Vol. 47, No. 2, February 1999 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
pages 239-249

ROLES OF NITRATE, NITRITE AND AMMONIUM ION IN PHYTOCHROME REGULATION OF NITRATE REDUCTASE GENE EXPRESSION IN MAIZE

Nandula Raghuram[1] and Sudhir K. Sopory[2]

[1]*Department of Life Sciences, University of Mumbai, Vidyanagari, Mumbai - 4000 098, India*
[2]*International Centre for Genetic Engineering and Biotechnology, New Delhi, India*

Received August 4, 1998
Accepted September 29, 1998

Abbreviations: 5HT, 5-hydroxytryptamine; NR, nitrate reductase; NiR, nitrite reductase; PEP, phospho enol pyruvate; Pfr, phytochrome; PI, phosphoinositude(s); PKC, protein kinase C; PMA, phorbol myristate acetate

SUMMARY

The influence of nitrate and its metabolites on the nitrate reductase (NR) gene expression and its relationship with phytochrome (Pfr) regulation of NR in etiolated maize leaves is examined. Nitrate induction and Pfr stimulation are brought about by independent signalling phenomena. Phorbol myristate acetate (PMA), a stimulator of protein kinase C (PKC), mimicked the effect of red light but could not replace the nitrate requirement for the induction of NR transcript accumulation. This suggests that while PKC-type enzymes may be involved in mediating the Pfr signal, nitrate may follow an independent signalling mechanism. Experiments with 5-hydroxytryptamine (5HT) and lithium ions (Li^+), which are known to modulate phosphoinositide (PI) turnover, indicated that in addition to generating Pfr-induced second messengers for PKC activation, PI cycle may also generate other signals which mediate nitrate induction of NR gene expression in the dark. The products of nitrate reduction ie., nitrite and ammonium ion had inhibitory and stimulatory effects respectively, on NR transcript accumulation. They work mainly at the level of nitrate induction.

Key words: nitrate reductase, phytochrome, signal transduction, PI cycle, PKC, PMA, 5HT, lithium, *Zea mays*

INTRODUCTION

Higher plant nitrogen metabolism involves the reduction of nitrate to nitrite which inturn is converted to ammonium ion by the sequential actions of the enzymes, nitrate reductase (NR) and nitrite reductase (NiR). Ammonium ions are subsequently incorporated into amino acids via the glutamine synthetase/glutamate synthase cycle. These enzymes are induced by nitrate and regulated by light, carbon (C) and nitrogen (N) metabolites, hormones and developmental factors (1). Nitrate reductase is one of the most studied enzymes of the N assimilatory pathway (3). The primary inducer of NR gene expression is nitrate (3,4). Nitrate

1039-9712/99 $12.00 + 0.00

responsive elements have been identified in the upstream regions of NR and NiR genes (5,6). However, the molecular mechanism underlying the nitrate response is not yet established.

Light exerts a strong positive influence on NR gene expression (1,9). The regulation operates primarily at the transcriptional level (3), though post-translational enzyme modulation by phosphorylation/dephosphorylation is also known (8). In green plants, light effects are mimicked by photosynthetic C-metabolites such as hexoses, which are thought to mediate the light effect (5). In etiolated plants, light regulation of NR operates via phytochrome (7). The Pfr-mediated light signalling mechanism that leads to NR gene regulation probably involves the phosphoinositide (PI) cycle (9) and a PKC-type protein kinase (10-12).

The exact mechanism of induction of NR gene expression by light/nitrate and the biochemical basis of co-operation between these two factors is not well understood. Further, the precise role of nitrite and ammonium ion in this process is not yet clear. This paper is an attempt to address these deficiencies in the knowledge of NR regulation. The approach used assumes that the NR regulatory pathway may be conceptually separated into two parts viz., nitrate induction and light stimulation. By identifying the factors/molecules that influence these two regulatory processes, it should be possible to delineate the roles of light and nitrate.

MATERIALS AND METHODS

Plant material and light regimes: Excised leaves were used from 9 day old etiolated and N-starved seedlings of maize (*Zea mays* cv. Ganga 5), grown on moist paper in plastic trays at 27±1°C. Red light was given as a saturating 5 min. pulse from four 100W tungsten lamps filtered through a CBS-650 filter (Carolina Biological Supply Co. USA; emission maximum 650 nm). The intensity of red light at the plant level was 1.47 Wm^{-2}. All the manipulations were done in green safe light (emission maximum 500 nm, intensity 0.01 Wm^{-1}).

Treatments and chemicals: Leaves were excised and floated on water or nitrate (60 mM KNO_3 or NH_4NO_3) with or without KNO_2 (10 mM), PMA (5 ng/ml), 5HT (30 mM) or LiCl (5 mM) and incubated in the dark, along with respective red light-irradiated controls. These concentrations were standardised specifically for excised leaves, though whole plants respond to much lower levels. Samples were collected at appropriate intervals, frozen in liquid N_2 and processed for RNA isolation. The chemicals used were obtained from Sigma (Mo, USA).

Northern and dot blot analysis of RNA: Total RNA was isolated using the guanidine-HCl-phenol-chloroform extraction method and dot blot/northern blot hybridisation was carried out as described (11). Typically, 10 µg each of the RNAs were denatured in 6% formaldehyde and 50% formamide at 50°C for 1 h and dot blots/ northern blots generated using the standard procedures (13) on Gene Screen Plus nylon membranes (DuPont NEN, USA). Radiolabelled probes were prepared by the random primer method (13) using a homologous NR cDNA

probe obtained from Prof. Wilbur Campbell, USA. The specific activity of the probe ranged between 0.8 to $2x10^9$ CPM/µg. Hybridisations were performed at 65°C in a solution containing 0.5 M NaCl, 0.1 M NaH_2PO_4, 0.1 M Tris base, 2 mM EDTA, 1% SDS and 100 µg/ml denatured salmon sperm DNA, together with the denatured probe to 10^6 CPM/ml. Washing was performed at 65°C using 10 mM sodium phosphate buffer (pH 7.0), 2 mM EDTA and 1% SDS, and autoradiography was carried out using intensifying screens (13).

Data analysis and presentation: Autoradiograms were scanned at 523 nm covering the entire area of the hybridisation signal using Ultroscan XL densitometer (Pharmacia-LKB, USA). The baseline was defined as an average of 16 lowest data points in each scanning lane. The areas under the peaks (arbitrary units) were plotted either directly, or as percent values relative to an appropriate control. NR enzyme assays were performed (14) in crude extracts prepared as described (15). All the experiments were repeated at least twice and the data presented from representative experiments or as average values. Variations in the data recorded are indicated either directly in the figures, or in the text.

RESULTS AND DISCUSSION

As NR gene expression is regulated by several factors, the experiments used were designed to specifically address the role of Pfr and the nitrogen source. The use of etiolated plants eliminated the role of plastidic factor and photosynthetic sugars (7). Using excised leaves eliminated variables associated with the uptake and reduction of nitrate in the roots and its transport to the shoots. The use of red light ensured the elimination of blue light effects (7).

Co-operation between Pfr and nitrate

Earlier studies indicated that light stimulation of NR is under the control of phytochrome in maize, and that light and nitrate act independently to bring about the *de novo* synthesis of NR (15). By separating the red light irradiation and nitrate supply in time, it was shown that the photoactivated phytochrome (Pfr) generates secondary biochemical signals or transmitters in the absence of nitrate and that once this process was initiated, the state of the phytochrome no longer affected the induction of NR activity by nitrate (16).

In this investigation, the kinetics of NR transcript induction (Fig. 1) indicates the *de novo* accumulation of NR mRNA in response to nitrate, consistent with earlier reports (3,10). The co-operation between red light and nitrate is indicated by the observation that maximal induction of NR is achieved only when both are supplied, regardless of whether they are supplied together or separated by a dark gap (dT) of 2 h. Thus, nitrate can act independently as well as interact with some component(s) of Pfr signal transduction, indicating that nitrate

induction of NR gene expression is light-independent, whereas light stimulation is strictly nitrate-dependent. As far as the Pfr signalling mechanism is concerned, earlier studies using cholera toxin, serotonin (5HT) and PMA showed that the light stimulation of NR gene expression is mediated by G proteins (Raghuram, Chandok and Sopory, submitted), PI cycle (9) and a protein kinase C type enzyme (11,12), which has been recently purified (17). However, the role of nitrate remained unaddressed in this system.

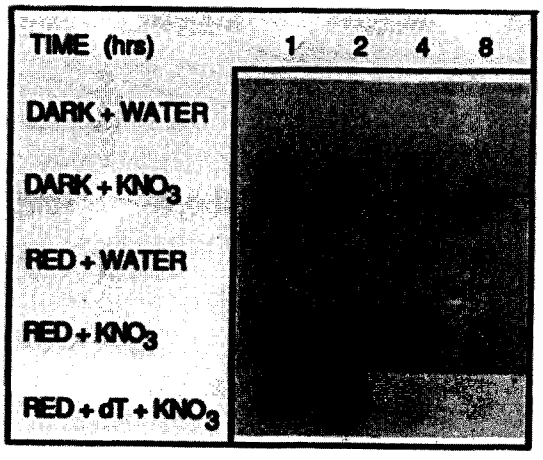

Figure 1. Kinetics of KNO_3-induced NR transcript accumulation. Leaves were excised and floated either on water or 60 mM KNO_3 and exposed to red light for 5 min with appropriate controls. Samples were collected at 1, 2, 4 and 8 h and processed for total RNA isolation and dot blot hybridisation with NR cDNA probe, as described in the Materials and Methods section. "dT" denotes a dark gap of 2 h separating red light irradiation and nitrate treatment.

Role of nitrate

Nitrate, in addition to being a substrate for NR, also acts as a biochemical signal for N-metabolism (4,18), which means that a signalling pathway must exist in order to mediate the nitrate-induced responses. While the components of this pathway are not known, it was suggested that they are constitutively expressed, and do not require *do novo* protein synthesis (4,19). The identification of nitrate responsive elements in the upstream regions of the NR and NiR genes lends further support to the existence of an independent signalling mechanism (5,6).

In this study, an approach using various agonists and antagonists of signal transduction to delineate the relationship between nitrate induction and light stimulation yielded some clues to unravel the mechanism of nitrate signalling. Figure 2 highlights the differential effects of PMA, 5HT and Li^+ ions with respect to NR regulation by Pfr and nitrate. PMA alone was ineffective in the induction of NR in the absence of nitrate, indicating that PMA mimicked only light stimulation and not nitrate induction. On the other hand, 5HT alone could induce NR gene expression in the absence of nitrate, though the optimum level of induction is achieved only in the presence of nitrate. In other words, 5HT mimics both nitrate

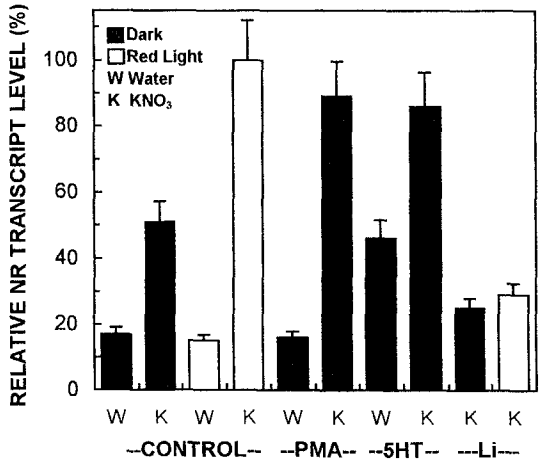

Figure 2. Effect of PMA, 5HT and Li^+ ions on nitrate-induced NR transcript levels. Leaves were floated on PMA (5 ng/ml) or 5HT (30 mM), prepared in water (W) or KNO_3 (K) and incubated in the dark for 2 h. Appropriate dark and red light controls were maintained. For lithium pre-treatment, leaves were floated on water (W) or 5 mM LiCl for 1 h before red light irradiation and nitrate supply. Steady state NR transcript levels were analysed by total RNA dot blot hybridisation and densitometric scanning of the autoradiogram. The areas under the peaks (arbitrary units) were plotted as percent values relative to the peak level of NR obtained with red light and KNO_3. Average data from three independent experiments is shown.

induction (in the dark) as well as red light stimulation (in the presence of nitrate) in a quantitative manner, indicating that both these processes may require messengers generated through the PI cycle. This was further corroborated by the lithium data, which show that Li^+ ions interfere with nitrate-induction of NR gene expression in the dark, albeit to a lesser extent in comparison to that in light. The specificity of lithium action was verified by rehybridising the RNA blots with a chick actin probe, which revealed that Li^+ ions did not significantly alter the levels of the maize actin RNA (data not shown). These results suggest the involvement of PI metabolites in nitrate-induced signal transduction. Recently, a lithium sensitive inositol monophosphatase from was cloned from tomato (20), demonstrating that Li^+ can be used as an effective tool for studying the role of the PI cycle in plant signalling pathways.

Nitrate enhances the activity of a protein kinase that brings about a phosphorylation-dependent activation of PEP carboxylase and the inactivation of sucrose phosphate synthase (21). Recently, nitrate-dependent phosphorylation of cellular proteins was found in maize (Goel and Sopory, unpublished results), suggesting the involvement of protein kinases in nitrate signalling. In this study, PMA could not replace the requirement of nitrate, indicating that the protein kinase induced by nitrate may belong to a category different from the PKC-type enzymes induced by light. There are indications that calcium-dependent protein kinases may be involved in nitrate signalling (19). Thus, nitrate and light may operate via different protein phosphorylation cascades, activated by messengers derived from the PI cycle.

Role of nitrite and ammonium

One of the most striking features of nitrate reductase gene regulation is that apart from nitrate, the inducer and substrate for NR, all the products of nitrate reduction, i.e., nitrite and ammonium ion, glutamine and aspargine influence NR mRNA levels or NR activity or both (22). Of these, aspargine and glutamine have been shown to repress NR gene expression (3,5,23). But the precise role of nitrite and ammonium ion in NR regulation is not clear.

Nitrite is an end product of nitrate reduction. A stringent regulation of NR gene expression is essential in order to avoid nitrite accumulation, which is toxic to the cell (2). Nitrite was found to inhibit NR activity in maize, without affecting the uptake or availability of nitrate (24). In this study, nitrite alone did not have a significant effect on NR RNA levels, but it inhibited nitrate induction of NR transcript accumulation by up to 28% in the dark and 64%

in red light irradiated-leaves (Fig. 3). These results are consistent with the above report, and suggest that the inhibition operates at the RNA level. Moreover, nitrite inhibition of NR induction seems to override light stimulation, leading to higher levels of inhibition in red light-irradiated leaves. This could mean that nitrite inhibition is a feed back control that counteracts nitrate induction, and renders light ineffective.

The role of ammonium ion in the regulation of nitrate reductase gene expression in higher plants is not well understood. In several higher plants, when ammonium ions were the sole N source, there was no detectable NR mRNA, protein or activity (2). When given along with nitrate, the reported effect of ammonium ions on nitrate-induced NR activity varied between no effect (25), stimulation (24, 26, 27) and inhibition (23) in different systems.

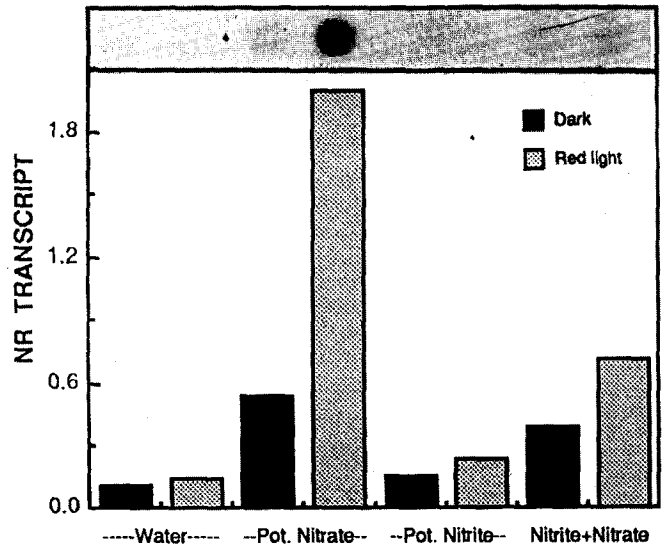

Figure 3. Effect of nitrite ions on NR transcript levels. Leaves were floated on 10 mM KNO_2 with or without 60 mM KNO_3 and exposed to red light for 5 min with appropriate controls. After 2 h, the leaves were processed for NR steady state transcript level analysis by total RNA dot blot hybridisation and densitometric scanning of the autoradiogram. The areas under the peaks (arbitrary units) were plotted against their respective treatments. The inhibition by nitrite at peak levels of induction varied by a maximum of 15% in three independent experiments.

However, in most plants, inhibition was observed mainly in roots, and not in shoots, probably because in intact plants, almost all the ammonium ion supplied is assimilated in the roots. These conflicting results seem to be at least partly due to the differences in the experimental conditions namely etiolated/green plants, whole plants/excised parts, prior nutrition etc.

In this study, NH_4NO_3 consistently yielded higher levels of NR transcript (Figs. 4 & 6) and NR activity (Fig. 5) as compared to KNO_3 in the dark as well as in red light. This indicates a stimulatory role for ammonium ions in the induction of NR gene expression by nitrate in maize. The experimental conditions were similar to those of pea (26) and wheat (27), in which a stimulatory effect of ammonium ions on NR activity was shown earlier (i.e., treatments were given to etiolated, N-starved and excised leaves). Data from six independent experiments revealed that ammonium ions caused a two fold increase in the nitrate-induced NR RNA in red-light irradiated leaves, whereas in the dark incubated leaves the stimulation was almost three fold (Fig. 4). Ammonium ions alone (in the absence of nitrate) did not have any effect on the levels of NR RNA or activity (data not shown). Earlier studies demonstrated that ammonium ion did not act by enhancing nitrate uptake (24,27), indicating its independent role. Northern blot hybridisation experiments under high stringency conditions revealed that the transcripts induced by NH_4NO_3 and KNO_3 are of the same size (Fig. 6) and that therefore, they may originate from the same gene. Moreover, there is no evidence in the literature indicating the presence of multiple genes for NADH:NR in maize.

The data in Figs. 4,5 and 6 also show that the ammonium effect was more pronounced in the dark than in red light, which means that it is not a part of the Pfr signal transduction and is presumably linked to nitrate induction. Moreover, ammonium ion does not seem to replace the light effect, since ammonium and light (or ammonium and PMA) together had an additive effect in stimulating nitrate-induced NR transcript accumulation even further (Figs. 4,6). Similarly, NO_2^- or Li^+ ions could neutralise the stimulatory effect of ammonium ions on the induction of NR by nitrate (data not shown). Together, these findings suggest that the products of nitrate reduction may act by influencing the nitrate signalling mechanism, rather than phytochrome phototransduction. The reason for the stimulatory role of ammonium is not clear, since both nitrite and glutamine inhibit NR induction. One possibility could be that NH_4^+ balances the negative effects of nitrite and glutamine, when the conditions are optimal for efficient nitrate assimilation.

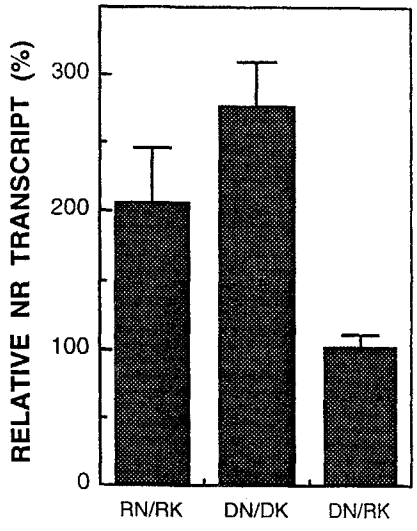

Figure 4. Effect of ammonium ions on nitrate-induced NR transcript levels. Leaves were floated on water, 60 mM KNO_3 or 60 mM NH_4NO_3 and exposed to red light for 5 min with appropriate controls. Samples were collected after 2 h and NR transcript levels were analysed by total RNA dot blot hybridisation and densitometric scanning of the autoradiogram. Relative values of the transcript levels obtained with NH_4NO_3 were calculated taking the corresponding KNO_3 control values as 100% and mean values from 6 independent experiments were plotted. RN/RK indicates the % RNA levels obtained with red light + NH_4NO_3 relative to those obtained in red light + KNO_3. Similarly DN/DK indicates dark + NH_4NO_3 relative to dark + KNO_3 and DN/RK indicates dark + NH_4NO_3 relative to red light + KNO_3.

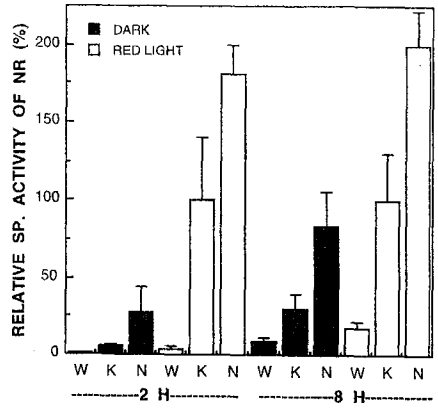

Figure 5. Effect of ammonium ions on nitrate-induced NR activity. Leaves were floated on water (W), 60 mM KNO_3 (K) or NH_4NO_3 (N) and exposed to red light for 5 min with appropriate controls. Samples were collected at 2 h and 8 h and assayed NR. The specific activity of NR is defined as nanomoles of nitrite produced per mg protein per h. The specific activity data was converted into percent values relative to the corresponding red light + KNO_3 controls and plotted on the x-axis. Mean data from three independent experiments is shown.

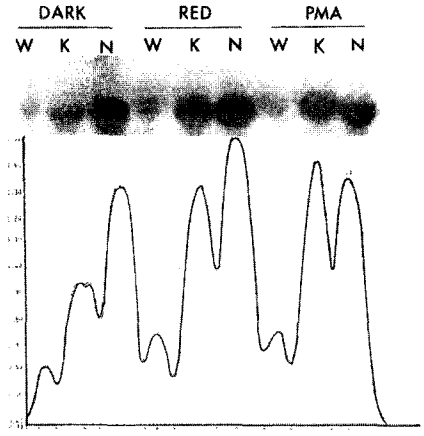

Figure 6. Effect of nitrate and ammonium ions on NR transcript levels in red light and PMA. Leaves were floated on water (W), 60 mM KNO_3 (K) or 60 mM NH_4NO_3 (N) with or without 5 ng/ml PMA and incubated in the dark for 2 h. Appropriate controls were maintained. Total RNAs were isolated and analysed by northern blot hybridisation. Note the higher intensity of the NR mRNA (3.2 Kbp) band in the presence of ammonium nitrate (lanes marked N).

ACKNOWLEDGEMENT: We thank Prof. Wilbur Campbell, USA, for providing the cDNA clone for the maize NR gene and for his valuable comments on the draft manuscript.

REFERENCES

1. Lam, H.-M., Coschigano, K.T., Oliviera, I.C., Melo-Oliviera, R and Coruzzi, G.M. (1996). Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**: 569-593.
2. Solomonson, L.P. and Barber, M.J. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**: 225-253.
3. Callaci, J. and Smarrelli, J. (1991) Biochim. Biophys. Acta **1088**: 127-30.
4. Gowri, G., Kenis, J.D., Ingemarsson, B., Redinbaugh, M.G. and Campbell, W.H. (1992) Plant Mol. Biol. **18**: 55-64.
5. Vincentz, M., Moureaux, T., Leydecker, M., Vaucheret, H. and Caboche, M. (1993) Plant J. **3**: 315-324.
6. Hwang, C.-F., Lin, Y., D'Souza, T. and Cheng, C.-L. (1997). Plant physiol. **113**: 853-862.
7. Raghuram, N. and Sopory, S.K. (1995) Physiol. Mol. Biol. Plants **1**: 103-114.
8. Kaiser, W.M. and Huber, S.C. (1994) Plant Physiol. **106**: 817-821.
9. Chandok, M.R. and Sopory, S.K. (1994) FEBS Lett. **356**: 39-42.
10. Sharma, A.K., Raghuram, N., Chandok, M.R., Das, R. and Sopory, S.K. (1994) J. Exp. Bot. **45**: 485-490.
11. Raghuram, N. and Sopory, S.K. (1995) Plant Mol. Biol. **29**: 25-35.
12. Chandok, M. R., Raghuram, N. and Sopory, S.K. (1997) in Plant Molecular Biology and Biotechnology (Tewari, K.K. and Singhal, G.S. Eds.) pp. 77-85. Narosa publishing house, London.
13. Sambrook, J. Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

14. Hageman, R.H. and Huckelsby, D.P. (1971) Methods Enzymol. **23**: 491-503.
15. Rao, L.V.M, Datta, N., Sopory, S.K and Guha-Mukherjee, S. (1980) Physiol. Plant. **50**: 208-212.
16. Sharma, A.K. and Sopory, S.K. (1984) Photochem. Photobiol. **39**: 491-493.
17. Chandok, M.R. and Sopory, S.K. (1998) J. Biol. Chem. (In press).
18. Scheible, W.-R., Gonzalez-Fontes, A., Laurer, M., Müller-Röber, B. Caboche, M. and Stitt, M. (1997) Plant Cell **9**: 783-798.
19. Sakakibara, H., Kobayashi, K., Deji, A. and Sugiyama, T. (1997) Plant Cell Physiol. **38**: 837-843.
20. Gillapsy, G.E., Kedde, J.S., Oda, K and Gruissem, W. (1995) Plant Cell 7: 2175-2185.
21. Van-Quy, L. and Champigny, M.L. (1992) Plant Physiol. **99**: 344-347.
22. Sivasankar, S. and Oaks, A. (1996) Plant Physiol. Biochem. **34**: 609-620.
23. Deng, M.D., Moureaux, T., Chérel, I., Boutin, J.P. and Caboche, M. (1991) Plant Physiol. Biochem. **29**: 239-247.
24. Ivashikina, N.V. and Sokolov, O.A. (1997) Plant Sci.**123**: 29-37.
25. Crawford, N.M., Smith, M., Bellissimo, D. and Davis, R.W. (1988) Proc. Natl. Acad. Sci., USA. **85**: 5006-5010.
26. Sihag, R.K., Guha-Mukherjee, S. and Sopory, S.K. (1979) Physiol. Plant. **45**: 281-287.
27. Datta, N., Rao, L.V.M., Guha-Mukherjee, S. and Sopory, S.K. (1981) Plant Sci. Lett. **20**: 305-313.