Pergamon

PII: S0031-9422(96)00624-3

Phytochemistry, Vol. 44, No. 5, pp. 775–780, 1997

0031-9422/97 $17.00+0.00

RAPID BLUE-LIGHT-INDUCED PHOSPHORYLATION OF PLASMA-MEMBRANE-ASSOCIATED PROTEINS IN WHEAT

VIJAY K. SHARMA, PRADEEP K. JAIN, SATISH C. MAHESHWARI* and JITENDRA P. KHURANA†

Department of Plant Molecular Biology, University of Delhi, South Campus, Dhaula Kuan, New Delhi-110021, India; * Department of Botany, University of Delhi, Delhi-110007, India

(*Received in revised form* 29 *April* 1996)

Key Word Index—*Triticum aestivum*; Gramineae; wheat; protein phosphorylation; plasma membrane; blue light; staurosporine.

Abstract—When the microsomal membranes isolated from 4-day-old etiolated wheat seedlings are irradiated *in vitro* with blue light and subjected to phosphorylation with [γ-^{32}P]ATP *in vitro*, the polypeptides of 110, 102, 82 and 70 kDa are heavily phosphorylated. The induction of phosphorylation is extremely rapid as it can be triggered by even 5 sec blue light irradiation, although maximal response is obtained with 15 sec blue irradiation. The response is observed most strongly in the microsomal fraction isolated from the highly photosensitive tip portion of the seedlings, and its intensity is higher in the leaf than in the tubular coleoptile fraction. The aqueous two-phase partitioning of the microsomal fraction has shown that all these four blue-light-responsive phosphopolypeptides are associated with the plasma membrane. The kinase involved in phosphorylating these polypeptides *in vitro* is sensitive to staurosporine. The presence of blue-light-responsive phosphopolypeptides in both the coleoptile sheath and the leaf encased inside, and the kinetics of induction of this response indicate that the phosphorylation of plasma membrane proteins may be one of the early steps in blue-light-elicited signal transduction chain, associated not only with phototropism but perhaps also with low-fluence blue responses in general.

INTRODUCTION

Blue light is known to regulate many diverse responses in higher plants, including phototropism, cessation of stem growth, stomatal opening and changes in gene expression [1]. Although the sensory chromoproteins involved in perceiving and transducing the blue light signal have not been characterized biochemically, available physiological data and molecular genetic analysis suggest that more than one receptor exists for blue light, with a flavin or a carotenoid, such as zeaxanthin, as the probable chromophore [2, 3]. The evidence for the existence of multiple photoreceptors for blue light in higher plants has also been provided through analysis of blue light response mutants of *Arabidopsis* [4–8]. Concomitant with these developments, rapid progress has also been made in the identification of the components of the signal transduction pathway which is initiated following photoexcitation of the presumptive blue light receptor [1, 9]. Besides the elucidation of signalling elements operative in stomatal opening/closing, blue-light-induced phosphorylation of membrane protein of several plants and stimulation of GTPase activity in pea seedling membranes have been claimed to be two of the early signalling events that may be associated with phototropism, a low-fluence blue response [1, 9, 10].

Rapid blue-light-induced phosphorylation of a M_r 120×10^3 polypeptide associated with the microsomal fraction of pea was first reported by Gallagher *et al.* [11]. Subsequently, it was demonstrated that the protein is associated with the plasma membrane, and the fluence required for threshold and saturation of the phototropic response matches that for blue-light-induced phosphorylation [12, 13]. Furthermore, the tissue that perceives the phototropic stimulus is the one that shows the strongest phosphorylation response to blue light [12, 14]. Studies employing phototropism mutants of *nph*1 (*non-p*hototropic *h*ypocotyl) class, including JK224 (now redesignated *nph* 1-2) [5, 15], which lack blue-light-induced phosphorylation of M_r 120 $\times 10^3$ polypeptide in crude homogenates [15, 16], provided credibility for the assumption that rapid blue-light-induced phosphorylation of a M_r 120×10^3 membrane polypeptide

† Author to whom correspondence should be addressed.

constitutes a signalling step in the phototropism transduction chain. Besides pea and *Arabidopsis*, blue-light-mediated phosphorylation of a membrane protein with an M_r of 100–130 $\times 10^3$ has also been reported from several other plant species (pea, zucchini, sunflower, *Arabidopsis*, tomato, barley, wheat, maize and oat) representing both dicots and monocots [16–19], indicating that this system is probably ubiquitous in higher plants.

In the present study, the effect of blue light on *in vitro* phosphorylation of membrane polypeptides isolated from young dark-grown wheat seedlings was investigated in detail. We report here that blue light enhances phosphorylation of at least four polypeptides in the M_r range 110–70 $\times 10^3$. The kinetics of phosphorylation and location of these polypeptides reflects their possible role in early steps of a blue-light-triggered signal transduction sequence.

RESULTS

Phosphorylation of membrane proteins—sensitivity to blue light

The microsomal and the plasma membrane fraction were isolated from 4-day-old etiolated wheat seedlings as described in the Experimental section. After extraction and solubilization, the protein samples were kept either in the dark or irradiated *in vitro* with blue light, and then subjected to phosphorylation *in vitro* by adding [γ-^{32}P]ATP. *In vitro* irradiation with blue light did not stimulate any detectable change in the gross polypeptide profile of the microsomal fraction resolved on 5–20% SDS–polyacrylamide gel and stained with Coomassie brilliant blue R-250 (Fig. 1(A)). Although short R and FR irradiations had no detectable effect (data not shown), *in vitro* irradiation with blue significantly enhanced the phosphorylation of a M_r 110 $\times 10^3$ polypeptide (Fig. 1(B)), and a few other polypeptides with M_r between 110 and 70 $\times 10^3$. The autoradiogram shown in Fig. 1(B) also shows that the maximum response was achieved with 15 sec blue irradiation, and longer irradiation of 1–2 min was not as effective, although the extent of phosphorylation was still higher than in the unirradiated control; even 5 sec of blue light exposure was sufficient to cause a detectable change in the phosphorylation (data not shown). In a separate experiment, blue irradiation for 1–2 min was found to be very effective in enhancing phosphorylation, but with 5 min blue irradiation the increase in response was negligible (data not shown).

Tissue distribution of blue-light-sensitive polypeptides

The distribution of the blue-light-sensitive polypeptides along the vertical axis of the seedling was examined. Microsomal fractions were thus isolated from different segments and phosphorylation performed after 2 min irradiation with blue light. As is evident from the autoradiogram shown in Fig. 2, blue-light-induced phosphorylation of M_r 110, 102, 82 and 70 $\times 10^3$ polypeptides was more intense in the top 1 cm segment (S1) compared with the immediately lower 1 cm segment (S2) of the seedling. The next two segments of the seedling did not show any significant increase in phosphorylation of blue-light-sensitive polypeptides (data not shown). This suggests that the tip of the seedling is more photoresponsive, thus resulting in heavy phosphorylation of polypeptides in this segment. Alternatively, these photosensitive polypeptides may be relatively more abundant in the top 1 cm segment of the seedling.

To localize these changes in different tissues, microsomal fractions were isolated from both the top 1 cm of coleoptiles (tubular sheath alone) and from leaves, and subjected to phosphorylation following the standard procedure. The autoradiogram shown in Fig. 3 shows that these blue-light-sensitive polypeptides are more heavily phosphorylated in the microsomal fraction isolated from the leaves than the coleoptiles devoid of leaves. Although the profiles of phosphorylated microsomal proteins from coleoptiles and leaves were slightly different (data not shown), besides these four polypeptides (M_r 110, 102, 82 and 70 $\times 10^3$) no other significant blue-light-induced change could be observed in the microsomal fraction isolated from leaves of dark-grown seedlings.

Intracellular location of blue-light-sensitive polypeptides

In order to find out the specific intracellular location of the polypeptides phosphorylated in response to blue irradiation, phosphorylation was also carried out in the plasma membrane and soluble fractions. The microsomal membrane preparation was fractionated by aqueous two-phase partitioning and the plasma membrane fraction characterized by a specific marker enzyme (β-1,3-glucan synthase II). It was found that plasma membrane fraction was about six times richer in glucan synthase II activity (292 pmol UDP-[^{14}C]glucose incorporated/mg protein/hr) compared with the microsomal fraction (45 pmol UDP-[^{14}C]glucose incorporated/mg protein/hr). The purified plasma membrane was subjected to phosphorylation. The phosphorylation profile shown in Fig. 4 shows that essentially the same polypeptides which are heavily phosphorylated in the microsomal fraction in response to blue were more intensely phosphorylated in the plasma membrane fraction. These phosphopolypeptides, however, could not be detected in the soluble protein fraction of wheat seedlings (data not shown).

Nature of the protein kinase—staurosporine sensitivity

The addition of staurosporine (10 or 100 nM, dissolved in DMSO), a potent protein kinase inhibitor, completely masked the blue-light-induced increase in

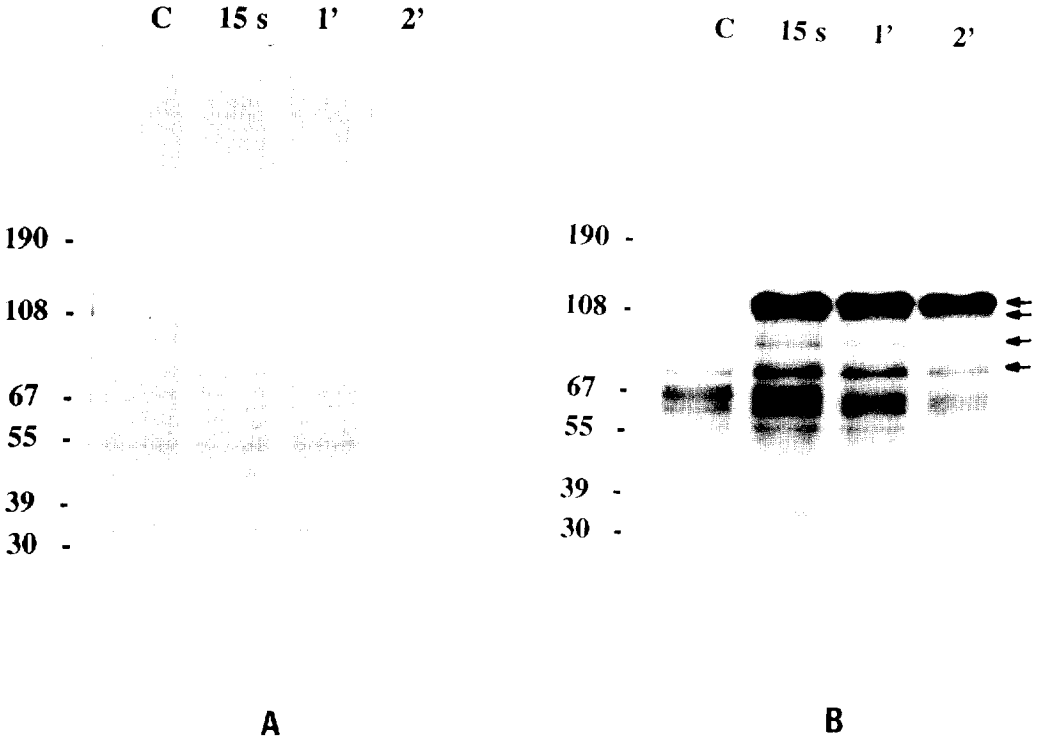

Fig. 1. Effect of blue light on the phosphorylation of proteins in the microsomal membrane fraction from 4-day-old dark-grown seedlings. (A) Coomassie brilliant blue stained gel showing the protein profile of microsomal fraction: (1) unirradiated microsomal fraction; (2–4) microsomal fraction irradiated with blue light for 15 sec, 1 min and 2 min. Protein (50 μg) was loaded in each lane and resolved on 5–20% gradient SDS–PAGE. (B) Autoradiogram showing the phosphorylation of polypeptides (indicated by arrows) associated with microsomal fraction after irradiation with blue light for different durations; for details see text. C, Control (unirradiated).

phosphorylation of all the relevant polypeptides (Fig. 5). However, staurosporine did not affect the basal level of phosphorylation of these or other polypeptides in the unirradiated samples.

DISCUSSION

The data presented here clearly demonstrate that a brief *in vitro* irradiation of microsomal fraction with blue light enhances phosphorylation of at least four polypeptides of M_r 110, 102, 82 and 70 $\times 10^3$, which in all probability are located in the plasma membrane. The phosphorylation of these polypeptides *in vitro* occurs rapidly and can be stimulated by even 5 sec of blue irradiation, but the response declines on prolonged irradiation of more than 2 min. These phosphoproteins are maximally labelled in the membranes from the most photosensitive tip portion of the wheat seedling. In general, these observations conform with earlier reports on pea [11, 20] and maize [14], except that wheat membranes clearly harbour a few more blue-light-sensitive phosphopolypeptides. In addition, the present study has also revealed that phosphorylation is more intense in the microsomal fraction polypeptides from a leaf (pulled out of the coleoptile) than from the corresponding fraction from the tubular coleoptile pool. Although the precise nature of the blue-light-induced protein kinase remains to be elucidated, the inhibition of blue-light-stimulated protein kinase activity by staurosporine indicates that a PKC (or PKA) type protein kinase is involved in phosphorylation of blue-light-sensitive polypeptides, a situation analogous to that reported for maize [21].

Blue-light-stimulated phosphorylation of membrane polypeptides(s) is of wide occurrence and is nearly ubiquitous in both monocot and dicot species, although the M_r of the polypeptide varies between 100 and 130×10^3 depending on the species [16–19]; in some species, the phosphorylation of more than one polypeptide is enhanced by blue light. In this general survey [17], preliminary work on wheat identified a blue-light-responsive polypeptide of *ca* M_r 114×10^3. In the present study, however, blue light was found to stimulate phosphorylation of at least four major polypeptides in the range M_r $110–70 \times 10^3$ which is at variance with the above observation on divergent species, including wheat. The reason for this discrepancy is not known, but it is unlikely that the four phosphopolypeptides detected in the wheat membranes are the degradation products of the M_r 110 $\times 10^3$ polypeptide, the largest moiety, since a proteolytic profile generated for pea M_r 120 $\times 10^3$ poly-

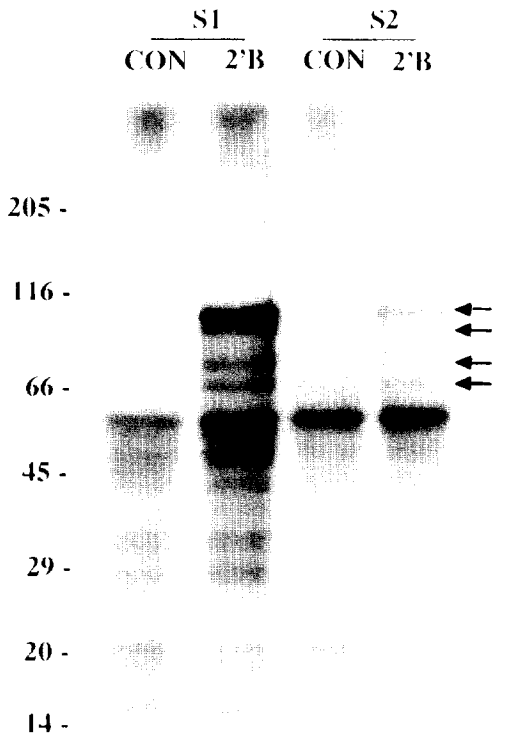

Fig. 2. The distribution of blue-light-induced phosphopolypeptides (indicated by arrows) in different segments of 4-day-old etiolated wheat seedling. S1, top 1 cm segment of wheat seedling; S2, next 1 cm below S1; CON, control (unirradiated); 2'B, 2 min blue irradiation.

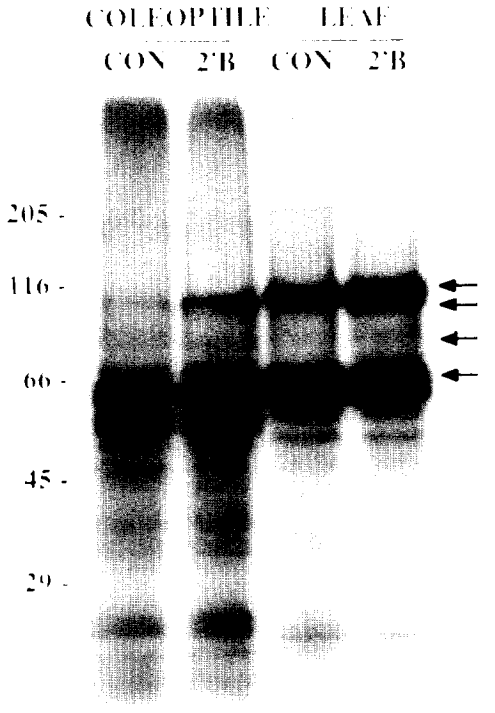

Fig. 3. The presence of blue-light-induced phosphopolypetides (indicated by arrows) in the microsomal fraction isolated from coleoptile and leaf tissue of 4-day-old etiolated wheat seedlings. The microsomal fractions were irradiated *in vitro* with blue light for 2 min (2'B), followed by phosphorylation, as outlined in the text.

peptide has revealed the existence of much smaller proteolytic fragments [20].

Although direct experimental evidence on wheat may be lacking, based on the tissue distribution and kinetics our studies support the idea proposed earlier by Short and Briggs [9] that enhanced phosphorylation of these polypeptides may be associated with some low-fluence, blue-light-induced responses, such as phototropism. This view has also gained substantial support from studies employing phototropism mutants of *Arabidopsis* [4, 15]. It has been demonstrated that blue-light-induced phosphorylation of the M_r 120 $\times 10^3$ polypeptide is completely lacking in the membrane preparations of the presumptive blue-light receptor mutants of the *nph*1 class, including JK224, now redesignated *nph*1–2 allele [15, 16]. However, the presence of blue-light-sensitive phosphopolypeptides even in the etiolated leaf tissue, and that too in greater abundance than in the coleoptile sheath, raises an interesting possibility that these phosphorylation changes may represent an early event of signal transduction chain leading to not only phototropism but many more low-fluence, blue-light-mediated responses. The analysis of other low-fluence, blue-light-induced responses, such as stomatal movement and expression of *cab*1 gene encoding chlorophyll a/b binding protein, in the phototropism mutants impaired also in phosphorylation of M_r 120 $\times 10^3$ polypeptide, should prove to be valuable in assessing the validity of this assumption.

EXPERIMENTAL

Source of plant material and growth conditions. Wheat (*Triticum aestivum* L. var. CPAN 1676) seeds were procured from the Wheat Directorate, Karnal. Seeds were soaked overnight under running tap water in the dark, and seedlings were grown in the dark at $27 \pm 1°$ in a BOD incubator. All operations starting with seed soaking onwards were carried out under dim-green safe light.

Isolation of microsomal fraction. The microsomal fraction was isolated from 4-day-old etiolated wheat seedlings following the procedure in ref. [16], with some modifications. The terminal 1 cm segments of wheat seedlings were harvested using scissors and floated on ice-cold water under a dim-green safe light. Harvested tissue (about 200 tips/sample) was homogenized in a prechilled mortar containing 2 ml homogenization buffer (25 mM MOPS, 0.2 M sucrose, 5 mM BAPTA, 0.1 mM $MgCl_2$, 8 mM L-cysteine, 120 mM *N*-methyl-D-glucamine; pH 7.8). The homogenate was filtered through 8 layers of muslin cloth and centrifuged for 10 min at 9700 *g*. The supernatant was centrifuged at 100 000 *g* for 30 min (80 Ti rotor,

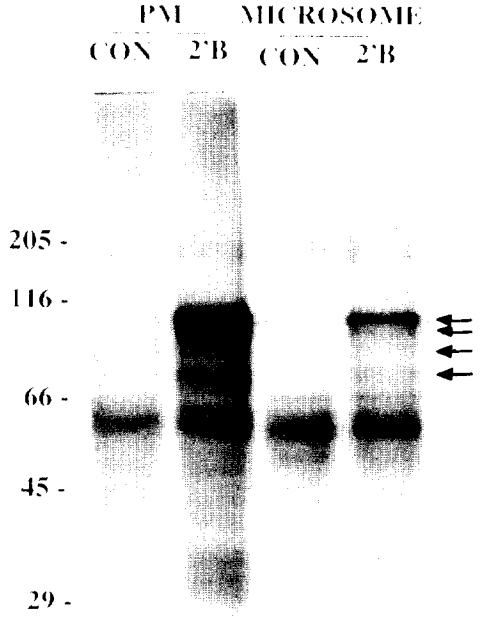

Fig. 4. Autoradiogram showing the presence of blue-light-induced phosphopolypeptides (indicated by arrows) in the plasma membrane enriched fraction. The plasma membrane was purified by aqueous two-phase partitioning of the microsomal fraction and subjected to phosphorylation *in vitro*. For details see text. PM, Plasma membrane fraction; Microsome, microsomal protein fraction; CON, control (unirradiated); 2'B, 2 min blue irradiation.

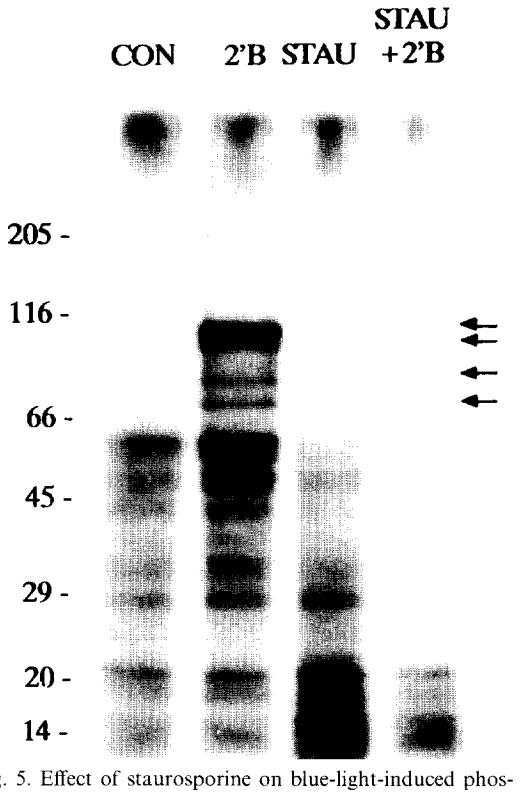

Fig. 5. Effect of staurosporine on blue-light-induced phosphorylation of microsomal fraction proteins (indicated by arrows) isolated from 4-day-old etiolated wheat seedlings. Samples preincubated for 5 min in the presence or absence of staurosporine (Stau) were irradiated with 2 min blue light (2'B) and subjected to phosphorylation.

Beckman L8–70M ultracentrifuge). The resultant pellet was resuspended in 150 μl resuspension medium (0.25 M sucrose, 4 mM KNO_3, 5 mM K_2PO_4 and 120 mM *N*-methyl-D-glucamine, adjusted to pH 7.8 with H_2SO_4).

Isolation of plasma membrane. The plasma membrane fraction was isolated employing aqueous two-phase partitioning following the procedure in [22] with minor modifications. About 100 g tissue was homogenized in 300 ml chilled isolation medium consisting of 50 mM Tris, 0.2 M sucrose, 1 mM EDTA, 0.1 mM $MgCl_2$ and 2.5 mM DTT, adjusted to pH 8.0. The homogenate was filtered through 2 layers of muslin cloth. The filtrate was centrifuged at 9700 *g* for 10 min and the supernatant recentrifuged at 100 000 *g* for 30 min. The pellet was resuspended in 5 mM K-Pi buffer, pH 7.8, containing 0.33 M sucrose and 3 mM KCl, and 0.9 g (3–4 mg protein) of the suspension was added to 2.7 g of the two-phase partitioning mixture, to give a final 3.6 g phase system, with a final composition of 6.5% (w/w) Dextran T500, 6.5% (w/w) PEG 3350, 0.33 M sucrose, 3 mM KCl and 5 mM K-Pi pH 7.8. The phase system was thoroughly mixed by 30–40 inversions of the tube, and phase settling was facilitated by centrifugation in a SW-28 swinging bucket rotor (Beckman L8-70M ultracentrifuge) at 1500 *g* for 5 min. Then the upper phase was removed with a Pasteur pipette and repartitioned twice with fresh lower phase to increase the purity. The final PEG phases were diluted at least two-fold with the resuspension medium consisting of 0.25 M sucrose, 4mM KNO_3, 5 mM K_2PO_4 and 120 mM *N*-methyl-D-glucamine, adjusted to pH 7.8 and centrifuged at 100 000 *g* for 30 min. The pellet was resuspended in an appropriate amount of resuspension medium. All operations were performed under dim-green safe light, at 4°.

Enzyme assay. β-1,3-Glucan synthase II activity was monitored in various fractions using, with modifications, the protocol described in [23]. Enzyme assay was done in final vol. of 100 μl containing 5 μg protein, 50 mM HEPES–KOH, pH 7.25, 0.33 M sucrose, 0.8 mM spermine, 16 mM cellobiose, 4 mM EGTA/4 mM $CaCl_2$, 1 mM DTT and 0.01% (w/v) digitonin. The reaction was initiated by adding UDP-[^{14}C]glucose (17.5 nCi) and diluted to give final conc. of 2 mM UDP–glucose. Samples were incubated at 25° for 30 min. After incubation, samples were immersed in a boiling water bath to terminate the reaction. The samples were transferred to paper filters (Whatman 3MM) which then were washed twice in 0.35 M NH_4OAc, pH 3.6, 30% (v/v) EtOH for 1 hr. The filters were dried

and the radioactivity incorporated was measured by liquid scintillation counting (Beckman, LS 1800). The enzyme activity was expressed as pmol UDP-[^{14}C]glucose incorporated/mg protein/hr.

Protein estimation. The amount of protein was estimated according to the method [24]. The standard curve was prepared using bovine serum albumin (BSA). For measurement of proteins, 10 μl of sample was mixed thoroughly with 5 ml of Bradford's reagent and *A* was read at 595 nm.

In vitro *protein phosphorylation.* The phosphorylation reaction was performed according to ref. [25], with a few modifications. Proteins (150 μg) of microsomal fraction or phase-purified plasma membranes were diluted to a final vol. of 100 μl reaction mixture in phosphorylation buffer (final concn: 30 mM Tris–MES (pH 7.0), 5 mM $MgCl_2$, 0.2 mM EGTA and 0.12 mM $CaCl_2$) and 0.5% Triton X-100 added to solubilize the membranes partially. The membrane mixture was allowed to remain for 15 min on ice, pre-equilibrated for 30 sec at 30°, and phosphorylation initiated by addition of 10 μCi [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol, BRIT, India) diluted to 100 μM final concn with unlabelled ATP. After 5 min incubation at 30°, the phosphorylation reaction was terminated by addition of 50 μl 3X Laemmli sample buffer and placed for 5 min in a boiling water bath and stored at 4° until electrophoresis.

SDS–PAGE and autoradiography. Radiolabelled polypeptides were resolved by SDS–PAGE following the method in ref. [26]; 50 μg protein was loaded in each lane. The separation gel contained 5–20% gradient acrylamide (w/v), while the stacking gel contained 5% acrylamide (w/v). Gels were stained with Coomassie brilliant blue R-250, destained, dried under vacuum, and autoradigraphed on Konica X-ray films.

Irradiations. For *in vitro* irradiation, a custom-built light source was employed using three 250 W tungsten–iodine lamps. For blue irradiation, an interference filter having λ_{max} 450 nm and 10 nm half-band width (Carl Zeiss, Germany) was employed. The incident energy of blue light reaching inside the Eppendorf tube (as measured by transmission through the plastic wall of a longitudinally cut tube) was 43 μmol m^{-2} sec^{-1}. The fluence was measured with a LI-COR (Lincoln, Nebraska) Model LI-1800 portable spectroradiometer.

Acknowledgements—The financial support by the University Grants Commission, New Delhi, and the Department of Science and Technology of the Government of India (through an equipment grant) is gratefully acknowledged. P.K.J. is thankful to CSIR, New Delhi, for the award of a Senior Research Fellowship.

REFERENCES

1. Kaufman, L. S., *Plant Physiology*, 1993, **102**, 333.
2. Ahmad, M. and Cashmore, A. R., *Nature*, 1993, **366**, 162.
3. Quinones, M. A. and Zeiger, E., *Science*, 1994, **264**, 558.
4. Khurana, J. P. and Poff, K. L., *Planta*, 1989, **78**, 400.
5. Konjevic, R., Khurana, J. P. and Poff, K. L., *Photochemistry and Photobiology*, 1992, **55**, 789.
6. Konjevic, R., Steinitz, B. P. and Poff, K. L., *Proceedings of the National Academy of Science, U.S.A.*, 1989, **86**, 9876.
7. Liscum, E. and Hangarter, R. P., *Planta*, 1993, **191**, 214.
8. Khurana, J. P., Kochhar, A. and Jain, P. K., *Genetica*, 1996, **97**, 349.
9. Short, T. W. and Briggs, W. R., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1994, **45**, 143.
10. Warpeha, K. M. F., Kaufman, L. S. and Briggs, W. R., *Photochemistry and Photobiology*, 1992, **55**, 595.
11. Gallagher, S., Short, T. W., Ray, P. M., Pratt, L. H. and Briggs, W. R., *Proceedings of the National Academy of Science, U.S.A.*, 1988, **85**, 8003.
12. Short, T. W. and Briggs, W. R., *Plant Physiology*, 1990, **92**, 179.
13. Short, T. W., Reymond, P. and Briggs, W. R., *Plant Physiology*, 1993, **101**, 647.
14. Palmer, J. M., Short, T. W. and Briggs, W. R., *Plant Physiology*, 1993, **102**, 1219.
15. Liscum, E. and Briggs, W. R., *Plant Cell*, 1995, **7**, 473.
16. Reymond, P., Short, T. W., Briggs, W. R. and Poff, K. L., *Proceedings of the National Academy of Science, U.S.A.*, 1992, **89**, 4718.
17. Reymond, P., Short, T. W. and Briggs, W. R., *Plant Physiology*, 1992, **100**, 655.
18. Hager, A. and Brich, M., *Planta*, 1993, **189**, 567.
19. Palmer, J. M., Short, T. W., Gallagher, S. and Briggs, W. R., *Plant Physiology*, 1993, **102**, 1211.
20. Short, T. W., Porst, M., Palmer, J., Fernbach, E. and Briggs, W. R., *Plant Physiology*, 1994, **104**, 1317.
21. Hager, A., Brich, M. and Bazlan, I., *Planta*, 1993, **190**, 120.
22. Larsson, C., Widell, S. and Kjellbom, P., *Methods in Enzymology*, 1987, **148**, 558.
23. Widell, S. and Larsson, C., in *The Plant Plasma Membrane*, eds C. Larsson and I. M. Moller. Springer, Berlin, 1990, p. 16.
24. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.
25. Short, T. W., Porst, M. and Briggs, W. R., *Photochemistry and Photobiology*, 1992, **55**, 773.
26. Laemmli, U. K., *Nature*, 1970, **227**, 680.