Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 9-14

INTRACELLULAR PROTEIN PHOSPHORYLATION IN OAT (Avena sativa L.) PROTOPLASTS BY PHYTOCHROME ACTION
(1) MEASUREMENT OF ACTION SPECTRA FOR THE PROTEIN PHOSPHORYLATION

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Received May 22, 1989

SUMMARY : The effects of red light and wavelength dependency of the protein phosphorylation in oat protoplasts were investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography. Red light(660 nm) irradiation of the protoplasts increased the phosphorylation of 15 different proteins, and the phosphorylation of 2 proteins(27 KDa, 32 KDa) out of 15 were observed to be dependent on the wavelength of the irradiating light. The phosphorylation densities of these two proteins increased up to two or three hundred percent during a three-minute period of irradiation. The phosphorylation of these two proteins revealed a red/far-red photoreversibility of phytochrome. When a calcium ion chelator(2 mM EGTA) was added into the cell suspension, the phosphorylations of all the proteins were reduced about 200%. These findings suggest that phytochrome action and Ca^{2+} influx are certainly involved in the in vivo phosphorylation of proteins in oat protoplasts.

A representative signal transduction in the plant kingdom is photomorphogenesis that begins with the absorption of red light by the photoreceptor phytochrome(1). It was reported that the physiologically active form of phytochrome(P_{fr}) might initiate the photomorphogenetic cell responses by controlling the cytosolic Ca^{2+} concentration(2). In addition to the role of ca^{2+}, the regulatory role of protein phosphorylation by the calcium-dependent protein kinases was also suggested as an important step in signal transduction in plant systems(3-7).

There have been several reports describing the action of phytochrome on the fluxes of Ca^{2+} through the plasma membrane(8,9). Two workers(8,9) reported that irradiation of the cells with red light enhanced ca^{2+} influx. In contrast, an efflux of ca^{2+} by irradiation of red light was observed in the mesophyll cells of Vallisneria gigantea(10).

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Abbreviations : DG, diacylglycerol ; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid ; ^{32}Pi, [^{32}P]orthophosphate ; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-291X/89 $1.50

Data obtained in this study suggested that phytochrome action was certainly involved in the processes of Ca^{2+} influx and the phosphorylation of proteins.

MATERIALS AND METHODS

Materials

Cellulase and pectinase used for the preparation of protoplasts were the products of Yakult Honsa. [^{32}P]orthophosphate(^{32}Pi:1 mCi/ml) was purchased from Amersham, and the rest of the chemicals used in these experiments were obtained from Sigma Chemical Company. Oat seed(Avena sativa L.CV Garry) was purchased from Stanford Seed Co., Buffalo, NY, U.S.A.

Oat seedlings

Fifty grams of oat seed was soaked at 4°C for 24 hours in darkness. The soaked seed was spread on wet vermiculite(100g/tray) in aluminum trays(35 x 45 cm), and the trays were put in an oat-growing box made of wood. The oats were grown at 25°C for 5 days in complete darkness.

Preparation of protoplasts

The preparation of protoplasts was mainly carried out as previously described(11,12). The washed oat tissue was cut into 1 mm pieces, which were then placed in the enzyme solution(0.6 M Sorbitol, 1mM $CaCl_2$, 0.05% BSA, 0.5% Cellulase, 0.5% Macerozyme, pH 6.5). After allowing the mixture to react at 25°C for 7.5 hours with gentle shaking(circular motion), it was centrifuged at 200 x g for 5 minutes and then filtered through a nylon mesh(100 µm). The enzyme solution was then removed by washing with the following buffer solution (0.6 M Sorbitol, 0.05% BSA, 1mM $CaCl_2$, pH6.5). After washing, the protoplasts were centrifuged at 1600 rpm for 10 minutes to remove the washing buffer. Sucrose density gradient centrifugations of the pelleted protoplasts were carried out at 1600 rpm for 8 minutes by using a stepwise gradient consisting of buffer I(0.6 M Sucrose, 1mM $CaCl_2$), buffer II(0.5M Sucrose, 0.1 M Sorbitol, 5 mM Mes, 1 mM $CaCl_2$), and buffer III(0.6M Sorbitol, 5 mM Mes, 1mM $CaCl_2$). The protoplasts banded between buffer II and buffer III.

In vivo protein phosphorylation

Purified protoplasts were suspended in the Pi-loading buffer(0.6 M Sorbitol, 25 mM Mes, 10mM $NaHCO_3$, 2 mM KH_2PO_4, 25mM Hepes, 1 mM $CaCl_2$, pH 5.8), and then ^{32}Pi(0.5 mCi) was added to the suspension. The suspension was incubated at 4°C for 2 hours ; gentle shaking was carried out every 10 minutes during the incubation. At the end of the incubation, the excess ^{32}Pi in the medium was removed by washing with ^{32}Pi-loading buffer.

Irradiation of light and SDS-PAGE

Protoplasts incorporated with ^{32}Pi were irradiated with monochromatic light of various wavelengths(400 nm, 500 nm, 660 nm, 730 nm ; the monochromatic light was generated by a 150 watt Xe lamp equipped in the spectrofluorometer, Hitachi MPF 3000) and the effect of the light was observed as a function of irradiation time. Reaction was terminated by adding SDS-PAGE sample buffer(0.125 M Tris, 4% SDS, 20% Glycerol, pH 6.5) to the irradiated protoplasts, and the proteins were reduced by the addition of 10 µl of 2-mercaptoethanol and heating at 100°C for 5 minutes. SDS-PAGE was performed by using 2% stacking gels and 10% resolving gels. After electrophoresis and drying the gels, autoradiography was carried out at -70°C for 7 days on X-ray film, and phosphorylation densities of the proteins were analyzed by a densitometer(LKB 2202).

RESULTS AND DISCUSSION

We investigated the effects of red light and Ca^{2+} on the phosphorylation of the intracellular proteins in oat protoplasts incorporated with ^{32}Pi. The

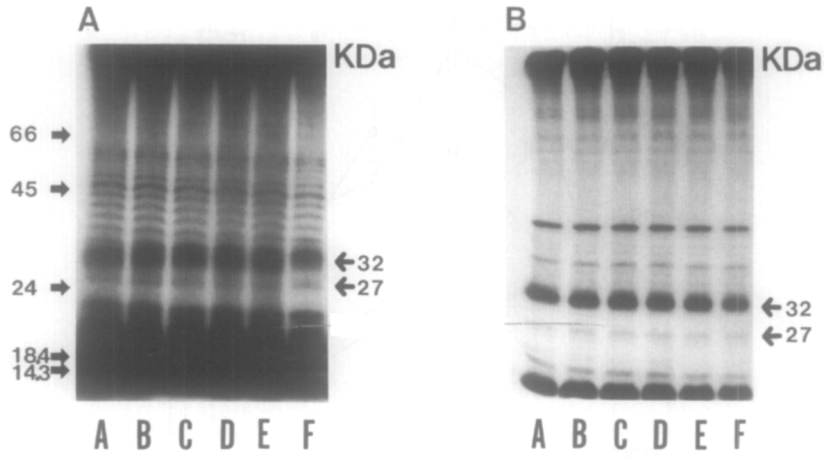

Fig.1. Autoradiograms of SDS-PAGE-separated proteins of irradiated oat protoplasts.
(A) Autoradiography was carried out after SDS-PAGE of the oat protoplasts (1 X 10^6/ml) that had been incorporated with ^{32}Pi in the presence of the external $CaCl_2$ (1 mM) in the suspension medium. The protoplast sample was irradiated with red light (660 nm) for different periods of time; lanes A to F are the autoradiograms of the sample irradiated with red light for 1,2,3,4 and 5 min. respectively.
(B) Autoradiography was carried out under the same conditions as (A) except for the removal of the external Ca^{2+} by the addition of 2 mM EGTA and 1 mM $MgSO_4$. Lanes A to F show the same irradiating times as in (A).

sample was irradiated with the red light for different times in the presence of 1 mM external Ca^{2+} (Fig.1A) or its absence (Fig.1B) and analyzed by SDS-PAGE, followed by autoradiography. The autoradiogram showed the presence of 15 protein bands, and their molecular weights ranged from 19-62.5 KDa. According to the densitometric analysis of these autoradiograms, red light irradiation in the presence of external Ca^{2+} increased the phosphorylations of all the proteins. Maximum phosphorylations were obtained after 2-3 minutes of red light irradiation in the presence of 1 mM external Ca^{2+} (Fig.1A), but when the Ca^{2+} influx was blocked by the addition of EGTA in the medium, the phosphorylations were drastically reduced (Fig.1B).

The degrees of phosphorylation of all the proteins were plotted as a function of the red light irradiation time in the presence of 1 mM external $CaCl_2$ or in its absence. Figure 2 shows the results obtained for two proteins (27 KDa, 32 KDa) ; these data are typical of the results observed for the other proteins (data not shown). As we can see, the phosphorylations of these two proteins were increased by irradiation of red light when the Ca^{2+} influx was possible. Phosphorylation was maximized with 2-3 minutes of red light irradiation, but a drastic decrease in the phosphorylation occurred when the Ca^{2+} influx was blocked by its chelator EGTA. These results certainly imply that phosphorylation of the intracellular proteins is controlled by Ca^{2+}, and the red light receptor (phytochrome ?) plays a role in the influx of Ca^{2+} into the cell.

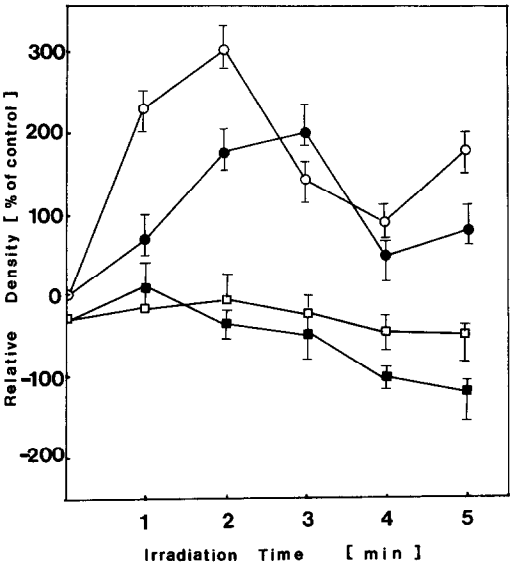

Fig.2. Change of phosphorylation densities of the two proteins (27 KDa, 32 KDa) by varying the irradiation time of red light(660 nm). Autoradiograms of the SDS-gels presented in Fig.1(A) and (B) (from lanes A to F) were analyzed by densitometry, and the relative densities of the two protein bands vs irradiation time were plotted [27 KDa(●) and 32 KDa(○) proteins in the presence of the external Ca^{2+} (1 mM) and 27 KDa(■) and 32 KDa(□) proteins in the absence of the external Ca^{2+}].

To obtain evidence to indicate that phytochrome participates in the protein phosphorylation process, we monitored the changes in the degree of phosphorylations brought about by varying the wavelength of the irradiating light. Figure 3 shows the autoradiograms obtained after the sample was irradiated

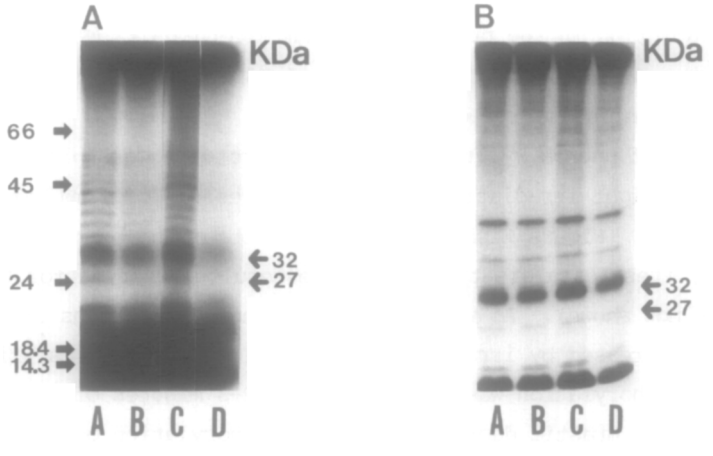

Fig.3.
(A) Autoradiogram of SDS-gel; the ^{32}Pi-incorporated protoplast sample containing 1 mM external Ca^{2+} was irradiated by light of various wavelengths for 3 min. (lane A, 400 nm; lane B, 500 nm; lane C, 660 nm; lane D, 730 nm). (B) These autoradiograms were obtained from the same sample under the same conditions as (A) except that all the external Ca^{2+} was removed by 2 mM EGTA.

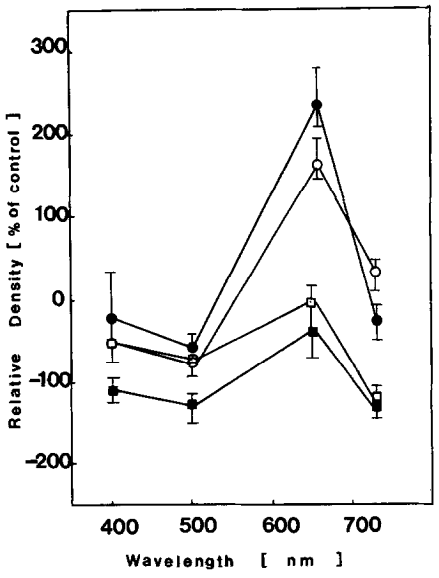

Fig.4. Change of the phosphorylation densities of the 27 KDa and 32 KDa proteins by varying the wavelength of the irradiating light. A red/far-red photoreversibility of phytochrome was observed regardless of the presence or absence of external Ca^{2+}[27 KDa(●) and 32 KDa(○) in the presence of 1 mM external Ca^{2+} and 27 KDa(■) and 32 KDa(□) without external Ca^{2+}].

with different wavelengths of light in the presence of external Ca^{2+} and in its absence. According to the densitometric analysis of these autoradiograms, only two proteins (27 KDa, 32 KDa) out of 15 revealed an action spectra similar to the absorption spectrum of phytochrome Pr(Fig.4). The rest of the proteins did not show photoreversibility by irradiation of far-red light(Figures are not shown in this paper). Lower phosphorylation by irradiating with blue light(400-500 nm) may be due to an involvement of the blue light receptor whose function for the Ca^{2+} fluxes is different from that of the red light receptor(13,14). However, a suppression of protein phosphorylation by irradiating with far-red light can be attributed to the photoreversible behavior of phytochrome ($Pr \underset{730\ nm}{\overset{660\ nm}{\rightleftharpoons}} Pfr$). It was observed that the phytochrome Pfr form stimulated the influx/mobilization of ca^{2+} into the cytosol of oat cells, and the Pr form functioned in the uptake of the cytosolic Ca^{2+} into the Ca^{2+} pools in the cell(15). The fact that phosphorylation of all the proteins was enhanced by red light irradiation and only two proteins out of fifteen showed photoreversibility in the phosphorylation requires further interpretation. A possible interpretation can be proposed under the assumption that phytochrome is the only red light receptor for controlling the Ca^{2+} influx by the red light, and the phosphorylation of the intracellular proteins is mainly dependent on the intracellular Ca^{2+} concentration. At the moment, we would like to consider two different pathways for the protein phosphorylation since Ca^{2+} is involved in this reac-

tion : one pathway is the phosphorylation by the Ca^{2+}/calmodulin-dependent protein kinase, and the other is phosphorylation by the Ca^{2+}/DG-dependent kinase. When we consider that the Ca^{2+} concentration required for the activation of the Ca^{2+}/DG-dependent protein kinase is much less than that for the former(16), it can be presumed that phosphorylation of the two proteins showing photoreversibility occurs because of the Ca^{2+}/calmodulin dependent protein kinase and the remaining proteins are phosphorylated through the Ca^{2+}/DG-dependent protein kinase. One further point that we must consider is the fact that the phosphorylations of the two proteins are photoreversible even after blocking the Ca^{2+} influx with EGTA. This agrees with the observation that Ca^{2+} mobilization and uptake can be caused by irradiation with the red/far-red light(15).

ACKNOWLEDGMENT

This work was supported by a grant from the Korea Science and Engineering Foundation.

REFERENCES

1. Roux,S.J. (1984) Bioscience. 34, 25-29.
2. Hepler,P.K. and Wayne,R.O. (1985) Annu. Rev. Plant Physiol. 36, 397-439.
3. Veluthambi,K. and Poovaiah,B.W. (1984) Plant Physiol. 76, 359-365.
4. Mcfadden,J.J. and Poovaiah,B.W. (1988) Plant Physiol. 86, 332-334.
5. Blower,D.P., Boss,W.F., and Trewavas,A.J. (1988) Plant Physiol. 86, 505-509.
6. Chen,Y.R. and Roux,S.J. (1986) Plant Physiol. 81, 609-613.
7. Veit Otto and Eberhard Schäfer (1988) Plant Cell Physiol. 29, 1115-1121.
8. Miyoshi,Y. (1986) Proceedings of the XVI Yamada Conference Photomorphogenesis in Plants, PP 101, Okazaki, Japan.
9. Acton,G.J. (1986) Proceedings of the XVI Yamada Conference Photomorphogenesis in Plants, PP 105, Okazaki, Japan.
10. Takagi,S. and Nagai,R. (1988) Plant Physiol. 88, 228-232.
11. Goldstein,A.H. and Hunziker,A.D. (1985) Plant Physiol. 77, 1013-1015.
12. Edwards,G.E., Robinson,S.P., Tyler,N.J.C., and Walker,D.A. (1978) Plant Physiol. 62, 1313-1319.
13. Briggs,W.R and Iino,M. (1983) Phil. Trans. R. Soc. B303, 345.
14. Shinkle,J.R. and Russel,L.J. (1986) Proceedings of the XVI Yamada Conference Photomorphogenesis in Plants, PP 127, Okazaki, Japan.
15. Chae,Q., Park,H.J. and Hong,S.D (1989) Biochim. Biophys. Acta, submitted for publication.
16. Rasmussen,H. and Barrett,P.Q. (1984) Physiol. Rev. 64, 938-984.