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In Vitro Phosphorylation of Proteins in IAA-Treated Primary Roots and Coleoptiles in *Zea mays*

Ryoichi Kato[†], Sachiko Takatsuna[†], Tsuyoshi Wada[†], Yumi Narihara[†] and Takashi Suzuki[‡]

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Abstract

Five-mm sections of elongation zones which were cut from primary roots or coleoptiles of *Zea mays* were incubated for designated periods with various concentrations of IAA. In vitro protein phosphorylation in the soluble fractions prepared from these sections was analyzed by SDS-PAGE. The phosphorylation of proteins in sections of primary roots incubated for 20 or 40 min in the presence of 10^{-7} M IAA was greater than that in the sections incubated in the absence of IAA. The phosphorylation of proteins in sections of primary roots incubated for 20 min or 2 h in the presence of 10^{-8}, 10^{-7} or 10^{-6} M IAA was higher than that in the sections incubated in the absence of IAA. An incubation for 20 min or 2 h with 10^{-4} M IAA inhibited the phosphorylation of proteins in sections of primary roots. The growth of the sections of primary roots incubated for 2 h in the presence of 10^{-7} M IAA or higher concentrations was lower than that of the sections incubated in the absence of IAA. These results suggest that the phosphorylation of proteins which was increased by IAA treatment is independent of an inhibition of the growth induced by IAA in maize primary roots. The phosphorylation of proteins in sections of coleoptiles incubated for 10, 20 or 40 min in the presence of 10^{-7} or 10^{-5} M IAA was equal to that in the sections incubated in the absence of IAA. These results show that IAA regulates growth of maize coleoptiles in no relation with phosphorylation of the proteins and that IAA regulates growth

[†]Biology Laboratory, Faculty of Education, Yamagata University, Yamagata, 990–8560 Japan

[‡]Science Laboratory, Faculty of Education, Yamagata University, Yamagata, 990–8560 Japan

of maize mesocotyls via phosphorylation of the proteins, comparing with earlier results [Kato et al. (1996) *Plant Cell Phsiol.* 37:667].

Abbreviations

EGTA, ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid; IAA, indole-3-acetic acid; MAPK, mitogen-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Key words

Coleoptile—IAA treatment—Primary root—Protein phosphorylation—*Zea mays.*

1 Introduction

Auxins control many aspects of plant growth and development. However, the mechanism by which auxin regulates diverse physiological processes is not clear. Some investigations have been reported regarding protein phosphorylation in relation with physiological responses in higher plants (Kato et al. 1983, 1984, Ranjeva and Boudet 1987, Kato and Fujii 1988, Briggs and Huala 1999, Harmon et al. 2000, Meszaros and Pauk 2000, Rock 2000, Leon et al. 2001, Muday and DeLong 2001). Auxin action has been associated with changes in the phosphorylation status of nuclear proteins (Murray and Key 1978, Schäfer and Kahl 1981), of membrane-located proteins (Morré et al. 1984, Varnold and Morré 1985), of ribosomal proteins (Pérez et al. 1990) and of soluble proteins (Veluthambi and Poovaiah 1986, Reddy et al. 1987). It has been indicated a possibility that auxin influences activation of a nuclear protein kinase CK2 (Hidalgo et al. 2001), expression of gene of a calmodulin like domain protein kinase (Davletova et al. 2001) and MAPK cascades (Mizoguchi et al. 1994, Nakashima et al. 1998, Morris 2001, Wrzaczek and Hirt 2001, Zwerger and Hirt 2001) which transduce a large variety of external signals in plants (Machida et al. 1997, Meskiene and Hirt 2000, Tena et al. 2001). Further, Chono et al. (1998) showed a protein kinase gene responsive to auxin and gibberellins in cucumber hypocotyls.

Kato et al. (1996) reported that IAA promotes cell elongation via protein phosphorylation that depends on calmodulin-dependent protein kinase and protein kinase C in maize mesocotyls. In this report, we determined which auxin affects phosphorylation of proteins in primary roots and coleoptiles of

Zea mays.

2 Materials and Methods

Plant materials—The procedures used were previously described by Kato et al. (1991) and Kato et al. (1995). Caryopses of *Zea mays* L., cv. Golden Cross Bantam 70 (Sakata Seed Co., Yokohama, Japan) were immersed in running tap water for 36 h in darkness. The imbibed caryopses were planted, embryo-upwards, on agar-solidified water (0.4% agar) in containers (286 × 190 × 50 mm^3). The containers were covered with a black vinyl sheet and kept in darkness at 25 ± 0.5°C. After an incubation for 24–36 h, the primary roots with 20–25 mm long were selected. Five-mm sections (elongation zone), starting from 1 mm below the root tip, were exactly cut from the roots. On the other hand, the imbibed caryopses were pierced on the surface of moist vermiculite in containers and covered with a vinyl sheet. The seedlings were allowed to elongate at 25 ± 0.5°C in the dark. Five-mm sections (elongation zone) were cut from 72-h-old coleoptiles starting from 5 mm below the tip. Primary leaves were discarded. Handling of the caryopses and other operations were performed under a very weak green light (intensity, less than 100 μW cm^{-2}; transmittance, 490–580 nm; peak, 530 nm), which was obtained by filtering the light from a fluorescent lamp through two sheets of green cellophane and two sheets of blue cellophane.

Phosphorylation of proteins—The procedures were those described by Kato et al. (1996). A group consisting of 30 sections of primary roots or coleoptiles was put into Petri dish (diameter, 51 mm; height, 22 mm) with 1 ml of 20 mM potassium-phosphate buffer (K-buffer; pH 5.8) containing various concentrations of IAA (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were incubated in darkness at 25 ± 0.5°C for various periods with shaking at 60 rpm. The sections were then rinsed twice with distilled water. The following procedures were performed at 4°C or in an ice-bath. The sections were homogenized by use of an ice-chilled mortar and pestle with 100 μl of 30 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM β-mercaptoethanol, 2 mM $MgCl_2$ and 8% sucrose (Buffer A). The above operations were performed under the weak green light (described above) and the following procedures were performed under the light condition. The mortar and pestle were rinsed three times with 100 μl of Buffer A and the rinses were combined with the homogenate. The homogenate was centrifuged at 3,000 ×g for 20 min. The resulting supernatant was then centrifuged at 85,000 ×g for 90 min. The supernatant obtained after the second centrifugation was mixed with 1.6 ml (in the case of primary roots) or 1.8 ml (in the case of

coleoptiles) of Buffer A. The supernatant was applied to a Centricon (Amicon, Inc., Beverly, U.S.A., Model: SR-10), which was centrifuged at 5,000 ×g for 90 min. A further 1.7 ml (in the case of primary roots) or 1.8 ml (in the case of coleoptiles) of Buffer A was added to the concentrated supernatant. The procedure was repeated twice. The final centrifugation was at 5,000 ×g for 120 min (in the case of primary roots) or 90 min (in the case of coleoptiles). Approximately 300 μl of concentrated and demineralized supernatant was finally obtained as a sample. The amount of proteins in each sample was determined by use of a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, California, U.S.A., Model: Kit II), with Bovine serum albumin as a standard.

The reaction mixture (final volume, 100 μl) contained 3.3×10^{-2} μM[γ-^{32}P]ATP (3.0–6.0 $\times$ 10^6 cpm pmol^{-1}; Radiochemical Centre, Amersham, Bucks., U.K.) and 80 μl of the sample. In some experiments, a cyclic nucleotide, a calmodulin antagonist or a protein kinase inhibitor was added to the mixture. The reactions were initiated by the addition of [γ-^{32}P]ATP, and were allowed to proceed for 4 min (in the case of primary roots) or 1 min (in the case of coleoptiles) at 30°C. Each reaction mixture was then combined with 25 μl of a Stop solution [50 mM Tris-HCl (pH7.0) containing 5 mM EDTA, 15% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 25% glycerol and 0.05% Bromophenol Blue] and heated at 95°C for 5 min. Each volume of the reaction mixture, containing exactly 30 μg (in the case of primary roots) or 20 μg (in the case of coleoptiles) of protein, was applied to lanes of SDS-PAGE. A portion of the reaction mixture was subjected to SDS-PAGE (concentration of polyacrylamide, 11%) according to the procedure of Laemmli (1970). The proteins in the gel were stained with Coomassie brilliant blue and the gel was dried and exposed to Kodak X-Omat AR Film (Eastman Kodak Co., Rochester, New York, U.S.A.) for 12–24 h. The dried gel and the exposed film were severally scanned by a scanner (Seiko Epson Co., Suwa-shi, Nagamo, Japan, Model: GT-9000). Total amounts of the gradations of the blue in the gel and of the darkness on the film were calculated with a personal computer and a software package for image processing (NIH image) at each lane. We confirmed that the same quantity of protein was present in each lane of the gel (data not shown).

Measurement of elongation of primary roots—A group consisting of 30 sections of primary roots was put into the Petri dish with 1 ml of K-buffer containing 10^{-12}〜10^{-4} M IAA. The sections were floated on the K-buffer and incubated in darkness at 25 ± 0.5°C for 2 h with shaking at 60 rpm. The length of each section was measured by use of a travelling microscope (Pika Seiko, Ltd., Tokyo, Japan, Model: PRM-D2).

3 Results

Phosphorylation of proteins in primary roots—Sections of elongation zones of primary roots were incubated for 10, 20 or 40 min in the presence or absence of 10^{-7} M IAA. At least nine phosphorylated proteins, with molecular masses of 53, 42, 41, 35, 33, 31, 21.5, 18.5 and 16.5 kDa, were detected in the soluble fraction prepared from the sections (Fig. 1). The extent of phosphorylation of these proteins in the sections incubated for 20 or 40 min with IAA was higher than that in the sections incubated without IAA (Fig. 1). But, a 10-min incubation with IAA had no effect on the phosphorylation of proteins (Fig. 1).

The root sections were incubated for 20 min in the presence of 10^{-8}∼10^{-4} M or 10^{-12}∼10^{-8} M IAA. The extent of phosphorylation of proteins in the soluble fraction prepared from the sections incubated with 10^{-8}, 10^{-7} or 10^{-6} M IAA was higher than that in the fraction from the sections incubated without IAA (control) (Fig. 2, 3). The extent of phosphorylation of proteins in the sections incubated with 10^{-12}∼10^{-9} M or 10^{-5} M IAA was nearly equal to that of the control (Fig. 2, 3). The phosphorylation of proteins in the sections incubated with 10^{-4} M IAA was lower than that of the control (Fig. 2).

The root sections were incubated for 2 h in the presence of 10^{-8}∼10^{-4} M IAA. The extent of phosphorylation of proteins in the soluble fraction prepared from the sections incubated with 10^{-8}, 10^{-7} or 10^{-6} M IAA was higher than that in the fraction from the sections incubated without IAA (Fig. 4). IAA at 10^{-4} M had the significant effect on the decrease of the phosphorylation of proteins (Fig. 4).

Phosphorylation of proteins in coleoptiles—Sections of elongation zones of coleoptiles were incubated for 10, 20 or 40 min in the presence or absence of 10^{-7} or 10^{-5} M IAA. Nine phosphorylated proteins, similar to proteins detected in primary roots, were detected in the soluble fraction prepared from the sections (Fig. 5, 6). The extent of phosphorylation of these proteins in the sections incubated with IAA was equal to that in the sections incubated without IAA (Fig. 5, 6).

Elongation of primary roots—Sections of elongation zones of primary roots were incubated for 2 h in the presence of 10^{-12}∼10^{-4} M IAA. The growth rates of the sections incubated with 10^{-7} M IAA or higher concentrations were lower than the rates of the sections incubated without IAA (Fig. 7). The inhibition of growth by IAA became stronger as the IAA concentration increased (Fig. 7).

All experiments were repeated three times with essentially identical results.

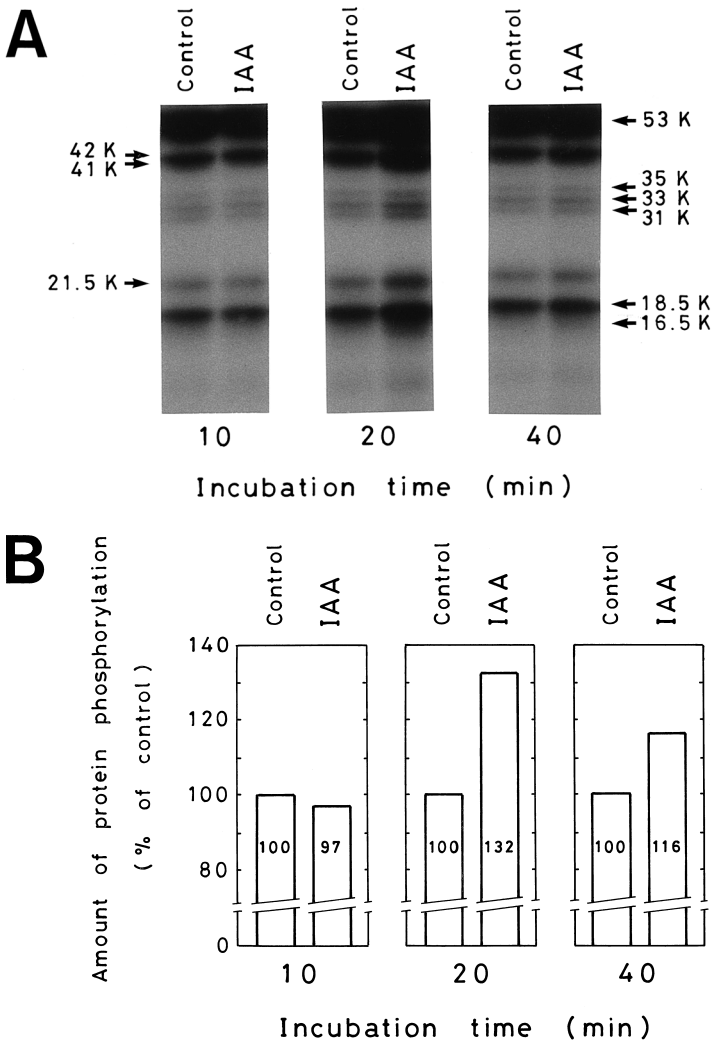

Fig. 1. Phosphorylation of proteins in sections of maize primary roots incubated for 10, 20 or 40 min in the presence of 10^{-7} M IAA. Thirty sections of the roots were incubated for 10, 20 or 40 min in the presence or absence of 10^{-7} M IAA. The sections were homogenized with an ice-chilled mortar and pestle. The homogenate was centrifuged at 3,000 ×g for 20 min. The obtained supernatant was centrifuged at 85,000 ×g for 90 min. The resulting supernatant was concentrated and demineralized with a Centricon, and each sample was obtained. The sample was incubated with [γ-^{32}P]ATP for 4 min at 30°C. After stopping the reaction by the addition of a Stop solution, just 30 μg proteins were separated by SDS-PAGE (concentration of polyacrylamide, 11%). The radioactivity of the proteins was determined by autoradiography. The exposed film was scanned with a scanner and the amount of the gradation of the darkness on the film was calculated with a personal computer at each lane. **A**: Autoradiography. The positions of 53 (53K), 42 (42K), 41 (41K), 35 (35K), 33 (33K), 31 (31K), 21.5 (21.5K), 18.5 (18.5K) and 16.5 (16.5K) k Da proteins are indicated by arrows. **B**: Total amounts of the gradations of the darkness on the film. Numbers in the bars indicate the relative extent of the amount.

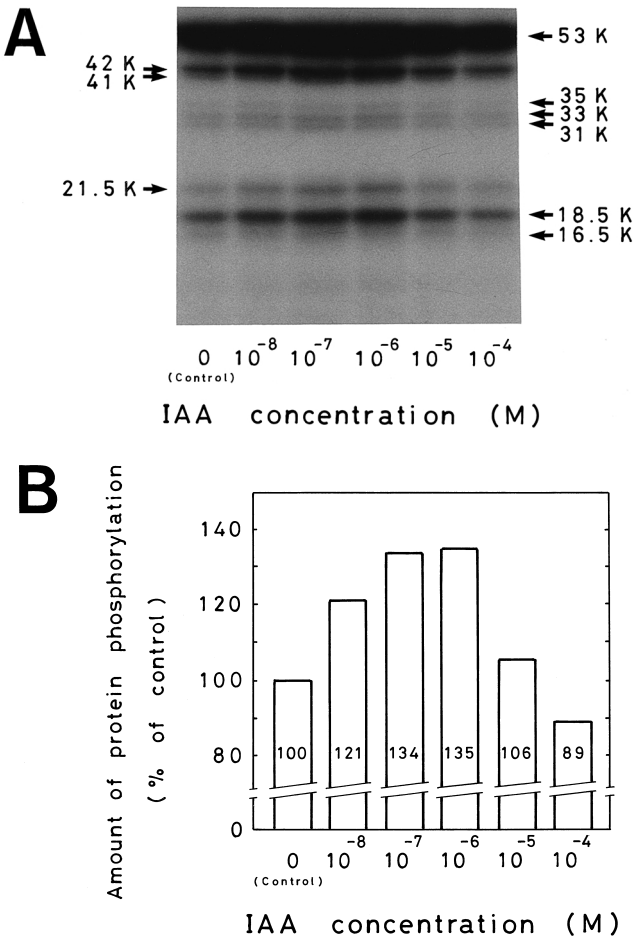

Fig. 2. Phosphorylation of proteins in sections of maize primary roots incubated for 20 min in the presence of 10^{-8}∼10^{-4} M IAA. Thirty sections of the roots were incubated for 20 min in the presence of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M IAA. The procedures were the same as those described in the legend of Fig. 1. **A**: Autoradiography. The symbols are the same as indicated in Fig. 1. **B**: Total amounts of the gradations of the darkness on the film. Numbers in the bars indicate the relative extent of the amount.

4 Discussion

It is well known that auxin regulates many physiological processes. However, these roles at the molecular level are not well understood. We previously reported phosphorylation of proteins relating to auxin-induced elongation in the sections of elongation zones of maize mesocotyls (Kato et al. 1996) whose growth is promoted by auxin (Vanderhoef and Brigges 1978, Walton and Ray 1981, Yahalom et al. 1988). This report presented evidence for the role of auxin in protein phosphorylation in the sections of elongation zones of maize primary roots whose growth is inhibited by auxin (Bonner and Koepfli 1939,

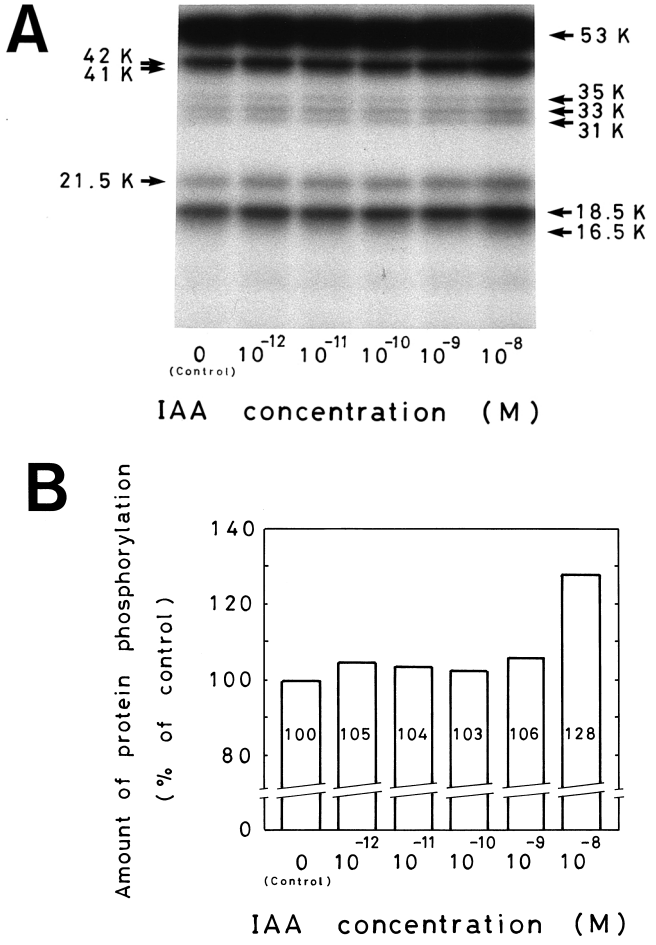

Fig. 3. Phosphorylation of proteins in sections of maize primary roots incubated for 20 min in the presence of 10^{-12}∼10^{-8} M IAA. Thirty sections of the roots were incubated for 20 min in the presence of 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} or 10^{-8} M IAA. The procedures were the same as those described in the legend of Fig. 1. **A**: Autoradiography. The symbols are the same as indicated in Fig. 1. **B**: Total amounts of the gradations of the darkness on the film. Numbers in the bars indicate the relative extent of the amount.

Pilet and Saugy 1987) and of maize coleoptiles whose growth auxin promotes (Kato and Fujii 1982, Karcz et al. 1990, Cleland 1991).

The phosphorylation of proteins in sections of primary roots incubated for 20 min or 2 h with 10^{-8}, 10^{-7} or 10^{-6} M IAA was higher than that in the sections incubated without IAA (Fig. 2, 4). The phosphorylation of proteins in sections of primary roots incubated for 20 min with 10^{-12}∼10^{-9} or 10^{-5} M IAA was almost the same as that in the sections incubated without IAA (Fig. 2, 3). The growth of sections of the roots incubated for 2 h with 10^{-7} M IAA or higher concentrations was lower than that of the sections incubated

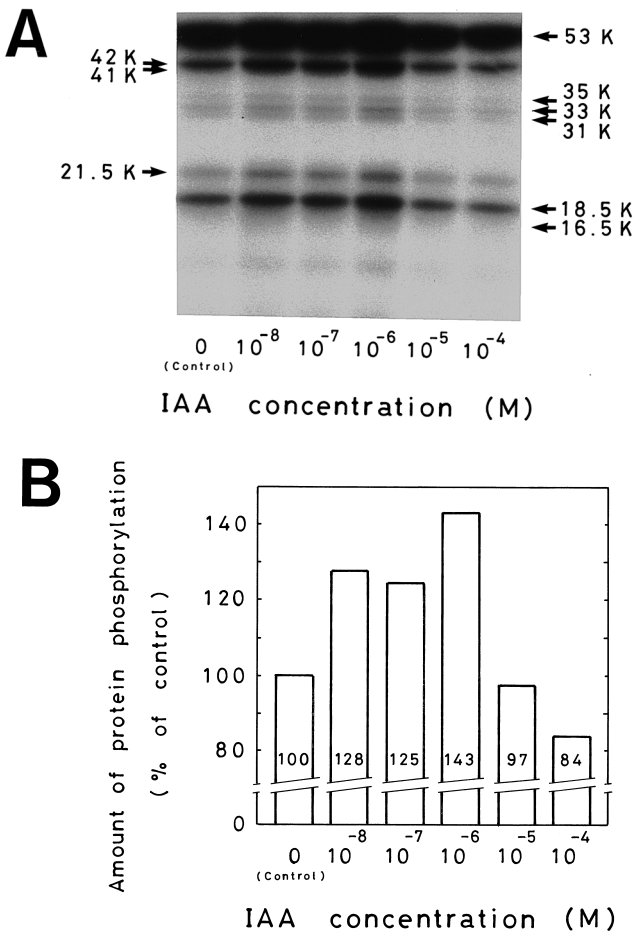

Fig. 4. Phosphorylation of proteins in sections of maize primary roots incubated for 2 h in the presence of 10^{-8}∼10^{-4} M IAA. Thirty sections of the roots were incubated for 2 h in the presence of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M IAA. The procedures were the same as those described in the legend of Fig. 1. **A**: Autoradiography. The symbols are the same as indicated in Fig. 1. **B**: Total amounts of the gradations of the darkness on the film. Numbers in the bars indicate the relative extent of the amount.

for 2 h without IAA (Fig. 7). These results suggest that the phosphorylation of 53, 42, 41, 35, 33, 31, 21.5, 18.5 and 16.5 kDa proteins is not related with the inhibition of growth induced by auxin in maize primary roots, although auxin regulates the growth via the phosphorylation of these proteins in maize mesocotyls (Kato et. al. 1996).

A 2-h incubation of sections of primary roots with 10^{-8}, 10^{-7} or 10^{-6} M IAA had the promotive effect on the phosphorylation of proteins in the root sections (Fig. 4). On the other hand, no changes in the phosphorylation of proteins were observed in sections of maize mesocotyls incubated for 2 h in the

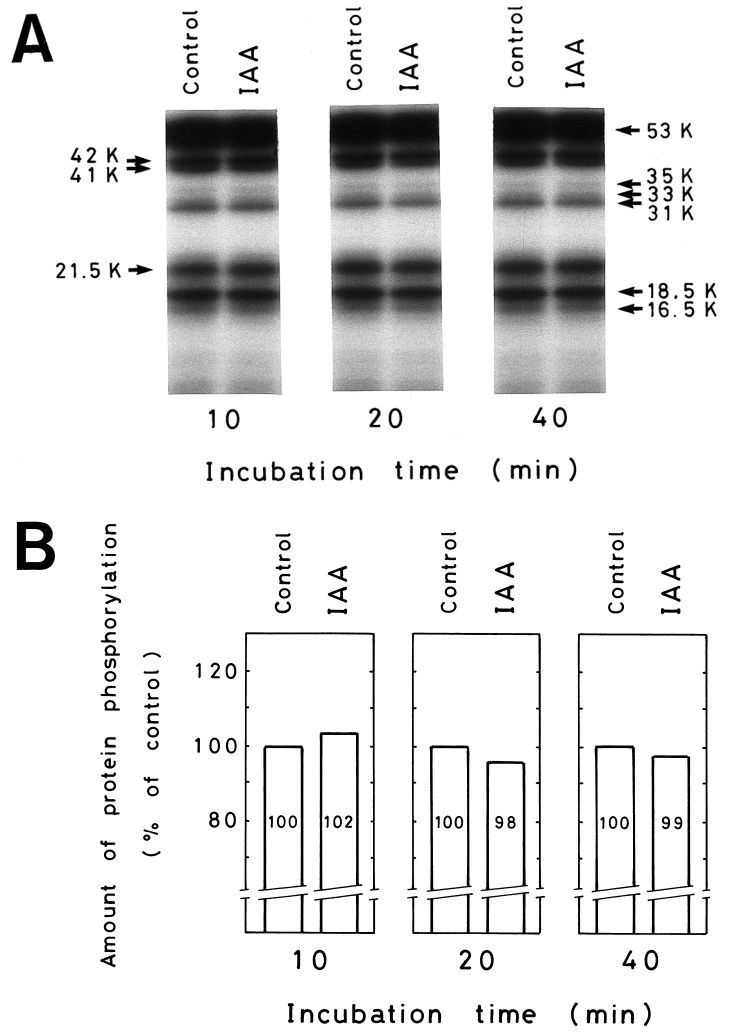

Fig. 5. Phosphorylation of proteins in sections of maize coleoptiles incubated for 10, 20 or 40 min in the presence of 10^{-7} M IAA. Thirty sections of coleoptiles were incubated for 10, 20 or 40 min in the presence or absence of 10^{-7} M IAA. The sections were homogenized, and the homogenate was centrifuged at 3,000 ×g for 20 min. The obtained supernatant was centrifuged at 85,000 ×g for 90 min. The resulting supernatant was concentrated and demineralized, and each sample was obtained. The sample was incubated with [γ-^{32}P]ATP for 1 min at 30°C. After stopping the reaction, just 20 μg proteins were separated by SDS-PAGE (concentration of polyacrylamide, 11%). The other procedures were the same as those described in the legend of Fig. 1. **A**: Autoradiography. The symbols are the same as indicated in Fig. 1. **B**: Total amounts of the gradations of the darkness on the film. Numbers in the bars indicate the relative extent of the amount.

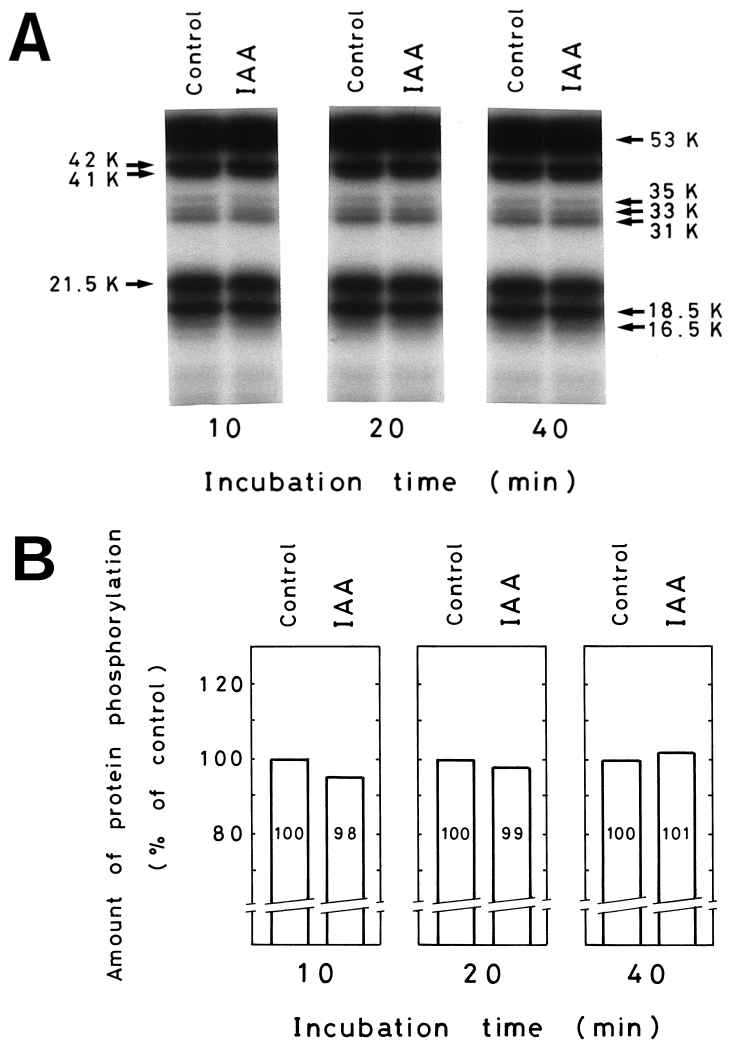

Fig. 6. Phosphorylation of proteins in sections of maize coleoptiles incubated for 10, 20 or 40 min in the presence of 10^{-5} M IAA. Thirty sections of coleoptiles were incubated for 10, 20 or 40 min in the presence or absence of 10^{-5} M IAA. The procedures were the same as those described in the legend of Fig. 5. **A**: Autoradiography. The symbols are the same as indicated in Fig. 1. **B**: Total amounts of the gradations of the darkness on the film. Numbers in the bars indicate the relative extent of the amount.

presence of IAA (Kato et al. 1996). The difference of the phosphorylation in primary roots from the phosphorylation in mesocotyls after a 2-h incubation in the presence of IAA may be caused by difference between response of primary roots and that of mesocotyls to IAA treatment.

The phosphorylation of proteins in sections of primary roots incubated for 20 min or 2 h with 10^{-4} M IAA was lower than that in the sections incubated for 20 min or 2 h without IAA (Fig. 2, 4). This is the first report to our

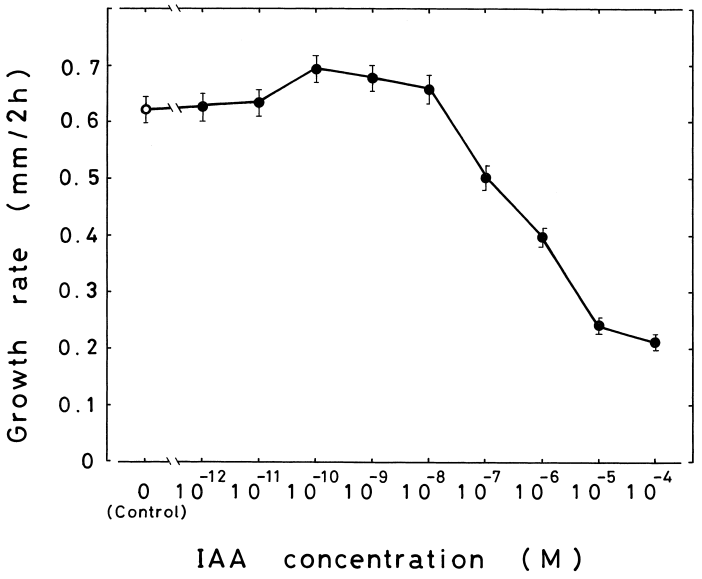

Fig. 7. Effects of IAA concentrations on the growth of sections of maize primary roots. Thirty sections of the roots were incubated for 2 h in the presence of 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M IAA. The length of each section was measured with a travelling microscope. Each point represents an average of values from 30 sections, with standard errors as indicated.

knowledge on the inhibition of protein phosphorylation induced by auxin.

The proteins in the soluble fraction (85,000 $\times$g supernatant) prepared from sections of primary roots which had been incubated for 20 min with 10^{-7} M IAA were phosphorylated in the presence of 10 μM cyclic nucleotide (cAMP or cGMP), 100 μM calmodulin antagonist (W-7 or W-5) or 20 μM protein kinase inhibitor (H-7 or HA1004). However, the addition of cyclic nucleotides or calmodulin antagonists had no effect on the phosphorylation of proteins, and there was no difference between the extent of phosphorylation of proteins in the presence of H-7 and that of phosphorylation of protein in the presence of HA1004 (data not shown). A calmodulin-dependent protein kinase and protein kinase C phosphorylate the proteins when auxin promotes the growth in maize mesocotyls (Kato et. al. 1996), however, these kinases might not be involved in the phosphorylation of the proteins increased by auxin in maize primary roots.

Mizoguchi et al. (1994) reported that the activity of the MAPKs increased when auxin-starved cultured cells of tobacco were treated with auxin for 5 or 10 min and that the activity did not increase after auxin treatment for 15 min or longer. Nakashima et al. (1998) showed that transcripts of a protein kinase gene which is related to MAPKKK were detected as early as 3 h after

the onset of incubation in the presence of both auxin and cytokinin in leaf discs of tobacco, but very limited amounts of the transcripts were found by an incubation in auxin alone. Chono et al. (1998) reported that the mRNA levels of a protein kinase began to increase 30 min after treatment with auxin in cucumber hypocotyls and reached a maximal level in 2 h. On the other hand, a 10-min incubation of the sections with 10^{-7} M IAA had no effect on the phosphorylation of proteins in maize primary roots, but a 20-min, 40-min or 2-h incubation with 10^{-7} M IAA had the promotive effect on the phosphorylation (Fig. 1, 4). A difference in respose time to auxin suggest that the phosphorylation of proteins which is enhanced by auxin in maize primary roots might be not related to the MAPK cascades in a tobacco plant and to the protein kinase in cucumber.

The phosphorylation of proteins in sections of coleoptiles incubated for 10, 20 or 40 min with 10^{-7} or 10^{-5} M IAA was not higher than that in the sections incubated for 10, 20 or 40 min without IAA (Fig. 5, 6). The growth of sections of coleoptiles incubated with 10^{-7} M IAA or higher concentrations was greater than that of the sections incubated without IAA and the promotion of growth of coleoptiles by IAA was greater at higher concentrations of IAA (Kato and Fujii 1982). On the other hand, the phosphorylation of proteins in sections of maize mesocotyls incubated for 10, 20 or 40 min with 10^{-5} M IAA was higher than that in the sections incubated for 10, 20 or 40 min without IAA and an incubation of maize mesocotyls for 20 min with 10^{-7} M IAA increased the phosphorylation of proteins (Kato et al. 1996). These results show that auxin regulates growth of coleoptiles in no relation with phosphorylation of the proteins and that auxin regulates growth of mesocotyls via phosphorylation of the proteins, in spite of auxin promoting both growth of coleoptiles (Kato and Fujii 1982, Karcz et al. 1990, Cleland 1991) and mesocotyls (Vanderhoef and Brigges 1978, Walton and Ray 1981, Yahalom et al. 1988), in *Zea mays*. There is a possibility that physiological process of auxin which promotes growth of coleoptiles was very different from the process of auxin which promotes growth of mesocotyls in *Zea mays*.

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