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Original Paper

Calcium-dependent protein phosphorylation in hairy roots of *Daucus carota*

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Abstract *In vitro* protein phosphorylation in soluble fractions (85,000×*g* supernatant) prepared from carrot hairy roots, which were induced by inoculation with *Agrobacterium rhizogenes* harboring the Ri plasmid, was analyzed by SDS-PAGE and autoradiography. The autoradiograms indicated that at least four proteins, with molecular masses of 65, 57.5, 52, and 38 kDa, were phosphorylated. The presence of Ca^{2+} enhanced the phosphorylation of the 65, 57.5, and 52 kDa proteins, but the presence of both Ca^{2+} and calmodulin did not enhance phosphorylation as much as Ca^{2+} alone. W-7 strongly inhibited the phosphorylation of the 65, 57.5, and 52 kDa proteins while W-5 mildly inhibited the phosphorylation of these proteins. The extent of phosphorylation of these proteins and the growth rate of the hairy roots became higher with increasing incubation temperature. W-7 had a considerable inhibitory effect on the growth of hairy roots, W-5 had a weak inhibitory effect on the growth of the roots, and a high concentration of TMB-8 inhibited growth. These results suggest that the 65, 57.5, and 52 kDa proteins are phosphorylated by CDPK and that the phosphorylation is connected with the increase in growth of carrot hairy roots.

Key words: Calcium-dependent protein phosphorylation, *Daucus carota*, hairy root.

Many responses of plants to environmental stress, light, phytohormones, and pathogen attack are mediated by changes in the cytoplasmic concentration of free calcium (Muto 1992, Pandey et al. 2000, Reddy 2001, Nayyar 2003, Malho et al. 2006). Further more, CDPKs are believed to affect various physiological processes in plants (Harmon et al. 2000, 2001, Cheng et al. 2002). For example, CDPK has been implicated in plasma-membrane H^+-ATPase activity (Lino et al. 1998, Nisi et al. 1999, Lino et al. 2006), plasmodesmata conductivity (Yahalom et al. 1998), control of stomatal aperture (Pei et al. 1996, Li et al. 1998, Mori et al. 2006), response to wounding (Chico et al. 2002, Szczegielniak et al. 2005), lamina inclination induced by brassinolide in rice (Yang and Komatsu 2000), response to salinity or cold stress in rice (Saijo et al. 2001, Yang et al. 2003, Abbasi et al. 2004), regulation of nodule number in legume (Gargantini et al. 2006), and embryogenesis, seed development, and germination in sandalwood (Anil et al. 2000). However, there have been no reports on the involvement of CDPK or protein phosphorylation by CDPK in the growth of higher plants, with the exception of pollen tube growth in maize (Moutinho et al. 1998).

It has been clearly demonstrated that the Ri plasmid present in *Agrobacterium rhizogenes* causes the transformation of plant cells via the introduction of the T-DNA from the Ri plasmid into the genomic DNA of plant cells. It has also been demonstrated that the transformed plant cells give rise to massive roots, known as hairy roots (White and Nester 1980, Tepfer 1984, Shanks and Morgan 1999, Giri and Narasu 2000). The transformed plant cells can continue to produce hairy roots even after *Agrobacterium* has been eliminated. Hairy roots grow vigorously in a phytohormone-free medium and provide a useful material for studies on secondary metabolite production (Kamada et al. 1986, Bourgaud et al. 2001, Facchini 2001, Kim et al. 2002, Srivastava and Srivastava 2007). However, there have been few physiological investigations of hairy root growth (Bonhomme et al. 2000, Tanaka et al. 2001, Hwang 2005).

In this report, we showed three proteins that were phosphorylated by CDPK, which might be connected with the growth of carrot hairy roots, that are materials

Abbreviations: CDPK, calcium-dependent protein kinase; EGTA, ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMB-8, 3,4,5 trimethoxybenzoic acid 8-(diethylamino)-octyl ester; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride.
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having a vigorous elongation.

Materials and Methods

Plant material

Seeds of carrot (*Daucus carota* L. cv. US-Harumakigosun) were sown on vermiculite at 25°C under conditions of continuous light (ca. 3 klux). One-week-old seedlings were surface-sterilized with 1% sodium hypochlorite solution for 15 min, and then rinsed three times with sterilized distilled water. The hypocotyls were cut into 20-mm-long segments and placed with the basal part uppermost on MS agar (1%) medium containing 3% sucrose without phytohormones. The cut ends of the segments were inoculated by needle with *Agrobacterium rhizogenes* strain 1724 that harbored the Ri plasmid (pRi 1724), which had been grown on YEB agar medium (Vervliet et al. 1975). Hairy roots appeared at the sites of inoculation three weeks later. The growing tips of the hairy roots were cut off and cultured on phytohormone-free MS agar (1%) medium containing 3% sucrose and an antibiotic (carbenicillin, 1 mg ml^{-1}) at 25°C under 16-h light (ca. 3 klux)/8-dark conditions. The root tips were subcultured three more times at two-week intervals on fresh MS medium supplemented with the same antibiotic. The hairy roots were transferred to an MS agar (1%) medium that contained 3% sucrose without antibiotic and subcultured at monthly intervals.

Phosphorylation of proteins

The procedures were described by Kato et al. (1996). A 0.8–1.0 g sample of hairy roots was rinsed five times with distilled water. The following procedures were performed at 4°C or in an ice-bath. They were homogenized by an ice chilled mortar and pestle with 200 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 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 1.4–1.6 ml of Buffer A. The supernatant was applied to a Centricon (Amicon, Inc., Beverly Hills, California, U.S.A., Model: SR-10 or SR-30), which was centrifuged at 5,000×g for 90 min. A further 1.8 ml of Buffer A was added to the concentrated supernatant. The procedure was repeated twice. The concentrated and demineralized supernatant was obtained finally as a sample. In some experiments, the amount of proteins in the sample was determined by using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, 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 ($3.0–6.0\times10^{6}$ cpm $pmol^{-1}$) (Radiochemical Centre, Amersham, Little Chalfont, Bucks., U.K.) and the sample. In some experiments, EGTA, $CaCl_2$, spinach calmodulin (Sigma-Aldrich Co., Louis, Missouri, USA, P-5779), or a calmodulin antagonist (W-7 or W-5, Seikagaku Co., Tokyo, Japan) was added to the mixture. The reactions were initiated by the addition of [γ-^{32}P]ATP, and were allowed to proceed for 45 sec 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. The same amount of protein per reaction mixture was applied to each lane of SDS-PAGE 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 2–6 days. The dried gel and the exposed film were separately scanned by a scanner (Seiko Epson Co., Suwa, Nagano, Japan, Model: GT-9000). The densitometry values of the dye in the gel and of the exposed areas on the film were calculated using a personal computer and a software package for image processing (NIH image). We confirmed that the same quantity of protein was present in each lane of the gel (data not shown).

Measurement of growth rate

Segments (length: 15 mm) of root tips were cut from the hairy roots. Ten segments of hairy roots were put side by side in a Petri dish (diameter: 94 mm) on an MS medium containing 3% sucrose, 0.2% Gellan Gum, and various concentrations of W-7, W-5, TMB-8 (Sigma-Aldrich Co., Louis, Missouri, U.S.A.) or no additive (control). The dishes were covered with a double thickness of aluminum foil. They were incubated at 4, 16, or 25°C for appropriate intervals. The elongation of each hairy root was measured with a slide caliper.

Results

Phosphorylation of proteins

The 85,000×g supernatant, prepared from a homogenate of the hairy roots, was concentrated and demineralized with a Centricon SR-30. The sample was incubated with [γ-^{32}P]ATP in the presence of 1 mM EGTA, 1 mM $CaCl_2$, and/or 800 nM spinach calmodulin, and then the proteins were separated by SDS-PAGE. The radioactivity of the proteins was determined by autoradiography. The autoradiograms indicated that at least four proteins, with molecular masses of 65, 57.5, 52, and 38 kDa, were phosphorylated (Figure 1). The extent of phosphorylation of the 65, 57.5, and 52 kDa proteins in the presence of Ca^{2+} was higher than that of phosphorylation of the same proteins in the presence of EGTA (Figure 1). The extent of phosphorylation of the 65, 57.5, and 52 kDa proteins in the presence of both Ca^{2+} and calmodulin was less than that with Ca^{2+} alone (Figure 1). The extent of phosphorylation of the 38 kDa protein in the presence of Ca^{2+} or in the presence of both Ca^{2+} and calmodulin was lower than that in the presence of EGTA (Figure 1).

The 85,000×g supernatant, prepared from a homogenate of the roots, was concentrated and demineralized with a Centricon SR-10, incubated with [γ-^{32}P]ATP in the presence of 200 μM W-7 or W-5, and then the proteins were separated by SDS-PAGE and the

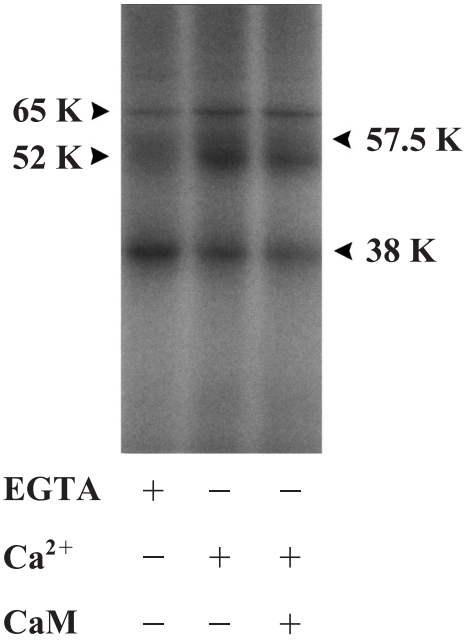

Figure 1. Phosphorylation of proteins in the presence of EGTA, in the presence of Ca^{2+}, or in the presence of both Ca^{2+} and calmodulin in carrot hairy roots. A 0.8 g sample of the hairy roots was rinsed and homogenized with an ice-chilled mortar and pestle. The homogenate was centrifuged at 3,000×*g* 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 to yield the samples. Samples were incubated with [γ-^{32}P]ATP in the presence of 1 mM EGTA, 1 mM $CaCl_2$, and/or 800 nM calmodulin for 45 sec at 30°C. After the reaction was stopped by the addition of Stop solution, 30 μg of proteins per lane was separated by SDS-PAGE. The radioactivity of the proteins was determined by autoradiography. The positions of the 65 (65 K), 57.5 (57.5 K), 52 (52 K), and 38 (38 K) kDa proteins are indicated by arrows.

radioactivity was determined by autoradiography. The extent of phosphorylation of the 65, 57.5, and 52 kDa proteins in the presence of W-7 was lower than that of phosphorylation of these proteins in its absence (control) (Figure 2). The extent of phosphorylation of these proteins in the presence of W-5 was slightly lower than that in the control (Figure 2). The extent of phosphorylation of the 38 kDa protein in the presence of W-7 was equal to that in the presence of W-5, and these antagonists slightly inhibited the phosphorylation of the protein (Figure 2).

The hairy roots were incubated on MS medium containing 3% sucrose, 0.2% Gellan Gum at 4, 16, or 25°C for 12 h. Each 85,000×*g* supernatant, prepared from a homogenate of the roots, was concentrated and demineralized with a Centricon SR-10, incubated with [γ-^{32}P]ATP, and subjected to SDS-PAGE and autoradiography. The extent of phosphorylation of the 65, 57.5, and 52 kDa proteins became higher with increasing incubation temperature (Figure 3). However, that of phosphorylation of the 38 kDa protein had no connection with temperature (Figure 3).

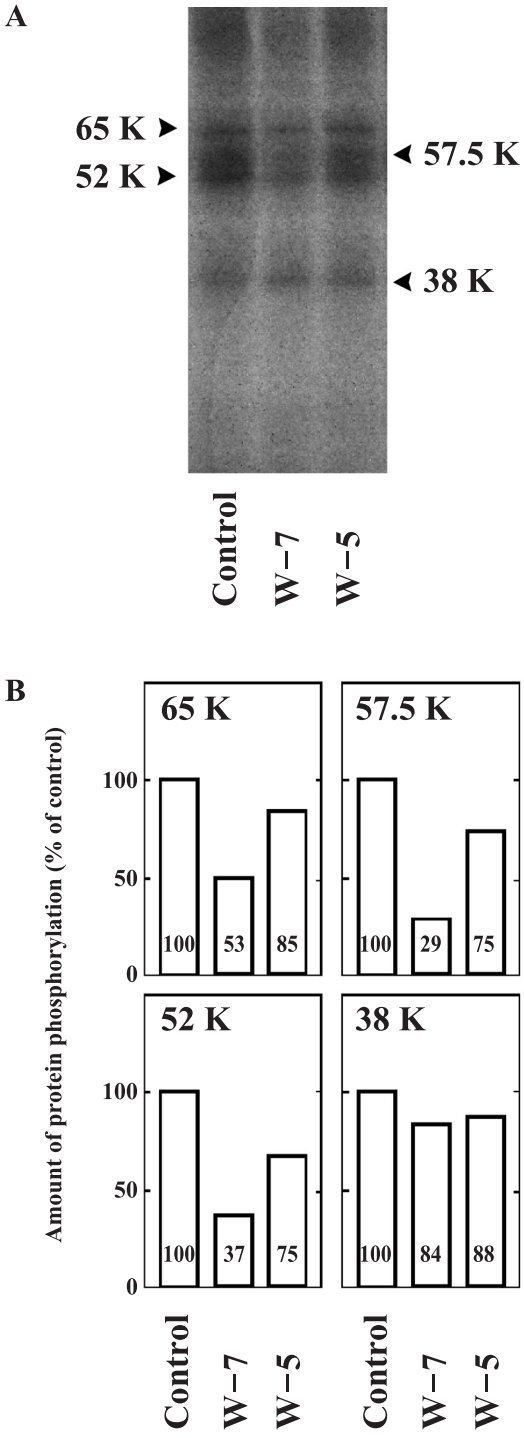

Figure 2. Phosphorylation of proteins in the presence of W-7 or W-5 in carrot hairy roots. The sample was incubated with [γ-^{32}P]ATP in the presence of 200 μM W-7 or W-5. The other procedures were the same as those described in the legend of Fig. 1. The exposed film was scanned with a scanner and the densitometry values of the exposed areas on the film were calculated for each protein of 65, 57.5, 52, or 38 kDa using a personal computer. (A) Autoradiography. The symbols are the same as indicated in Fig. 1. (B) The densitometry values of the exposed areas of the 65, 57.5, 52, and 38 kDa proteins on the film. Numbers in the bars indicate the relative values.

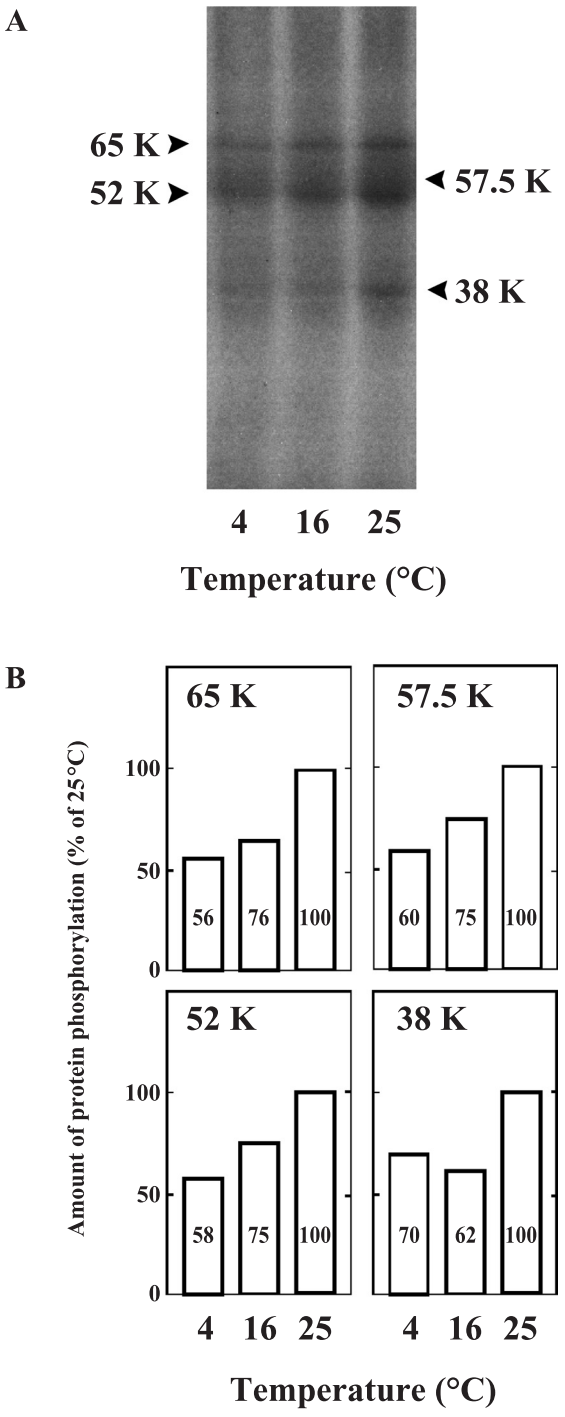

Figure 3. Phosphorylation of proteins in carrot hairy roots that had been incubated at 4, 16, or 25°C. The hairy roots were incubated on MS medium containing 3% sucrose and 0.2% Gellan Gum at 4, 16, or 25°C for 12 h. The sample was incubated with [γ-^{32}P]ATP. The other procedures were the same as those described in the legends of Figs. 1 and 2. A: Autoradiography. The symbols are the same as indicated in Fig. 1. B: The densitometry values of the exposed areas of the 65, 57.5, 52, and 38 kDa proteins on the film. Numbers in the bars indicate the relative values.

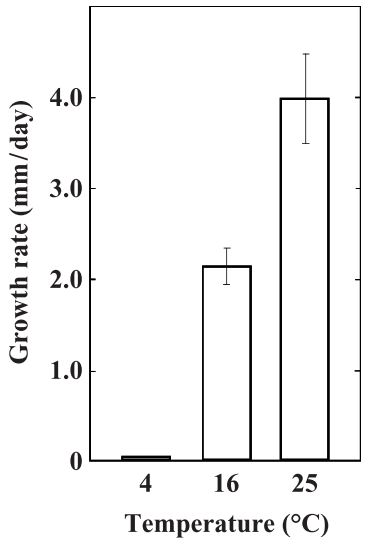

Figure 4. Growth rate of carrot hairy roots that had been incubated at 4, 16, or 25°C. Segments (length: 15 mm) of root tips, which were cut from the hairy roots, were put side by side on MS medium containing 3% sucrose and 0.2% Gellan Gum and incubated at 4, 16, or 25°C for 12 days in the dark. The length of each elongation was measured with a slide caliper. Each point represents an average value from at least 20 segments, with standard errors as indicated.

Measurement of growth rate

Segments of root tips which were cut from hairy roots were incubated on MS medium containing 3% sucrose and 0.2% Gellan Gum at 4, 16, or 25°C for 12 days. The growth rate of the segments incubated at 25°C was higher than the rate of the segments incubated at 16°C (Figure 4). The segments could not elongate at 4°C (Figure 4).

The segments of root tip of the hairy roots were incubated on medium containing various concentrations of W-7 or W-5 at 25°C for 12 days. The growth rates of the segments cultured on media containing 0.1, 1, and 10 μM antagonists were equivalent to that of the segments cultured in its absence (Figure 5). Thirty micro-molar W-5 did not inhibit the growth of segments, but 30 μM W-7 did (Figure 5). The 100 μM W-7 inhibited the growth of segments more than 100 μM W-5 did (Figure 5).

The segments of hairy roots were incubated on medium containing various concentrations of TMB-8 at 25°C for 12 days. The growth rate of the segments incubated in the presence of 10 μM TMB-8 was slightly lower than the rate of the segments incubated in its absence, and the rate of those incubated in the presence of 100 μM TMB-8 was just half of that in its absence (Figure 6).

Discussion

It is well known that free calcium and CDPK regulate many physiological process in plant cells (Muto 1992,

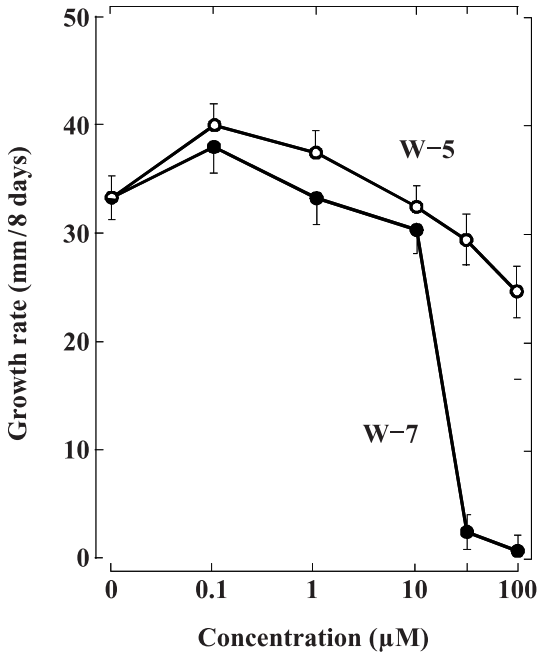

Figure 5. Effects of W-7 and W-5 on the growth rate of carrot hairy roots. The segments of root tips, which were cut from the hairy roots, were put side by side on MS medium containing various concentrations of W-7 or W-5, 3% sucrose, and 0.2% Gellan Gum and incubated at 25°C for 12 days in the dark. The length of each elongation was measured with a slide caliper. Each point represents an average value from at least 20 segments, with standard errors as indicated.

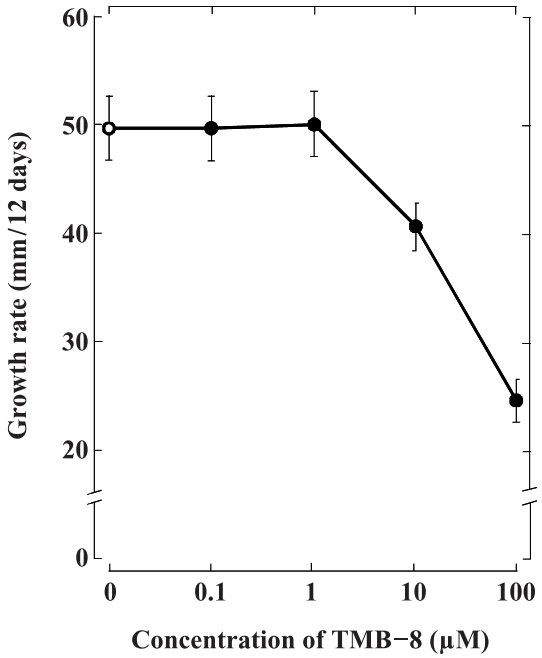

Figure 6. Effect of TMB-8 on the growth rate of carrot hairy roots. The segments of root tips, which were cut from hairy roots, were put side by side on MS medium containing various concentrations of TMB-8, 3% sucrose, and 0.2% Gellan Gum and incubated at 25°C for 12 days in the dark. The length of each elongation was measured with a slide caliper. Each point represents an average value from at least 20 segments, with standard errors as indicated.

Harmon et al. 2000, 2001, Pandey et al. 2000, Reddy 2001, Cheng et al. 2002, Nayyar 2003, Malho et al. 2006). However, there have been no reports of CDPK or protein phosphorylation by CDPK influencing growth in plants. In this report, we present a possibility of the role of protein phosphorylation by CDPK in the growth of carrot hairy roots.

The presence of Ca^{2+} enhanced the phosphorylation of 65, 57.5, and 52 kDa proteins, but the presence of both Ca^{2+} and calmodulin did not enhance the phosphorylation of these proteins as much as Ca^{2+} alone (Figure 1). These results show that CDPK phosphorylates the 65, 57.5, and 52 kDa proteins while calmodulin-dependent protein kinase does not phosphorylate these proteins. The 65, 57.5, and 52 kDa proteins were slightly phosphorylated in the presence of EGTA (Figure 1). There is a possibility that these proteins were phosphorylated by other protein kinase(s) that were contained in the reaction mixture (85,000×*g* supernatant) in the absence of Ca^{2+}.

W-7 is a calmodulin-binding compound and an inhibitor of Ca^{2+}/calmodulin-regulated enzymes, and penetrates the cell membrane (Hidaka et al. 1981). W-7 has a considerable inhibitory effect on the activity of not only calmodulin-dependent protein kinase but also CDPK (Harmon et al. 1987). W-5, a less potent analog of W-7 (Hidaka et al. 1981), has a weak inhibitory effect on the activities of these kinases (Harmon et al. 1987, DasGuota 1994). W-7 strongly inhibited the phosphorylation of the 65, 57.5, and 52 kDa proteins and W-5 mildly inhibited the phosphorylation of these proteins (Figure 2). These results and the data in Figure 1 strongly suggest that the 65, 57.5, and 52 kDa proteins are phosphorylated by CDPK.

The extent of phosphorylation of the 65, 57.5, and 52 kDa proteins after incubation at 25°C was higher than that of phosphorylation of these proteins after incubation at 16°C (Figure 3). The three proteins were weakly phosphorylated after incubation at 4°C in comparison with incubation at 16°C (Figure 3). The growth rate of the hairy roots became higher with increasing incubation temperature (Figure 4). These results show that phosphorylation of the 65, 57.5, and 52 kDa proteins is connected with the increase in growth of carrot hairy roots, but there is a possibility of temperature-dependent phosphorylation.

The phosphorylation of the 38 kDa protein was not enhanced by the presence of Ca^{2+} (Figure 1), was slightly inhibited by W-7 or W-5 at the same concentrations (Figure 2) and was not enhanced at higher the growth rates caused by increasing temperature (Figure 3, 4). Therefore, we think that the phosphorylation of the 38 kDa protein has no connection with CDPK or with the growth of carrot hairy roots.

W-7 had a considerable inhibitory effect on the growth of carrot hairy roots while W-5 had a weak inhibitory effect (Figure 5). TMB-8 is commonly used as an inhibitor of intracellular Ca^{2+} mobilization (Chiou and Malagodi 1975), and is known to prevent the release of Ca^{2+} from intracellular stores (Rittenhouse-Simmons and Deykin 1978). A high concentration of TMB-8 inhibited the growth of carrot hairy roots (Figure 6). There is a possibility that W-7 or W-5 inhibits CDPK activity and the decrease of Ca^{2+} by TMB-8 induced down of CDPK activity in the cells and that the decrease of the phosphorylation of the 65, 57.5, and 52 kDa proteins caused inhibition of the growth of carrot hairy roots.

Considering all the results in this study, we conclude that the phosphorylation of the 65, 57.5, and 52 kDa proteins by CDPK may be connected with the increase in growth of carrot hairy roots. This is just one instance, out of very few reports, of protein phosphorylation by CDPK being related to the growth of higher plants.

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