoelectric focusing (IEF) was carried out according to O'Farrel (1975) with modifications. Eighty μg of labeled protein was loaded onto the alkaline end of IEF gels containing 4.8% (w/v) acrylamide, 10 M urea, 3% (v/v) Nonidet P-40, and 2.4% (v/v) ampholines (pH 4-6.5: pH 3-10=5 : 1). Then the gels were covered with protection solution (10% glycerol, 2% ampholine), followed by IEF for 16 h at 700 V. After IEF, the gels were equilibrated in equilibration buffer [10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.8] for less than 30 min, and loaded onto SDS-polyacrylamide gels.

The second dimension was prepared as follows: the separating gel consisted of a 10 to 15% gradient of acrylamide and a stacking gel contained 4% acrylamide. After electrophoresis, for fluorography, the gels were fixed and dehydrated and soaked in 20% 2,5-diphenyloxazole (PPO) containing acetic acid for 2 h, then washed and the gels were dried onto Whatman 3MM paper under reduced pressure and exposed to X-ray film at −70℃ for 3 d.

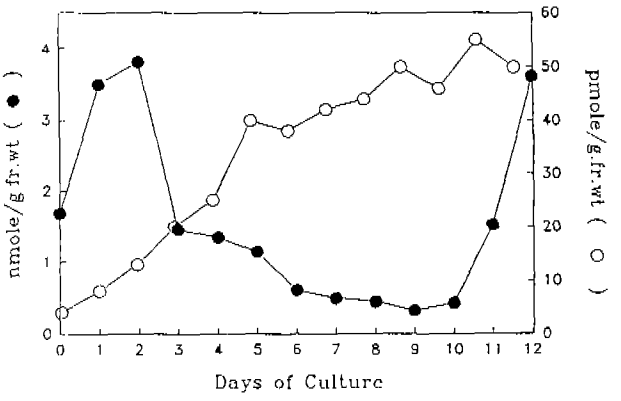

Fig. 1. IAA (●) and *t*-ZR (○) contents in the midvein segments of poplar forming adventitious bud. Midvein segments were cultured in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA. Values were averages from three independent experiments.

RESULTS

IAA and *t*-ZR contents in midvein segments of poplar during culture

Endogenous IAA content increased at a high rate and then decreased rapidly at the initial culture stage, followed by rapid increment again at the late culture stage. In the case of *t*-ZR known as active cytokinin in poplar (Letham and Palni, 1983), rapid increment occurred at the initial culture stage, and then its content increased slowly until the adventitious bud was formed (Fig. 1).

Changes in pattern of protein synthesis during culture in one-dimensional SDS-PAGE

As shown in Fig. 2, gradual accumulation of the 40 kD band was significant by Coomassie Blue staining during culture, while there was no 40 kD band in the adventitious root-inducing medium (Fig. 3). The 40 kD band reached its maximum concentration after 10 d of culture. Because of low resolution in one-dimensional SDS-PAGE, it is possible that the band includes a number of different proteins. Also, the protein pattern of 0 d of culture using ^{35}S-methionine was similar with 10 d of culture ,which meant that the band clearly appeared before the cul-

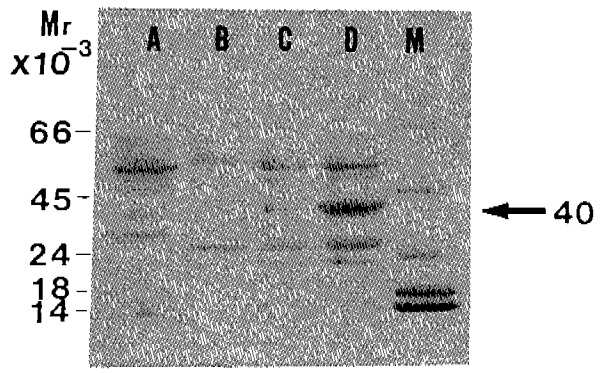

Fig. 2. Protein patterns analyzed by one-dimensional SDS-PAGE stained with Coomassie Blue in the midvein segments of poplar forming adventitious bud according to the culture time for 0 d (A), 6 d (B), 8 d (C), and 10 d (D). Mol wt markers were indicated as M. Eighty μg of protein was loaded on each lane. Arrow indicates the protien band increased in concentration during the culture. Midvein segment were cultured in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA.

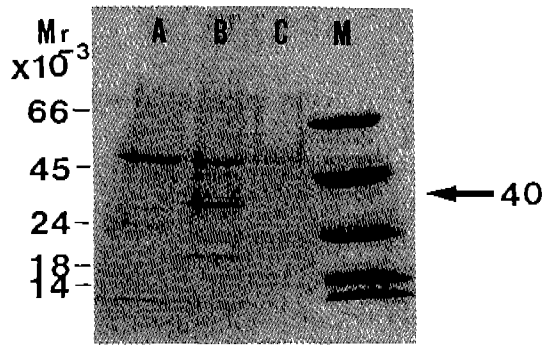

Fig. 3. Protein patterns analyzed by one-dimensional SDS-PAGE stained with Coomassie Blue in the midvein segments of poplar forming adventitous bud according to the medium composition. A, 0 d; B, 0.01 mg/L NAA and 0.2 mg/L BA after 12 d of culture; C, NAA only (0.1 mg/L) after 12 d of culture. Mol wt markers were indicated as M. Eighty μg of protein was loaded on each lane. The arrow indicates the protien band increased in concentration during the culture.

ture (Fig. 4).

Two-dimensional distribution of newly synthesized proteins

The 40 kD band mentioned above seemed to have adventitious bud-related proteins. For the detection of adventitious bud-specific proteins, the midvein segments after 7 d of culture were selected, at which

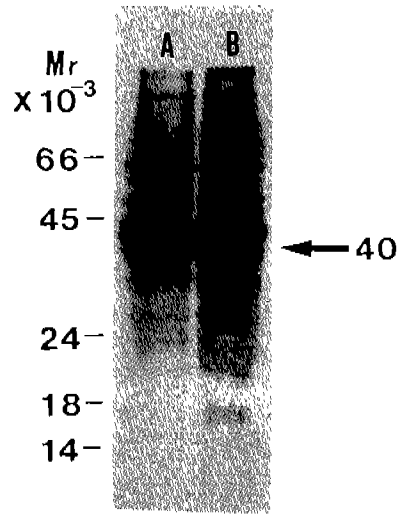

Fig. 4. Newly synthesized proteins analyzed by one-dimensional SDS-PAGE in the midvein segments of poplar forming adventitious bud from 0 d (A) and 10 d (B) of culture. Midvein segments were cultured in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA.

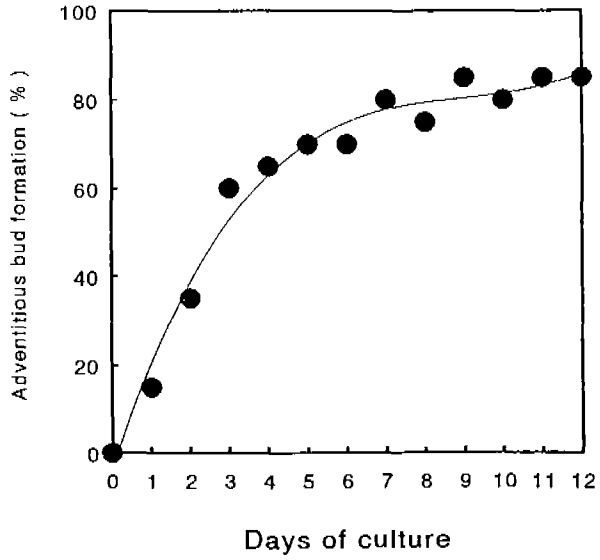

Fig. 5. Adventitious bud formation when the poplar midvein segments cultured in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA were transferred at each day to the same medium with actinomycin D (2 μM). Adventitious bud formation was calculated as follows.

$$\frac{\text{Number of adventitious bud-formed segments}}{\text{Number of total segments}} \times 100$$

The values were averages of adventitious bud-formed segments from three independent experiments with 10 segments each.

time the segments acquired an ability enough to form the adventitious bud against the actinomycin

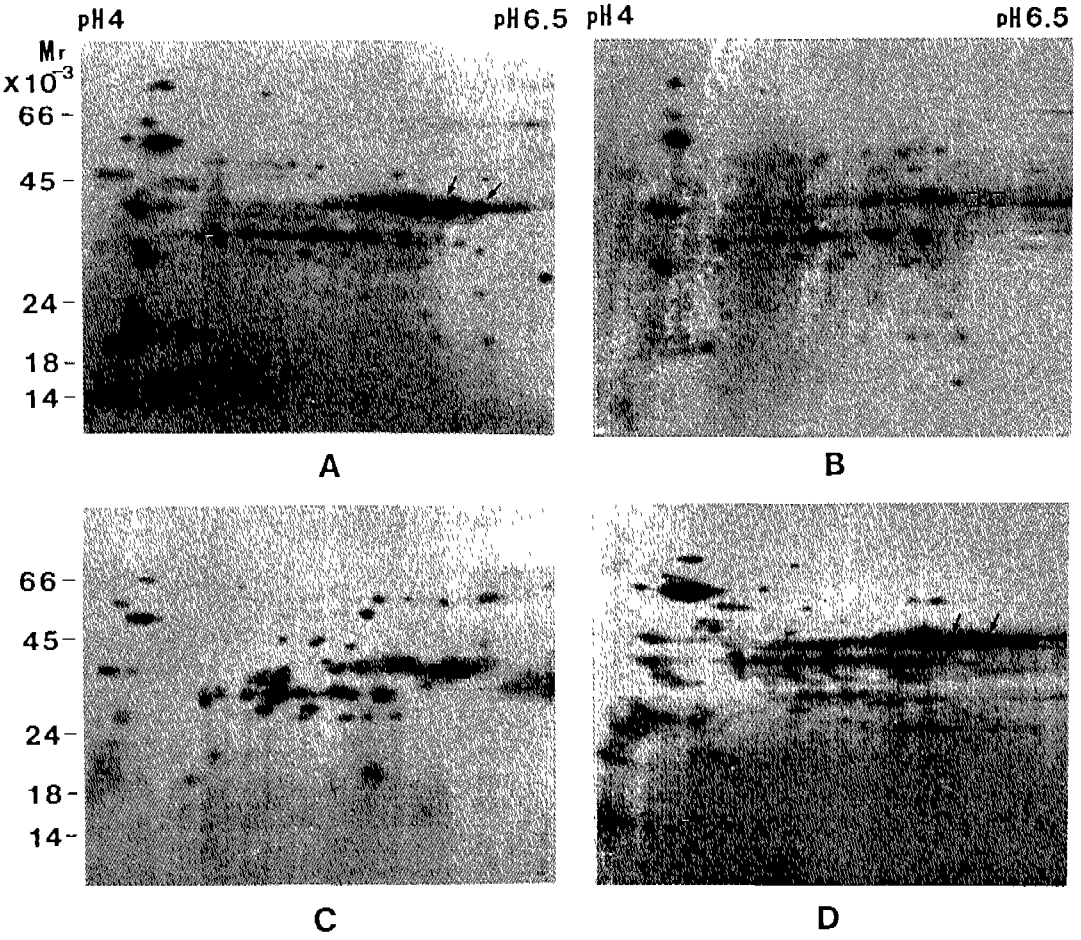

Fig. 6. Newly synthesized proteins analyzed by two-dimensional SDS-PAGE in the midvein segments of poplar forming adventitious bud cultured for 7 d in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA (A) with 0.01 mg/L NAA only (B) and in the midvein segments of poplar not forming adventitious bud cultured for 7 d in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA (C) and in the midvein segments of poplar forming adventitious bud at 0 d of culture (D).

D, an RNA synthesis inhibitor (Fig. 5). That is to say, sufficient mRNA for adventitious bud-related proteins were supposed to have been synthesized and accumulated. Segments were cultured on normal medium and BA-free medium for 7 d, and their protein extracts were subjected to two-dimensional SDS-PAGE. Comparing two-dimensional gel fluorography detected two distinctive proteins whose mol wt was 40 kD in the midvein segments cultured on normal medium, but not in those cultured on BA free-medium (Fig. 6A, B). The difference could not be discriminated by one-dimensional SDS-PAGE of ^{35}S-methionine-labeled proteins, because the mol wt of the ABRPs was the same with the rest of the proteins located in the same line.

And we used the midvein segments of poplar not forming adventitious bud (*Populus deltoides*×*Populus trichocarpa*) to confirm that they were not the proteins formed simply by cytokinin treatment but the real ABRPs by performing the two-dimensional SDS-PAGE. As supposed, none of these two distinctive proteins was observed (Fig. 6C). To know whether the ABRPs appear before the begining of culture, midvein segments of 0 d culture were selected and investigated. From its result, ABRPs were preexistent before the culture (Fig. 6D).

In the case of initial culture stages, most of the protein spots including the ABRPs increased in intensity, but decreased at the late culture stage (Figs. 7 and 8). By transferring the midvein segments from the normal medium to actinomycin D-containing medium, we realized that the activities of two pro-

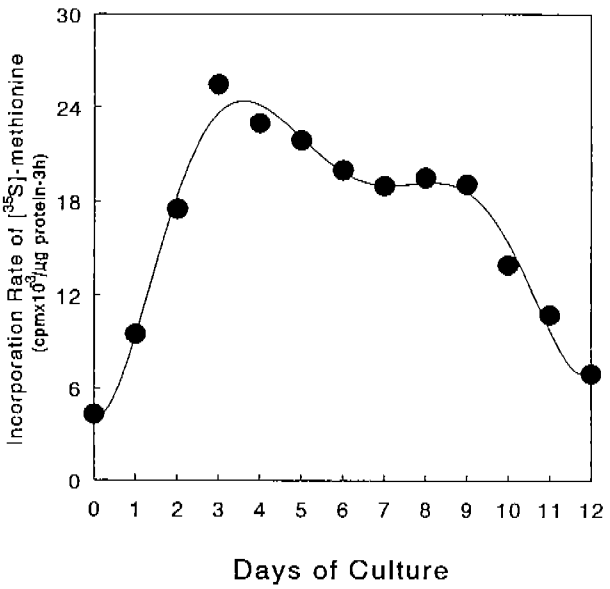

Fig. 7. Incorporation rate of [^{35}S]-methioninc *in vivo*. Each midvein segment was labeled for 3 h.

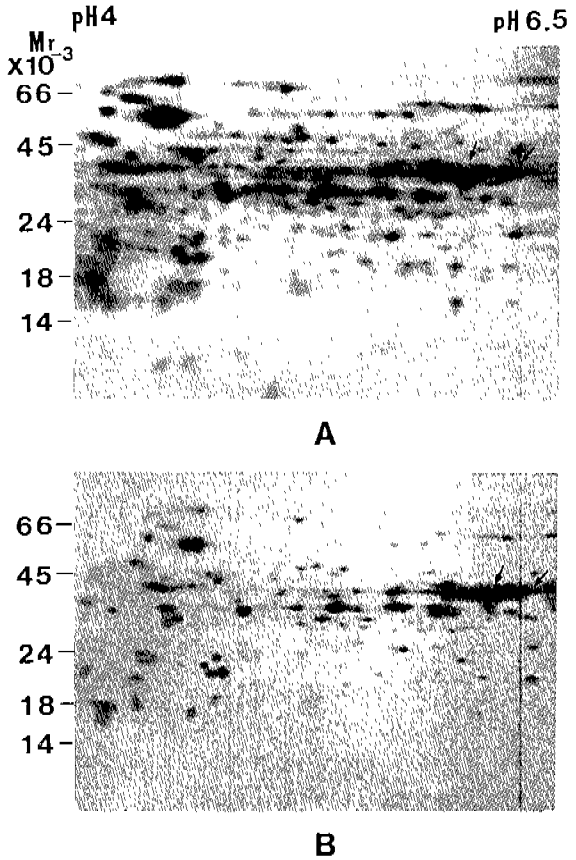

Fig. 8. Newly synthesized proteins analyzed by two-dimensional SDS-PAGE in the midvein segments of poplar forming adventitious bud cultured for 3 d (A) and 12 d (B). Midvein segments were cultured in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA.

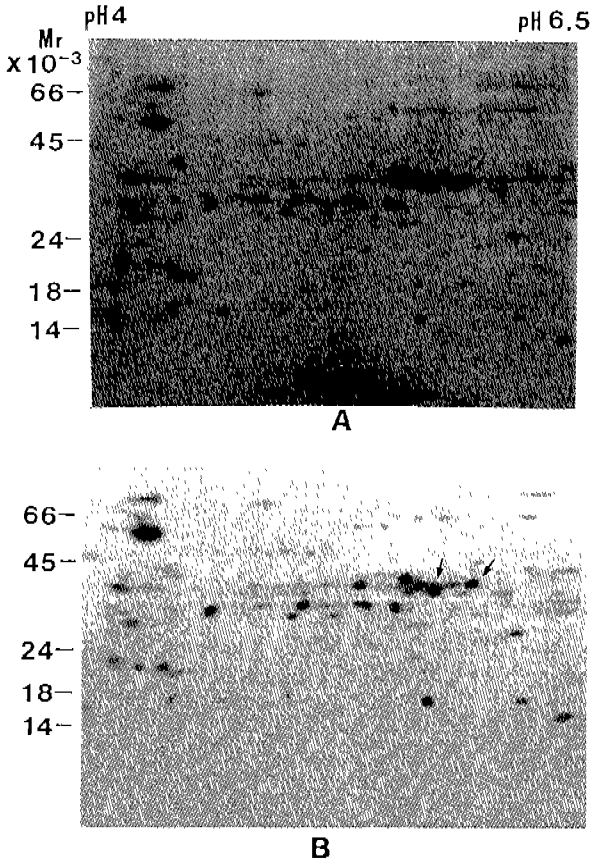

Fig. 9. Newly synthesized proteins analyzed by two-dimensional SDS-PAGE in the midvein segments of poplar forming adventitious bud. Actinomycin D (2 μM) was treated for the last 1 d of 3 d of culture (A) and for the last 2 d of 3 d of culture in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA (B).

teins were not fully repressed but decreased to some degree in proportion to the period of the actinomycin D treatment (Fig. 9). Actually, the actinomycin D treatment affected the incorporation rate significantly. After the midvein segments were cultured for 3 d, incorporation rate of the ^{35}S-labeled methionine (100 μCi/mL) to the proteins for 3 h was 25,500 cpm/μg protein, but in the case of the actinomycin D treatment for the last 1 d of 3 d, its incorporation rate decreased to 14,124 cpm/μg protein and for the last 2 d of 3 d, its incorporation rate was 5,930 cpm/μg protein (data not shown).

One distinguishable fact was the continuous synthesis of ABRPs in spite of transferring the midvein segments from the adventitious bud-inducing medium after 7 d culture to adventitious root-inducing medium for 1 d culture, the spots of most proteins

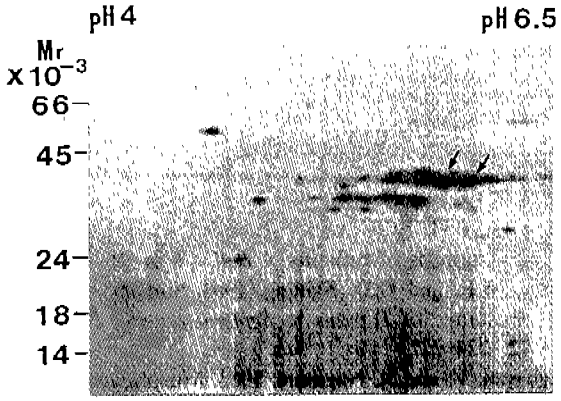

Fig. 10. Newly synthesized proteins analyzed by two-dimensional SDS-PAGE in the midvein segments of poplar forming adventitious bud cultured in WPM supplemented with 0.01 mg/L NAA and 0.2 mg/L BA for 7 d and then transferred to the WPM supplemented with 0.1 mg/L NAA only for 1 d.

decreased significantly in intensity but those of ABRPs still remain in intensity (Fig. 10). Actually, the adventitious buds formed normally in spite that the midvein segments were transferred to root-inducing medium (data not shown).

DISCUSSION

The changes of endogenous hormone concentration showed that the auxin was required for callus initiation and proliferation which enhanced the competence of regeneration, but must be exchanged for cytokinin before differentiation would occur (Douglas, 1985; Reynolds, 1986). And it was likely to be accompanied by the synthesis of ABRPs.

The 40 kD band detected by Coomassie Blue staining seemed to have ABRPs. To verify this assumption, cytoplasmic soluble proteins synthesized during the culture stages were analyzed by two-dimensional SDS-PAGE of ^{35}S-methionine-labeled proteins. And also we could lead to the expectation that if the synthesis of the ABRPs required for the adventitious bud formation were enhanced or induced by cytokinin, we may expect a difference in the protein population between cytokinin-treated and cytokinin-untreated midvein segments. From their results, we have known that the ABRPs were preexistent and increased during the culture by *de novo* synthesis. By Coomassie Blue staining, the 40 kD band was observed after 6 d of culture and increased in concentration, whereas in two-dimensional gel as well as one-dimensional gel using autoradiography, the 40 kD proteins were clearly observed before the culture and observed continuously. This means fresh midvein segment contains a store of presynthesized adventitious bud-related RNAs and proteins (Hussey and Wakeley, 1994). The late appearance of 40 kD band by Coomassie Blue staining would be the results of both the less sensitive staining method and the reflection of the current protein composition, whereas the autoradiography was more sensitive and it provides current information on which proteins are being translated (Choi and Sung, 1984). Therefore, it was certain that the 40 kD band which had not appeared in Coomassie Blue staining showed clearly in fluorography.

Cytokinin synthesis and the ABRPs formation were deeply correlated. Namely, the preexistent ABRPs were synthesized continuously until the late culture stage corresponding to the cytokinin synthesis, but the ABRPs were not observed in BA-free medium, suggesting that these proteins be regulated by cytokinin.

By the transfer of the midvein segments from normal medium to the actinomycin D-containing medium, the spots of the ABRPs were weakened in intensity in proportion to the period of the actinomycin D treatment. And so, this fact indicates that the genes coding for the ABRPs are being synthesized continuously during culture period and regulated to some degree at the transcriptional level. Yet, it is not clear whether the regulation also occurs at the translational level (Chen *et al.*, 1987).

One of the striking results of this study was the formation of adventitious bud when the midvein segments were transferred to adventitious root-inducing medium after 7 d of culture. This means that the midvein segments had the competence of adventitious bud organogenesis. The intensity of the ABRPs was not much weakened than that of other proteins in adventitious root-inducing medium where the radioactivity of most proteins decreased, and so it must be certain that continuous synthesis of the mRNAs coding for the ABRPs have occurred or the mRNAs were very stable. But it would be more probable that the mRNA had already had the stability because the spots of the ABRPs did not disappeared even when the actinomycin D was treated

for a long time (Fig. 9).

For the maximum formation of adventitious bud, a longer culture period —more than 12 d— was required, which was perhaps the factor responsible for the accumulation of the ABRPs to a critical level required for adventitious bud induction. And it would be necessary to identify and characterize the ABRPs for a further study about adventitious bud differentiation.

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포플라의 잎 절편 培養時 內生호르몬 含量의 變化와 부정아 形成에 關與하는 蛋白質의 檢出

宋 在 眞*† · 金 明 苑[1] · 康 榮 熹
延世大學校 理科大學 生物學科, [1]文理大學 生命科學科

적 요

식물 호르몬인 NAA와 benzyladenine(BA)를 각각 0.01 mg/L와 0.2 mg/L의 농도로 목본 식물 배지에 첨가한 다음 포플라의 잎 절편을 배양하여 부정아를 형성하였다. 이때 내생 IAA의 양은 배양 초기에 급속히 증가하다가 감소하였으며 배양 후기에 다시 빠르게 증가하였다. 그러나 포플라에서 활성이 있는 사이토키닌으로 알려져 있는 *trans*-zeatin riboside (*t*-ZR)는 배양기간 동안 계속 증가하였다. 부정아로의 분화시 수용성 단백질의 양상에 변화가 있는지를 알아 보고자 1차원 전기영동을 한 결과 분자량이 40,000인 단백질의 양이 증가하였다. 동위원소를 사용한 2차원 전기영동시에는 분자량이 40,000인 두 개의 특징적인 단백질이 검출되었고 이들 단백질은 부정아가 형성되기 전까지 지속적으로 합성되었다. 중록절편을 actinomycin D가 들어 있는 배지로 옮겨 주었을 때 부정아 형성에 관여하는 단백질은 사라지지는 않았으나 그 강도는 약해졌다. 따라서 부정아 형성에 관여하는 단백질을 암호화하는 유전자들은 전사단계에서 어느 정도 조절받는 것으로 보이며 BA가 없는 배지에서는 전혀 나타나지 않는 것으로 보아 이들 단백질은 사이토키닌에 의하여 조절 받으면서 부정아 형성에 관여하는 것으로 생각된다.

주요어 : 포플라 잎 절편, 부정아, *in vivo* labeling, 부정아 관여 단백질

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