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A GSK-3/SHAGGY-Related Protein Kinase is Involved in Phytochrome Signal Transduction Pathway

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Phosphorylation of cellular proteins is a key regulatory mechanism for signal transduction pathway in living cells. Phytochrome, a red/far-red light photoreceptor in plants, is known to employ protein phosphorylation for its light signaling, although its detailed mechanism is still ambiguous. This study is intended to identify the phosphoproteins and protein kinases that are regulated by phytochrome, by employing transgenic rice seedlings that overexpress *Arabidopsis* phytochrome A. Red light stimulated phosphorylation of a 70 kDa protein and far-red light negated the effect. The red light induced phosphotylation of the 70 kDa protein was strongly activated by heparin and inhibited by poly-L-lysine, suggesting that the 70 kDa protein phosphorylating kinase belongs to GSK-3/SHAGGY protein kinase that has functional roles in establishing cell fate and pattern formation in *Drosophila*. Taken together with the fact that phytochrome controls plant development, these results may suggest that a GSK-3/SHAGGY-related protein kinase in plant (ASK) is likely to be involved in phytochrome signal transduction.

key words: ASK, kinase, phosphoprotein, phytochrome, 70 kDa protein, transgenic rice

INTRODUCTION

Phytochrome controls plant growth and development by perceiving red and far-red lights [1]. As a major photoreceptor for plant photomorphogenesis, phytochrome has been extensively studied for its molecular structure, light perception and physiological responses [2]. However, its signal transduction pathway to evoke the responses is still ambiguous. Protein phosphorylation and dephosphorylation are known to be a good and effective tool for transmitting signals to target sites of cells to regulate physiological activities of plants. Phytochrome is also likely to transmit light signal through protein phosphorylation and dephosphorylation, as evidenced by the facts that the red and far-red lights change the levels of second messengers for protein kinases, such as cGMP, phosphoinositides and calcium ion [3], and that several cytosolic and nuclear proteins are phosphorylated by red light [4, 5].

Although PKS1 (phytochrome kinase substrate 1) has been identified as a possible signal transmitter of phytochrome [6], the presence of other phosphoproteins than the PKS1 has been warranted since several proteins have been shown to be phosphorylated by red light treatments. However, a crucial evidence for the identity of phosphoproteins and the protein kinases that are involved in the phytochrome signal transduction has not been provided so far. Difficulties to identify the signaling components involved in the phytochrome signal transduction pathway partly arise from the fact that the intermediate signaling molecules in the pathway exist at extremely low concentrations. A phytochrome overexpressor that exhibits an exaggerated phenotype is expected to have an amplified light signal transmission system and, thus, higher contents of intermediate signaling molecules [7]. Increase in the intermediate signaling components of the transgenic plants may facilitate to identify the intermediate molecules in the phytochrome signal transduction pathway.

The present study is intended to identify the phosphoproteins and protein kinases that are regulated by phytochrome, by employing transgenic rice plants that overexpress *Arabidopsis* phytochrome A and display a typical phenotypic trait of phytochrome overexpressor.

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MATERIALS AND METHODS

Chemicals. Ammonium sulfate and Tris were purchased from United States Biochemical Co. (Cleveland, OH, USA). Phenylmethylsulfonylfloride (PMSF), leupeptin, poly-L-lysine, heparin, dithiothreitol (DTT), polyethyleneimine (PEI), ampholyte (pH 3-10), bis-acrylamide, sodium dodecyl sulfate (SDS) and X-ray film were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Coomassie brilliant blue R-250 was purchased from Bio-Rad Biochemical Co. (Richmond, CA, USA). The [γ-

^{32}P] ATP (3000 Ci/mmol) and scintillation cocktail were obtained from NEN Research Products (Boston, MA, USA) and Packard Bioscience Co. (Meriden, CT, USA), respectively. KN-62 was obtained from Calbiochem (San Diego, CA, USA). Phorbol-12-myistate-13-actate (PMA), bisindolylmaleimide (BIS), 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB), staurosporine, H-89, PD 98059, AG 1487, rapamycin were provided by Dr. Young-Seuk Bae and Dr. Young-Sup Lee (Kyungpook National University, Korea).

PHYA transgenic rice plants. Transgenic rice plants that overexpress *Arabidopsis* phyA cDNA (*PHYA*) were obtained by using an expression vector driven by maize *ubiquitin*-1 promoter (unpublished results in this laboratory). The transgenic plant had one copy of *PHYA* and the expression levels of PHYA in the transgenic rice seedlings grown in darkness were at least 3 times higher than that in the non-transgenic seedlings, when analyzed by Southern blot and by Western blot. The transgenic rice plant displayed a typical phenotype of phytochrome transformants, that is, reduction in plant height, culm length and panicle length by *ca*. 20% over the wild type.

Growth of plants and crude extract preparation. Seeds of transgenic and wild type rice (*Oryza sativa* cv. Nagdong) were placed at 4℃ for 1 day, sowed on vermiculite in the plastic petridish and germinated at 28℃ for 5～6 days in the dark. Etiolate tissues of both wild type and transgenic (U1 line) rice were harvested under green safety light. The rice tissues (0.5g) were pulverized in liquid nitrogen using a mortar and pestle and homogenized with 1 ml of the homogenization buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.25 M sucrose, 1 mM DTT, 1 mM PMSF and 1μg/ml leupeptin). The homogenates were centrifuged at 23,000 x g for 20 min and the resulting supernatant was used for assays as crude extracts.

Light treatment. Samples were irradiated with LED lamps (LED plant radiation system, Model GF-320S, Good Feeling, Korea) and the fluence rates were 163 μmol/m^2/s for red light (660nm) and 2 μmol/m^2/s for far-red light (730 nm).

Assay of protein phosphorylation. An assay mixture for protein phosphorylation contained 5 μl of 5-fold concentrated stock solution of kinase assay buffer containing 10nM [γ-^{32}P] ATP (2.5 μCi) and 20 μl of the crude extract in a total volume of 25 μl. The crude extract of both the wild type and transgenic (U1 line) etiolated rice seedlings was irradiated with red and far-red lights for an appropriate time. The stock solution of kinase assay buffer contained 0.1 M HEPES-NaOH, pH 6.8, 7.5 mM $MgCl_2$, 0.5 mM EDTA and 0.1 M NaCl. After incubation at 28 ℃ for an appropriate time, the reaction was terminated by boiling the incubation mixture with 6.25 μl of 5-fold concentrated SDS-PAGE sample buffer containing 0.5 M Tris-Cl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 14.4 mM β-mercaptoethanol and 0.05% (w/v) bromophenol blue. The solution was sujected to 10% SDS-PAGE and autoradiographied on Kodak X-Omat AR film.

To characterize the protein kinase that phoaphorylated the 70 kDa protein, the effects of protein kinase inhibitors or activator on the 70 kDa protein phosphorylation were evaluated. The assay mixture for that evaluation comprised 6 μl of ATP solution, 5 μl of effector solution and 19 μl of the crude extract (20 μg protein) in a total volume of 30 μl. The effectors used were: phorbol-12-myistate-13-actate (PMA), bisindolylmaleimide (BIS), 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB), staurosporine, H-89, PD 98059, AG 1487, rapamycin, KN-62, poly-L-lysine and heparin.

Gel electrophoresis and autoradiography. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method described by Laemmli [8]. Protein samples were electrophoresed on a 4% stacking and 10% separating polyacrylamide slab gel in the presence of 0.1% SDS. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 or silver nitrate [9]. The stained gel was dried onto Whatman 3MM paper and autoradiographied by layering on Kodak X-Omat AR film with a Dupont Cronex intensifying screen at -70℃ for 7 days.

Isoelectric focusing was performed in a rod gel of 1.5 mm diameter and 12 cm long that was made of 8 M urea, 3% (v/v) ampholytes, pH 3.5-10, 5% (v/v) acrylamide stock, 15% (v/v) glycerol, 0.33% (w/v) ammonium peroxodisulfate and 0.05% (v/v) TEMED, as described by O´Farrell [10]. The gel was serially prerun at 200 volts for 15 min, 300 volts for 30 min, and 400 volts for 30 min. After loading the sample, the gel was run at 400 volts for 18 hours, 500 volts for 6 hours and then at 800 volts for 1 hour. The focused gel was cut into 1 cm long and measured for pH or stained for protein with Coomassie brilliant blue.

Determination of protein concentration. Protein concentration was determined by the method of Bradford [11] using BSA as a standard.

RESULTS AND DISCUSSION

To determine the optimum condition to phosphorylate proteins in the crude extract of the etiolated rice seedlings by red light, the effect of the order of light treatment and [γ-^{32}P] ATP addition on the protein phosphorylation was evaluated. The etiolated tissue extract was phospho-labeled by the radioactive ATP before and after red light irradiation. The radioactively labeled extracts were analyzed by SDS-PAGE and autoradiography to visualize phosphorylated proteins. As shown in Fig.1, the extent of protein phosphorylation in the autoradiogram was drastically different between the two treatments, although their electrophoretic profile of the SDS-PAGE was essentially the same. In general, the extract that received red light before ATP addition was highly phosphorylated compared to the one that received red light after ATP addition. In both cases, a 50 kDa protein was the major phosphorylated band and a 70 kDa protein was the

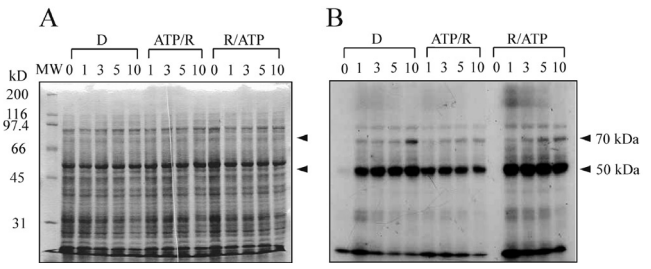

Figure 1. Effects of red light irradiation on protein phosphorylation of the etiolated rice seedlings. The crude extracts of the etiolated rice seedlings were phosphorylated with [γ-^{32}P] ATP before or after red light irradiation for 1 min. The crude extract of 20 μl (20 μg protein) was irradiated with red light and mixed with 5 μl of 5-fold concentrated stock solution of a kinase assay buffer containing 10 nM [γ-^{32}P] ATP (2.5 μCi). Or the crude extract of 20 μl (20 μg protein) was mixed with 5 μl of the stock solution of a kinase assay buffer containing 10 nM [γ-^{32}P] ATP (2.5 μCi, NEN) and the mixture was irradiated with red light for 1 min. The reaction mixtures were incubated at 28℃ for the time indicated and boiled with the SDS-PAGE sample buffer and electrophoresed in a 4% stacking and 10% separating polyacrylamide gel. The gel was dried and subjected to autoradiography. Molecular weight markers (MW) are shown on the left. A: SDS-PAGE, B: autoradiogram. D: etiolated seedlings kept in the dark, ATP/R: extract incubated with [γ-^{32}P] ATP before red light irradiation, R/ATP: extract incubated with [γ-^{32}P] ATP after red light irradiation. 0: no added [γ-^{32}P] ATP. 1, 3, 5 and 10 indicate the incubation time (min) with [γ-^{32}P] ATP. 70 kDa and 50 kDa on the right indicate the MWs of the bands.

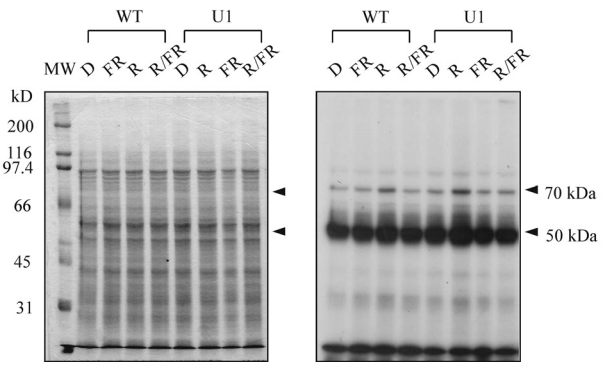

Figure 2. Red and far-red light dependent protein phosphorylation of the wild type and transgenic rice seedlings. The crude extract were prepared from both the wild type and transgenic (U1 line) etiolated rice seedlings and irradiated with red and far-red lights for an appropriate time. Twenty μl (20 μg protein) of the light-treated extract were mixed with 5 μl of 5-fold concentrated solution of a kinase assay buffer containing 10 nM [γ-^{32}P] ATP and incubated for 5 min. The mixture was electrophoresed and autoradiographied as described in Fig. 1. Molecular weight markers used are shown on the left. A: SDS-PAGE, B: autoradiogram. WT: wild type seedlings, U1: transgenic rice seedlings. D: etiolated seedlings kept in the dark, R: red light irradiated for 3 min, FR: far-red light irradiated for 3 min, R/FR: red light for 3 min followed by far-red light for 5 min. 70 kDa and 50 kDa on the right indicate the MWs of the bands.

next highly phosphorylated band except the bands on the front line of the electrophoresis. The 50 kDa major protein band was intensely phosphorylated by the red light treatment before ATP addition but the phosphorylation remained constant during incubation up to 10 min. On the other hand, the phosphorylation of the 70 kDa band increased gradually after red light irradiation as the incubation continued. We were focussed on the 70 kDa protein as a red light-responsive phosphoprotein in this study since its phosphorylation gradually increased after perceiving red light, while the phosphorylation of the 50 kDa phosphoprotein remained constant during the prolonged incubation. The incubation time for the phosphorylation of the crude extract was fixed to 5 min in the further experiments.

Under the conditions above mentioned, the crude extracts of both the wild type and the *PHYA* transgenic U1 line rice seedlings were phosphorylated to determine photo-responsiveness of the 70 kDa protein phosphorylation to red and far-red light treatments. The etiolated crude extracts were irradiated with red or far-red light for 3 min, respectively, or red light for 3 min followed by immediate far-red light for 5 min. A solution of [γ-^{32}P] ATP was added to the extracts that perceived light treatments, and incubated for 5 min to phosphorylate proteins. As shown in Fig. 2, red light increased the 70 kDa phosphorylation, while far-red light maintained the 70 kDa protein phosphorylation to the same level of the etiolated seedlings. The red light-induced stimulation of 70 kDa protein phosphorylation was negated by the immediate far-red light irradiation of the red light-treated extract. Such a red light stimulation of the 70 kDa protein phosphorylation was significantly higher in the transgenic rice compared to the wild type, showing that the phytochrome overexpressed transgenic plant exhibits an amplified light signal transduction. These results clearly indicated that the 70 kDa protein phosphorylation was responsive to red and far-red lights, in other words, the phosphorylation of the 70 kDa protein was controlled by phytochrome.

The 70 kDa phosphoprotein in the crude extract that was under the control of phytochrome was precipitatable with 40%-saturated ammonium sulfate and had a pI value between 6.3 and 7.0 in isoelectric focussing gel (data not shown).

To characterize the protein kinase that phosphorylated the 70 kDa protein, the effects of several inhibitors and activators of different protein kinases on the 70 kDa phosphorylation had been evaluated. As shown in Fig. 3, the phosphorylation of the 70 kDa protein was not affected by inhibitors of PKA such as H-89, staurosporine, BIS and PMA, indicating that the kinase did not belong to the family of PKC. The kinase activity was not also affected by DRB and KN-62 which are potent and specific inhibitors of casein kinase II and of Ca^{2+}/calmodulin-dependent protein kianse II, respectively. The phosphorylation activity remained constant by the addition of PD 98059 and rapamycin which are inhibitors of MEK and p70 kinase, respectively. It was unlikely that

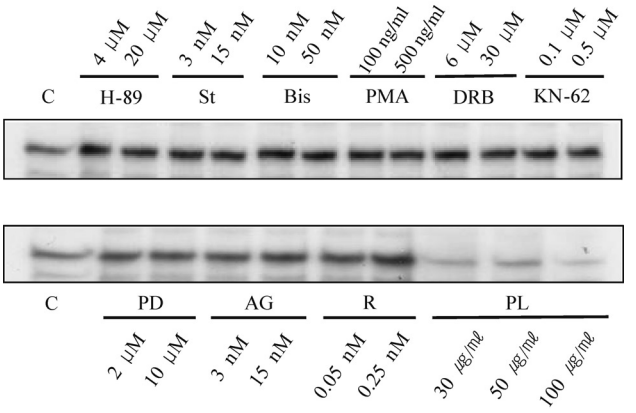

Figure 3. Phosphorylation of the 70 kDa protein in the presence of the effectors of different protein kinase family. The assay mixture in a total volume of 30 μl contained 6 μl of 5-fold concentrated solution of kinase assay buffer containing 10nM [γ-^{32}P] ATP (2.5 μCi, NEN), 19 μl of the crude extract (20 μg) of the etiolated seedlings and 5 μl of protein kinase effector solution. After incubation at 28°C for 5 min, the reaction mixture was boiled with 7.5 μl of 5-fold concentrated SDS-PAGE sample buffer, electrophoresed and autoradiographied as described in Fig. 1. C: without inhibitor (H_2O added). The effectors used were: H-89, St (staurosporine), Bis (bisindolylmaleimide), PMA (phorbol-12-myistate-13-actate), DRB (5,6-dichloro-1-β-ribofuranosylbenzimidazole), KN-62, PD (PD 98059), AG (AG 1487), R (rapamycin) and PL (poly-L-lysine). The concentrations of the effectors used are indicated in the figure.

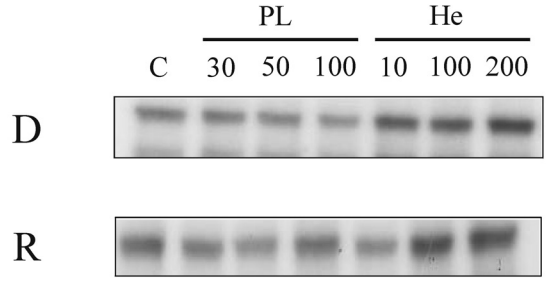

Figure 4. Effects of poly-L-lysine and heparin on the phosphorylation of the 70 kDa protein. Red light was irradiated to a mixture of 19 μl of the crude extract (20 μg), containing 5 μl of poly-L-lysine or heparin for 3 min. The mixture was incubated with 6 μl of 5-fold concentrated solution of kinase assay buffer containing 10nM [γ-^{32}P] ATP (2.5 μCi, NEN), and assayed for protein phosphorylation as described in Fig. 3. D: etiolated tissues kept in the dark, R: extract irradiated with red light for 3 min., C: without inhibitor (H_2O added). PL: poly-L-lysine, He: heparin. The numbers on the top of the figure indicate the concentrations of PL and He used (μg/ml).

the kinase is a tyrosine kinase since the kinase activity was not affected by AG 1487 which is a specific inhibitor of tyrosine kinase.

On the other hand, poly-L-lysine strongly inhibited the kinase activity of the 70 kDa protein phosphorylation, suggesting that the kinase may belong to glycogen synthase kinase-3 (GSK-3) which is strongly inhibited by polycations, especially by poly-L-lysine [12]. Of several protein kinase

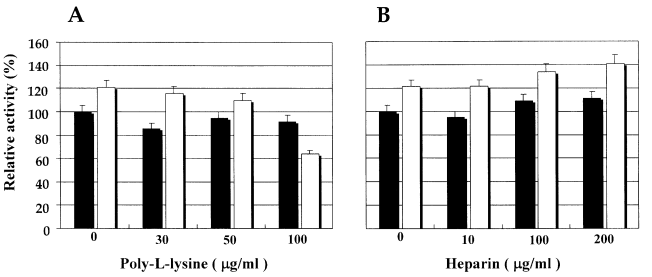

Figure 5. Histogram of the effects of poly-L-lysine and heparin on the 70 kDa protein phosphorylation. The radioactively phosphorylated band of the 70 kDa in Fig. 4 was cut from the dried gel and subjected to liquid scintillation counting. The percent radioactivity was calculated from three independent measurements and plotted as a histogram. The bars indicate standard deviations of the measurements. The percent value was calculated by counting the radioactivity of the etiolated tissues without perceiving red light as 100%. A: effect of poly-L-lysine, B: effect of heparin. ■: tissue extract kept in the dark, □: tissue extract irradiated with red light.

family, GSK-3 was strongly deactivated by poly-L-lysine and activated by heparin, but not affected by other second messengers such as cyclic nucloetide, Ca^{2+} and phosphatidyl inositol phosphate. Heparin stimulates GSK-3 by 3- to 5- fold at a concentration of 3 μg/ml and reverses the inhibitory effect of poly-L-lysine [13, 14]. In the present study, heparin also activated the kinase activity to phosphorylate the 70 kDa protein. Since both poly-L-lysine and heparin affected the kinase activity, their effects on the 70 kDa protein phosphorylation were evaluated at different concentrations with both the etiolated tissues and the red light-irradiated tissues (Fig. 4). As determined by scintillation counting of the phosphorylated bands of the SDS gel, red light increased the phosphorylation of the 70 kDa band by *ca*. 20%. The effects of poly-L-lysine and heparin on the 70 kDa protein phosphorylation were much more pronounced with the red light-irradiated tissues than the etiolated tissues. As diagramed in Fig. 5, poly-L-lysine decreased the kinase activity by 5% in the etiolated tissues and 15% in the red light irradiated tissues at a concentration of 50 μg/ml, and heparin increased the activity by 10% in the etiolated tissues and by 20% in the red light irradiated tissues at a concentration of 100 μg/ml. For the phosphorylation of the 70 kDa protein, the red light-stimulated kinase activity was more sensitive to poly-L-lysine and heparin than the kinase activity in the crude extract that did not received red light. These results indicate that the protein kinase phosphorylating the 70 kDa protein was under the control of phytochrome and had similar properties with GSK-3.

GSK-3 that was originally identified as a regulator of glycogen synthase in mammals is known to phosphorylate and regulate transcription factor such as *c-jun* and *c-myb*, and thus, regulating cell cycle. SHAGGY that originally found in Drosophilla is a protein kinase homologous to the mammalian GSK-3 and has been identified in several organisms such as

Sacchromyces spp. [15, 16], *Dictyostelium discoideum* [17] and *Xenopus laevis* [18]. The mammalian GSK-3 and *Drosophila* SHAGGY kinase are functional homologues that are 85% identical in the catalytic domain [19] and likely to have a key role in signal transduction pathway that controls cell fate and/or pattern formation [20].

A plant homologue of the SHAGGY/GSK-3 has been identified in *Arabidopsis thaliana* [21], Medicago [22] and *Nicotiana* [23] and *Petunia* [24], and named as ASK (*Arabidopsis* shaggy-related protein kinase). Although biological function of ASK in plant is unknown, it seems to play an important role in plant development such as flowering, seed development and somatic embryogenesis [25]. It is interesting that ASK, a GSK-3/SHAGGY homologue in plants, that controls plant development is regulated by phytochrome that is a major photoreceptor to control plant photomorphogenesis. Taken all these together, it can be suggested that a protein kinase related with GSK-3/SHAGGY is involved in phytochrome signal transduction.

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