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# LIGHT- AND CALCIUM-MODULATED PHOSPHORYLATION OF PROTEINS FROM WHEAT SEEDLINGS

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**Key Word Index**—*Triticum aestivum*; wheat; Gramineae; light; calcium; phosphorylation; soluble fraction proteins.

**Abstract**—*In vivo* white light irradiation (for 1 hr or more) of four-day-old etiolated wheat seedlings followed by *in vitro* phosphorylation decreased the phosphorylation of 52 and 48 kDa polypeptides in the proteins of the soluble fraction; short pulses of white light or red/far-red were not effective. Studies using norflurazon, a bleaching herbicide, suggest that dephosphorylation of this polypeptide may be linked with light-dependent development of plastids. Studies employing a $Ca^{2+}$ chelator, EGTA, and several calmodulin (CaM) inhibitors indicate that phosphorylation of 52, 48, 34 and 31 kDa polypeptides, both in the dialysed and undialysed soluble fractions, is $Ca^{2+}$–CaM dependent. The depletion of $Ca^{2+}$ also retarded the mobility of a 52 kDa polypeptide by 4–6 kDa, particularly in the undialysed fraction, which was restored to control level by increasing the $Ca^{2+}$ level, a property unique to $Ca^{2+}$-binding proteins. Strikingly, the phosphorylation status of a doublet (17 and 15 kDa phosphopolypeptides), visible primarily in the dialysed fraction, was not affected by light and/or $Ca^{2+}$–CaM antagonists. The results suggest the existence of $Ca^{2+}$/CaM-dependent protein kinase(s) in young wheat seedlings, whose activity, directly or indirectly, is down-regulated by white light.


## INTRODUCTION

Light is an important environmental factor that profoundly influences plant growth and development. In higher plants, light is perceived by at least three sensory photoreceptors: the red/far-red reversible phytochromes, UV-A/blue light receptors and UV-B receptor(s) [1]. Although the role of G-proteins, $Ca^{2+}$-calmodulin (CaM) and cGMP in eliciting light-induced responses has recently been demonstrated [2], in comparison with animal systems, however, not much is known about the signal transduction steps triggered by the photo-excitation of these regulatory receptors in plants, leading eventually to the generation of more overt response(s). In an effort to elucidate the mechanism of action of light, particularly through phytochromes, some earlier studies demonstrated that light modulates $Ca^{2+}$ fluxes in a red/far-red reversible manner [3–5]. Since the effect of second messengers like $Ca^{2+}$ is invariably mediated through activation of specific enzymes, such as protein kinases, several attempts have been made to study light- and/or $Ca^{2+}$-mediated changes in phosphorylation of proteins in higher plants. However, only in a few cases has it been possible to demonstrate red/far-red reversibility of phosphorylation of specific proteins. This has been achieved either by directly irradiating the tissue supplied with $^{32}$Pi (oat coleoptiles [6], oat protoplasts [7] or wheat protoplasts [8]) or irradiating the tissue, followed by phosphorylation *in vitro* with [$\gamma$-$^{32}$P]ATP (*Sorghum* coleoptiles [9] or evacuolated parsley protoplasts [10]) or else by irradiating the nuclear extracts, before phosphorylation *in vitro* (pea plumules [11] or oat seedlings [12]). Similar to these reported effects of red light on phosphorylation, sufficient evidence is also available on the involvement of $Ca^{2+}$ in regulation of phosphorylation [13–18]. However, there is only one report where phytochrome-induced changes in phosphorylation have been shown to be directly related to changes in $Ca^{2+}$. Fallon *et al.* [8] have shown that red light-induced changes in phosphorylation of 60 and 70 kDa polypeptides, in wheat protoplasts, follows an essentially similar kinetics of increase as

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detected for transient increase in $[Ca^{2+}]_i$ levels induced by red light.

Earlier studies in our laboratory have also demonstrated that phytochrome regulates $Ca^{2+}$ fluxes in wheat protoplasts, probably by modulating the activity of ion channels and pumps [5]. In order to identify further steps in the signalling cascade of phytochrome, we have directed our efforts to study light- and $Ca^{2+}$-induced changes in phosphorylation of proteins of various cellular fractions of etiolated wheat seedlings. Recently, we have demonstrated that white light (probably through phytochrome) and $Ca^{2+}$ down-regulate the phosphorylation status of a 120 kDa mitochondrial polypeptide in wheat seedlings [19, 20]. Our studies also reveal that blue light rapidly enhances phosphorylation of at least four wheat plasma membrane polypeptides [21]; however, blue light acts independently of $Ca^{2+}$.

In the present communication, the effects of light and $Ca^{2+}$ on phosphorylation of wheat soluble fraction proteins have been reported. Although in this study short-term red, far-red or blue light treatments have apparently been of no consequence, white light and $Ca^{2+}$ have been found to influence the phosphorylation status of two prominent polypeptides of *ca* 52 and 48 kDa, and a few others. One of these phosphopolypeptides (52 kDa) also appears to have $Ca^{2+}$-binding capacity and could possibly be playing a role in plant development.

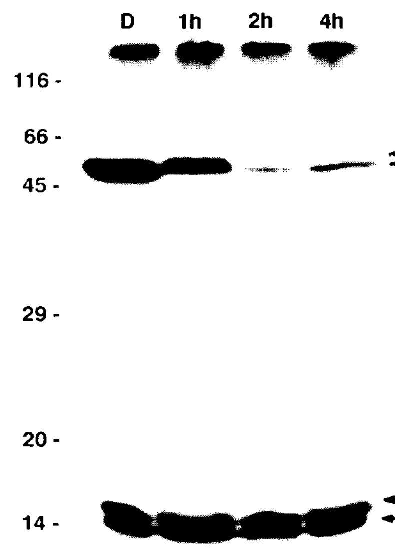


Fig. 1. Phosphorylation profile of soluble fraction proteins isolated from four-day-old dark-grown seedlings irradiated with white light for different times. Soluble proteins, isolated and dialysed overnight, were subjected to phosphorylation with [$\gamma$-$^{32}$P]ATP, resolved by 12.5% SDS–PAGE and autoradiographed. Lane 1, unirradiated dark control; lanes 2, 3 and 4 irradiated for 1, 2 and 4 hr, respectively.

## RESULTS AND DISCUSSION

The soluble fraction contains endogenous substrate proteins which are the primary target of protein kinase(s) and phosphatase(s) activated by the second messengers released upon stimulus perception. The effect of light, the primary stimulus, and $Ca^{2+}$, a possible second messenger in light action, was therefore examined in regulating the phosphorylation of soluble fraction proteins.

### *Light-induced changes in protein phosphorylation*

For studying the effect of light on protein phosphorylation, dark-grown seedlings were pre-irradiated with light for various times and the soluble fraction proteins were subjected to phosphorylation *in vitro* with the addition of [$\gamma$-$^{32}$P]ATP. White light irradiation for 1 hr resulted in *decreased* phosphorylation of two prominent polypeptides of 52, 48 kDa and a few other polypeptides, and the response was further accentuated with prolonged irradiation of 2 and 4 hr (Fig. 1). There was no apparent change in corresponding Coomassie-stained protein profiles (data not shown). However, short-term (up to 15 min) *in vivo* (or *in vitro*) irradiation with white, red, far-red or blue light had no detectable change in the phosphorylation profile of soluble fraction proteins (data not shown). This is in contrast to some earlier reports where short-term red irradiation *in vivo* and *in vitro* has been found to enhance the phosphorylation of 55, 67 and 70 kDa polypeptides of *Sorghum* coleoptiles [9], 60 and 70 kDa polypeptides of wheat protoplasts [8] and 40 kDa polypeptide of evacuolated parsley protoplasts [10].

To find out whether these white light dependent changes occurring in protein phosphorylation are coupled with plastid development usually triggered with the onset of light, experimental seedlings were grown in the presence of norflurazon, a bleaching herbicide. Norflurazon is known to inhibit the synthesis of carotenoids and, as a consequence, photobleaching of chlorophylls occurs under high intensity illumination, leading to arrest of plastid development [22]. The Coomassie-stained protein profile of the soluble fraction isolated from seedlings grown in the presence of norflurazon does not show the accumulation of the large (*ca* 55 kDa) and the small (*ca* 14 kDa) subunits of Rubisco and this effect is more dramatic in seedlings grown in light (Fig. 2(A)). The autoradiogram in Fig. 2(B) shows that the phosphorylation of a doublet of 15 and 17 kDa in the dialysed fraction, which is not influenced by light, is marginally reduced in norflurazon-treated tissue extracts. Interestingly, norflurazon not only reversed the inhibitory effect of light, but even enhanced the phosphorylation of 52, 48, 34, 31 and 21 kDa polypeptides (compare lanes G and GN; Fig. 2(B)). It appears that the phosphorylation of some polypeptides is down-regulated both by light and/or development stimuli (even in the dark) and norflurazon is able to enhance the phosphorylation by obviating their repressive effects.

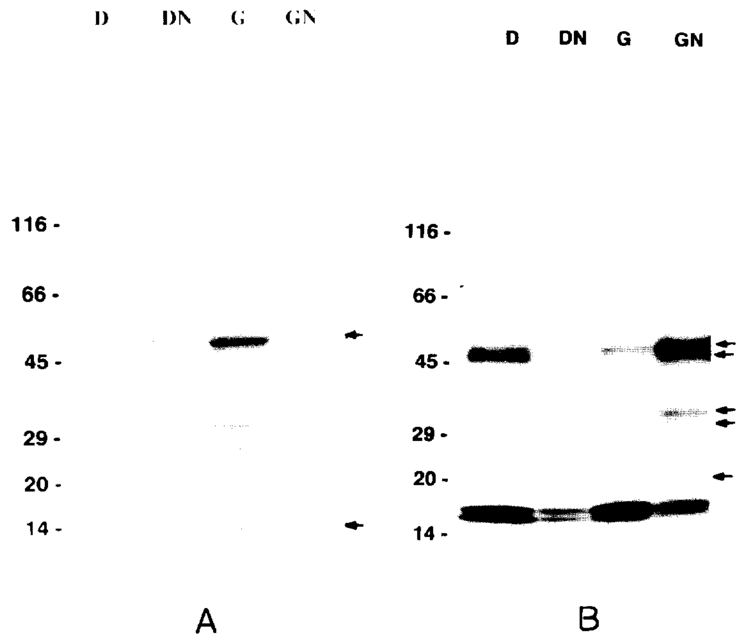


Fig. 2. Autoradiogram showing the effect of norflurazon on phosphorylation of soluble fraction proteins isolated from four-day-old wheat seedlings grown in dark (D) or light (G), with or without norflurazon. (A) Coomassie brilliant blue-stained gel resolved on 5–20% acrylamide gradient; (B) autoradiogram of the same gel. Lane D, dark control; lane DN, seedlings grown in dark with 100 $\mu$M norflurazon; lane G, seedlings grown under white light; lane GN, seedlings grown under white light with 100 $\mu$M norflurazon. For details, see legend to Fig. 1.

The effect of white light on the phosphorylation of undialysed soluble fraction proteins was also examined, but essentially similar results as with the dialysed fraction were obtained. However, the phosphorylation of some polypeptides was either enhanced or decreased upon dialysis of soluble fraction proteins, as would become obvious from the autoradiograms shown in Figs 3 and 5.

### *Effect of* $Ca^{2+}$ *and* CaM *antagonists*

The effect of $Ca^{2+}$ was investigated both on the dialysed and undialysed soluble fraction either by adding more $Ca^{2+}$ exogenously or by depleting the $Ca^{2+}$ by adding EGTA, a $Ca^{2+}$ chelator, in the reaction mixture.

### *Dialysed extracts*

In the dialysed fraction isolated from dark-grown and light-grown seedlings, depletion of $Ca^{2+}$ by adding 0.2 mM EGTA resulted in the dephosphorylation of 52, 48, 34 and 31 kDa polypeptides, and addition of 0.2 mM $Ca^{2+}$ restored the phosphorylation status of these polypeptides (Fig. 3). Strikingly, a doublet of 17 and 15 kDa was not affected at all by this concentration of EGTA and served as a good control for comparison.

To investigate the involvement of CaM, if any, in regulating the phosphorylation of these polypeptides, CaM and various CaM antagonists were added to the reaction mixture. Although CaM did not have any significant effect on the four phosphopolypeptides (mentioned above) whose phosphorylation was $Ca^{2+}$-dependent, the phosphorylation of both 52 and 48 kDa polypeptides was effectively inhibited by the CaM antagonists chlorpromazine (CPZ), trifluoperazine (TFP), $W_7$ and fluphenazine (FP), when tried at 100 $\mu$M (Fig. 4). Since these phenothiazine drugs are known to exert non-specific effects at higher

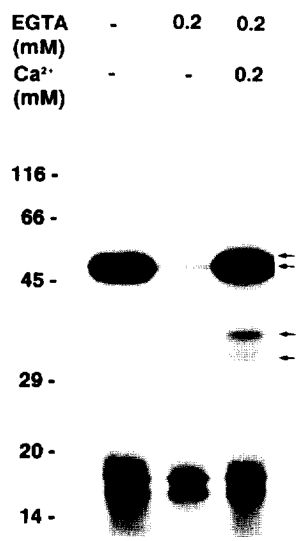


Fig. 3. Effect of $Ca^{2+}$ on *in vitro* phosphorylation of dialysed soluble fraction proteins isolated from four-day-old etiolated wheat seedlings. Lane 1, control; lane 2, 0.2 mM EGTA; lane 3, 0.2 mM EGTA and 0.2 mM $Ca^{2+}$. For details, see Experimental.

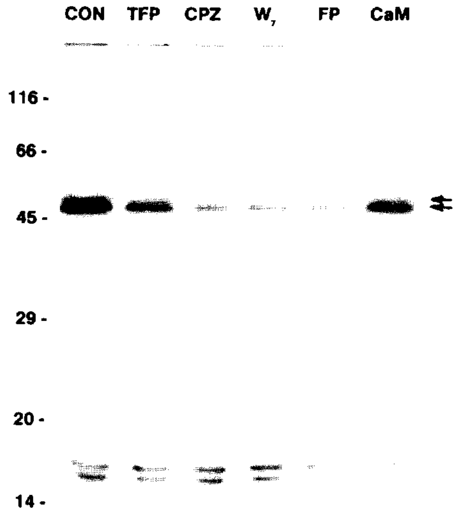


Fig. 4. Effect of CaM antagonists on phosphorylation of dialysed soluble fraction proteins of four-day-old etiolated wheat seedlings. CaM antagonists were added to reaction mixture and incubated for 5 min before the reaction was started by adding [$\gamma$-$^{32}$P]ATP. Lane 1, control; lane 2, 100 $\mu$M trifluoperazine (TFP); lane 3, 100 $\mu$M chlorpromazine (CPZ); lane 4, 100 $\mu$M $W_7$; lane 5, 100 $\mu$M fluphenazine (FP); lane 6, 2.5 units of spinach CaM.

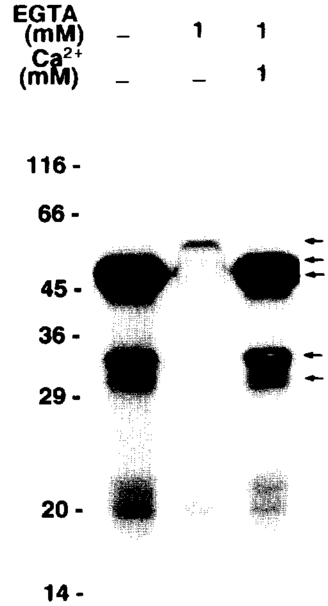


Fig. 5. Effect of $Ca^{2+}$ on phosphorylation of undialysed soluble fraction proteins isolated from four-day-old etiolated seedlings. Lane 1, control; lane 2, 1 mM EGTA; lane 3, 1 mM EGTA and 1 mM $Ca^{2+}$. (Note the shift in mobility of 52 kDa band in lane 2.)

concentrations [23], in this study, they were also tried at lower concentrations and at least TFP and FP were found to be effective even at the 10 or 50 $\mu$M level, although their inhibitory effect was less pronounced at these concentrations (data not shown). These data thus indicate the presence of endogenous substrates for both $Ca^{2+}$-dependent and $Ca^{2+}$/CaM-dependent protein kinases in the soluble fraction. In an earlier study [16] it was also observed that $Ca^{2+}$-stimulated phosphorylation of 49 and 47 kDa polypeptides of the soluble fraction (40 000 $g$ supernatant) from the endosperm of another species of wheat, *Triticum turgidum*, and also showed inhibition by CPZ and TFP; in this study too, CaM itself was ineffective. Similarly, Veluthambi and Poovaiah [14, 15, 24] have shown $Ca^{2+}$/CaM-dependent phosphorylation of soluble fraction proteins of maize, and the activity of soybean CDPK has also been reported to be inhibited by CaM antagonists [25].

*Undialysed extracts*

The effect of $Ca^{2+}$ was also studied on undialysed soluble protein fraction isolated either from etiolated or light-grown seedlings. In these fractions, depletion of $Ca^{2+}$ resulted in dephosphorylation of 52, 48, 34 and 31 kDa polypeptides. The addition of EGTA also retarded the mobility of 52 kDa polypeptide and it moved with $M_r$ of 56 kDa. The dephosphorylation of these polypeptides and shift in mobility of 52 kDa polypeptide was reversed by the addition of 1 mM $Ca^{2+}$ to the reaction mixture (Fig. 5). In the case of the undialysed fraction, a higher EGTA concentration was required to observe the optimum effect compared with the dialysed fraction and this may be attributed to the loss of ions during dialysis. In comparison with the dialysed fraction, a few more polypeptides in the range 20–23 kDa were phosphorylated in the undialysed fraction. However, the doublet of 15 and 17 kDa, which appeared more intensely in the dialysed fraction, was peculiarly missing in the undialysed fraction, indicating the presence of some low $M_r$ inhibitor(s). In an earlier study too the presence of a low $M_r$ inhibitory factor in the soluble protein fraction of coconut milk has been reported [26].

This novel 52 kDa polypeptide in the soluble fraction of dark-grown wheat seedlings may be a $Ca^{2+}$-binding protein or a $Ca^{2+}$-dependent protein kinase, as all $Ca^{2+}$-binding proteins exhibit mobility shift on the gel, depletion of $Ca^{2+}$ (by EGTA) retarding the mobility and excess of $Ca^{2+}$ enhancing the mobility [27]. It should, however, be mentioned that a $Ca^{2+}$-dependent protein kinase with this type of property has also been purified and characterized from suspension-cultured soybean cells [28], the membrane fraction of apple [29], oat [30], rice [31] and the soluble fraction of groundnut [32] and etiolated pea nuclei [33]. Our preliminary studies indicate the presence of a $Ca^{2+}$-dependent autophosphorylating protein kinase of an apparent $M_r$ of 52 kDa with an intrinsic CaM domain, since the phosphorylation of this protein is inhibited by CPZ and TFP, but is not stimulated by CaM. In fact, the existence of this unique group of protein kinases with an intrinsic CaM domain in higher plants has been (now rather convincingly) demonstrated in several plants [25, 34–37].

It is also striking that the phosphorylation of the 52 and 48 kDa polypeptides is stimulated by $Ca^{2+}$, but is down-regulated by illuminating the dark-grown seedlings with white light for 1 hr or more; short-term white, red, far-red or blue radiations are ineffective in this regard. This is in contrast to the view, now current, that light may act through a $Ca^{2+}$ messenger system. Of course, this idea has emerged largely from short-term red/far-red radiation effects [8]. It would be of interest to follow the effect of light given for times ranging between 5 and 60 min and also monitor changes *in vivo* by feeding inorganic $^{32}P$, to determine whether rapid *in vivo* phosphorylation of these phosphoproteins by endogenous unlabelled ATP, in response to light, may not be leaving any sites available for later phosphorylation *in vitro* with [$\gamma$-$^{32}$P]ATP, as has been found for rapid blue light induced changes in phosphorylation of membrane proteins [20, 38]. As of now, to our knowledge, this is the only report where light and $Ca^{2+}$ have been found to act antagonistically to each other for regulating phosphorylation of soluble fraction proteins.

## EXPERIMENTAL

*Source of plant material and growth conditions.* Wheat (*T. aestivum* L. var. CPAN 1676) seeds employed throughout the present study were procured from the Wheat Directorate, Karnal. Seeds were soaked overnight under running tap water in the dark and spread over a 12 mm layer of absorbent cotton, laid in plastic trays. Seedlings were grown in complete darkness at $27 \pm 1°$ in a BOD incubator for 4 days and watered on the 2nd and 3rd days. All operations subsequent to seed germination were carried out in dim-green safe light. For light-grown tissue, seedlings were grown in white light in a growth chamber. For studies employing norflurazon, seeds were soaked in 100 $\mu$M norflurazon and subsequent to germination seedlings were supplied with $H_2O$ containing 100 $\mu$M norflurazon.

*Isolation of soluble fraction proteins.* The soluble fr. proteins were isolated according to the procedure of ref. [24] with some modifications. About 20 g wheat coleoptiles were homogenized in a buffer containing 50 mM Tris–MES (pH 8.0), 0.5 M sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1% Dextran and 14 mM 2-mercaptoethanol. The homogenate was filtered through 8 layers of muslin cloth and the filtrate centrifuged at 13 000 *g* for 10 min (SS-34 rotor, Sorvall RC-5B centrifuge). The supernatant was collected and centrifuged at 100 000 *g* for 90 min (80 Ti rotor, Beckman L8-70M). The supernatant was collected and dialysed overnight against 50 mM Tris (pH 7). The dialysed fr. was concd by further dialysing it against PEG 8000. Throughout the isolation procedure, the temp. was maintained at 4° and all operations were performed under dim-green safe light.

In vitro *protein phosphorylation.* For phosphorylation of soluble proteins, the procedure described in ref. [24] was followed. The reaction mixt. consisted of 25 mM MES (pH 7), 5 mM $MgCl_2$ 50 $\mu$M DTT and 10 $\mu$Ci[$\gamma$-$^{32}$P]ATP diluted to 100 $\mu$M with unlabelled ATP. The phosphorylation reaction was started by adding 150 $\mu$g proteins [2] to the reaction mixt. (total vol. of reaction mixt. 100 $\mu$l). Incubation was carried out at 30° for 5 min and the reaction was then terminated by adding 50 $\mu$l 3X sample electrophoresis buffer and heating the sample to 100° for 5 min.

*Protein quantification.* The amount of protein was estimated using BSA as standard protein according to the method of ref. [39].

*Gel electrophoresis and autoradiography.* Radiolabelled phosphopolypeptides (*ca* 50 $\mu$g proteins) were resolved by SDS–PAGE following the method of ref. [40]. The sep. gel contained either 5–20% (w/v) gradient acrylamide or 12.5% (w/v) acrylamide while the stacking gel contained 5% (w/v) acrylamide. Gels were stained with Coomassie brilliant blue, destained, dried *in vacuo* and autoradiographed to Konica X-ray film at −20° for 7–10 days.

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## REFERENCES

1. Kendrick, R. E. and Kronenberg, G. H. M., *Photomorphogenesis in Plants.* Kluwer Academic, Dordrecht, The Netherlands, 1994.
2. Bowler, C. and Chua, N.-H., *Plant Cell*, 1994, **6**, 1529.
3. Tretyn, A. and Kendrick, R. E., *Photochemistry and Photobiology*, 1990, **52**, 123.
4. Shacklock, P. S., Read, N. D. and Trewavas, A. J., *Nature*, 1992, **358**, 753.
5. Mehta, M., Malik, M. K., Khurana, J. P. and Maheshwari, S. C., *Plant Growth Regulation*, 1993, **12**, 293.
6. Otto, V. and Schäfer, E., *Plant and Cell Physiology*, 1988, **29**, 1115.
7. Park M.-H. and Chae, Q., *Biochemical and Biophysical Research Communications*, 1990, **162**, 9.
8. Fallon, K. M., Shacklock, P. S. and Trewavas, A. J., *Plant Physiology*, 1993, **101**, 1039.
9. Doshi, A., Aneeta and Sopory, S. K., *Photochemistry and Photobiology*, 1992, **55**, 465.
10. Harter, K., Fröhnmeyer, H., Kircher, S., Kunkel, T., Muhlbauer, S. and Schäfer, E., *Proceedings of the National Academy of Science, U.S.A.*, 1994, **91**, 5038.

11. Datta, N., Chen, Y.-R. and Roux, S. J., *Biochemical and Biophysical Research Communications*, 1985, **128**, 1403.
12. Romero, L. C., Biswal, B. and Song, P.-S., *FEBS Letters*, 1991, **282**, 347.
13. Salimath, B. P. and Marmé, D., *Planta*, 1983, **158**, 560.
14. Veluthambi, K. and Poovaiah, B. W., *Science*, 1984, **223**, 167.
15. Veluthambi, K. and Poovaiah, B. W., *Plant Physiology*, 1984, **76**, 359.
16. Krishnan, H. B. and Pueppke, S. G., *Physiologia Plantarum*, 1988, **72**, 747.
17. Friedmann, M. and Poovaiah, B. W., *Plant Cell Physiology*, 1991, **32**, 299.
18. Sakamoto, H. and Shibata, S., *Phytochemistry*, 1992, **31**, 2251.
19. Sharma, V. K., Jain, P. K., Maheshwari, S. C. and Khurana, J. P., *Journal of Plant Biochemistry and Biotechnology*, 1995, **4**, 91.
20. Sharma, V. K., Ph.D. thesis, University of Delhi, India, 1995.
21. Sharma, V. K., Jain, P. K., Maheshwari, S. C. and Khurana, J. P., *Phytochemistry*, in press.
22. Chamovitz, D., Pecker, I. and Hirshberg, J. *Plant Molecular Biology*, 1991, **16**, 967.
23. Roufgalis, B. D., in *Calcium and Cell Function*, ed. W. Y. Cheung. Academic Press, New York, 1982, p. 129.
24. Veluthambi, K. and Poovaiah, B. W., *Plant Physiology*, 1986, **81**, 836.
25. Harper, J. F., Sussman M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H. and Harmon, A. C., *Science*, 1991, **252**, 951.
26. Janistyn, B., *Phytochemistry*, 1989, **28**, 329.
27. Klee, C. B. and Venaman, T. C., *Advances in Protein Chemistry*, 1982, **35**, 213.
28. Putnam-Evans, C. L., Harmon, A. C. and Cormier, M. J., *Biochemistry*, 1990, **29**, 2488.
29. Battey, N. H. and Venis, M. A., *Planta*, 1988, **176**, 91.
30. Schaller, G. E., Harmon, A. C. and Sussman, M. R., *Biochemistry*, 1992, **31**, 1721.
31. Abo-El-Saad, M. and Wu, R., *Plant Physiology*, 1995, **108**, 787.
32. DasGupta, M., *Plant Physiology*, 1994, **104**, 961.
33. Li, H., Dauwlder, M. and Roux, S. J., *Plant Physiology*, 1991, **96**, 720.
34. Harper, J. F., Binder, B. M. and Sussman, M. R., *Biochemistry*, 1993, **32**, 3282.
35. Suen, K.-L. and Choi, J. H., *Plant Molecular Biology*, 1991, **17**, 581.
36. Kawasaki, T., Hayashida, N., Baba, T., Shinozaki, K. and Shimada, H., *Gene*, 1993, **129**, 183.
37. Binder, B. M., Harper, J. F. and Sussman, M. R., *Biochemistry*, 1994, **33**, 2033.
38. Short, T. W. and Briggs, W. R., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1994, **45**, 143.
39. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.
40. Laemmli, U. K., *Nature*, 1970, **227**, 680.