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INFLUENCE OF CYTOKININS AND PHYTOCHROME ON NITRATE REDUCTASE ACTIVITY IN ETIOLATED LEAVES OF MAIZE

L. V. M. RAO, NEERAJ DATTA, M. MAHADEVAN, SIPRA GUHA-MUKHERJEE and SUDHIR K. SOPORY*

Plant Research Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

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Key Word Index—*Zea mays*, Gramineae; maize; cytokinins; phytochrome; nitrate reductase.

Abstract—Of the different hormones tested, cytokinins stimulated nitrate-induced nitrate reductase (NR) activity in the dark. The optimal stimulation was obtained at 16 hr and this was sensitive to tungstate, 6-methylpurine and cycloheximide. The cytokinin stimulation of NR activity was further enhanced by brief irradiation with red light, but this effect was not noticed when leaves were exposed to far-red light. Both kinetin and red light, when given together, or given with a darkness interruption, stimulated the NR activity more than with either of them alone.

INTRODUCTION

Investigations have been carried out in order to understand the interaction of phytochrome and hormones in plant development. However, the molecular relationship between these two factors is not well understood. The regulation of the hormonal level by phytochrome *in vivo* [1–4] and *in vitro* [5–7] had indicated that phytochrome action is mediated via hormones. However, in many responses, phytochrome and hormones have been shown to act as independent factors [8–12].

The activity of NR is regulated by both hormones, especially cytokinins [13–16] and phytochrome [17–24] but no detailed work has been done to check for the effect of their interaction on NR activity. In an earlier communication [24] we reported on the phytochrome control of NR activity in excised, etiolated leaves of maize. In the present paper we report on the stimulating effect of cytokinins on nitrate-induced NR activity and show that this effect of cytokinins is independent of the phytochrome effect.

RESULTS

The effect of hormones on NR activity was tested in leaves incubated either in total darkness or illuminated with short and prolonged red light treatments. When excised leaves floating in potassium nitrate were supplemented with various hormones and with acetylcholine and cAMP, no other hormone except kinetin showed any significant and consistent effect on the induction of NR activity in the dark (Table 1, experiment 1). Since in all the experiments an increase of *ca* 100–120% in NR activity was obtained by kinetin, the effect of two more cytokinins, benzylaminopurine and zeatin, was also tested (Fig. 1). The optimal concentration for NR stimulation was 10^{-4}, 10^{-5} and 10^{-6} M for kinetin, benzylaminopurine and zeatin, respectively. Although zeatin was more potent, as it enhanced the NR activity at lower concentration, maximum stimulation was achieved by kinetin. It was further found that the stimulatory effect of the cytokinins was not due to the adenine moiety, since it did not stimulate NR activity when supplied at an equimolar concentration (Table 1, experiment 2). The kinetics of NR induction in the dark, with the exogenous application of different cytokinins, showed a lag of 30 min. Only after 1 hr was an increase in NR activity discernible. Optimal stimulation was achieved around 16 hr, after which there was a decrease in the enzyme activity (Fig. 2). All three cytokinins showed a similar pattern.

Table 1. Effect of various hormones and adenine on the induction of NR in the dark

Treatment	NR activity	Relative activity
Experiment 1		
Control (60 mM KNO_3)	98 ± 17	100
+ IAA (10^{-5} M)	76 ± 43	78
+ GA_3 (10^{-6} M)	119 ± 23	121
+ kinetin (10^{-5} M)	205 ± 11	209
+ ACh (10^{-5} M)	105 ± 37	107
+ cAMP (10^{-5} M)	111 ± 61	113
Experiment 2		
Control (60 mM KNO_3)	125 ± 18	100
+ kinetin (10^{-4} M)	260 ± 15	208
+ adenine (10^{-4} M)	114 ± 17	91

Excised, etiolated maize leaves were incubated in nitrate supplemented with various hormones in complete darkness. After 4 hr, the enzyme activity was measured and expressed as nmol NO_2^- formed/mg protein per hr. Data given ± s.d. (n = 3–6).

When the effect of red light and continuous far-red light on NR activity was studied in the presence of different cytokinins, it was found that the leaves incubated with cytokinins showed a further enhancement in NR activity (Table 2) over and above the red light control. However, under continuous far-red irradiation, a slight inhibition in NR activity was observed. Furthermore, kinetin alone (in the absence of nitrate) had no effect on NR activity, whereas its stimulatory effect in the presence of potassium nitrate was further stimulated by short treatment of red

*To whom correspondence should be addressed.

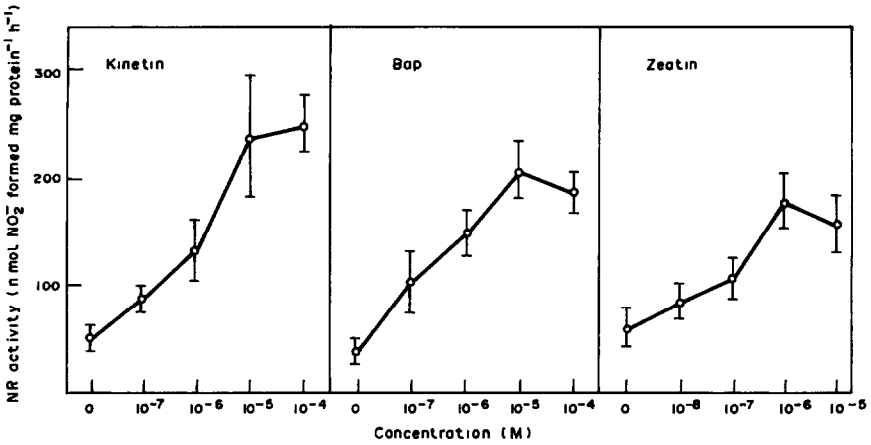

Fig. 1. Effect of different concentrations of kinetin, benzylaminopurine (BAP) and zeatin on nitrate reductase activity in maize. 60 mM KNO_3 was used.

light but not by far-red light. The far-red light also did not reverse the kinetin effect (Table 3).

We reported earlier [24] that phytochrome-mediated stimulation of NR activity reached a maximum at 4 hr. The effect of the interaction between kinetin and phytochrome was tested on NR stimulation after this period. The different sets of treatments employed are shown in Table 4. It was found that either red light (5 min) or kinetin enhanced NR activity after 4 hr in darkness. Furthermore, when the leaves were transferred to kinetin solution for 4 hr in darkness following 5 min of red-light irradiation, NR activity increased dramatically. In experiments where the NR activity was measured after 8 hr (Table 4), a similar trend was observed. After 5 min of red light treatment and 4 hr of dark incubation, when the leaves were irradiated again for 5 min with red light and left in darkness for a further 4 hr, the NR stimulation did not increase significantly as compared to leaves which were irradiated with 5 min of red light and left in darkness for 4 hr and then incubated in the dark in kinetin for 4 hr.

Since with cytokinins maximum stimulation in NR activity was reached by 16 hr, experiments were performed in which the interaction of red light, given at the beginning or after 8 hr, was tested with kinetin (Table 5). As seen earlier, 5 min of red light irradiation given at the beginning and after 8 hr of potassium nitrate treatment stimulated NR activity. However, when red light treatment at the beginning was followed by kinetin treatment after 8 hr the stimulation was greatly enhanced. Interestingly, pretreatment of leaves for 8 hr with kinetin followed by 5 min of red light irradiation also markedly affected the stimulation of NR activity.

To discover if the enhancement of NR activity by kinetin involved new synthesis of RNA and protein, inhibitors of RNA and protein synthesis were used. With 6-methylpurine and cycloheximide, the enhancement of NR by kinetin was inhibited by 80 and 60%, respectively (Table 6). Even tungstate (2 mM) inhibited hormone-mediated enhancement of NR activity. It was also found that the enhancement of NR by kinetin was not due to any enhanced uptake of nitrate in the tissue in its presence. In fact, in the presence of kinetin a slight decrease in nitrate accumulation was noticed (data not given).

DISCUSSION

Hormones and phytochrome influence a variety of similar developmental processes in plants. Although at-

Table 2. Effect of cytokinins on NR induction with a short pulse of red light and under continuous far-red light (CFR)

Treatment	Red light		CFR	
	NR activity	Relative activity	NR activity	Relative activity
Control (60 mM KNO_3)	220 ± 27	100	599 ± 4	100
+ BAP* (10^{-5} M)	289 ± 29	131	555 ± 12	93
+ kinetin (10^{-4} M)	314 ± 30	142	547 ± 24	91
+ zeatin (10^{-6} M)	275 ± 35	125	514 ± 9	66

Excised, etiolated maize leaves were floated in nitrate medium supplemented with suitable concentrations of kinetin, BAP and zeatin. Leaves were illuminated either with 5 min of red light or continuous far-red light. After 4 hr, the enzyme activity was measured and expressed as nmol NO_2^- produced/mg protein per hr. Data given ± s.d (n = 3–4).

*BAP = Benzylaminopurine.

Table 3. Effect of red and far-red light on kinetin (10^{-4} M) stimulated NR activity

Treatment	NR activity
Kinetin (alone)	10
KNO_3 (dark)	100
KNO_3 + kinetin	250
KNO_3 + kinetin + 5 min red light	325
KNO_3 + kinetin + 5 min far-red light	248

Experimental conditions as in Table 3. NR activity is expressed as nmol of NO_2^- formed/mg protein per hr.

Table 4. Effect of the interaction between kinetin and red light (5 min) on NR activity in maize

Treatment	NR activity	Relative activity
4 hr D*	121 ± 65	100
Kinetin + 4 hr D	380 ± 163	314
5 min R* + 4 hr D	345 ± 73	285
Kinetin + 5 min R + 4 hr D	580 ± 159	479
8 hr D	192 ± 33	159
Kinetin + 8 hr D	417 ± 35	345
5 min R + 8 hr D	322 ± 16	266
5 min R + 4 hr D + kinetin + 4 hr D	686 ± 65	567
5 min R + 4 hr D + 5 min R + 4 hr D	412 ± 44	340

Kinetin (10^{-4} M) was supplied to the leaves together with nitrate. The leaves were illuminated with red light for 5 min as indicated in the table. Enzyme activity was measured after 4 and 8 hr and is expressed as nmol NO_2^- formed/mg protein per hr. Data given ± s.d. ($n = 3$).

*D = Darkness, R = red light

Table 5. Effect of the interaction between kinetin and red light on NR activity in maize

Treatment	NR activity
16 hr D*	155
5 min R* + 16 hr D	218
Kinetin 16 hr D	276
5 min R + kinetin 16 hr D	361
8 hr D + 5 min R + 8 hr D	284
8 hr D + kinetin 8 hr D	310
5 min R + 8 hr D + 5 min R + 8 hr D	279
5 min R + 8 hr D + kinetin 8 hr D	455
Kinetin 8 hr D + 5 min R + 8 hr D	542

Experimental conditions as in Table 5. Enzyme activity was measured after 16 hr. Data are the mean of three experiments.

*D = Dark; R = red light.

Table 6. Effect of 6-methylpurine (6-MP), cycloheximide (CHI) and tungstate (WO_4^{2-}) on the enhancement of NR activity mediated by kinetin

Treatment	NR activity	Relative activity
KNO_3	114 ± 21	100
+ kinetin	265 ± 15	232
+ kinetin + 6-MP	100 ± 26	87
+ kinetin + CHI	51 ± 17	45
+ kinetin + WO_4^{2-}	14 ± 3	12

Kinetin (10^{-4} M), 6-methylpurine (1 mM), cycloheximide (20 μg/ml) and tungstate (2 mM) were supplied to excised, etiolated leaves together with induction medium and incubated in the darkness for 4 hr. After 4 hr, the enzyme activity was measured and is expressed as nmol NO_2^- formed/mg protein per hr Data given ± s.d. ($n = 3$–4)

tempts have been made to link phytochrome and hormone action in plants [1], the participation of hormones in phytochrome-mediated responses is still controversial [8, 25]. The results obtained in the present work suggest an independent mode of action of cytokinins and phytochrome in enhancing NR activity in maize.

None of the hormones tested (GA_3, IAA, acetylcholine and cAMP) had a significant effect on the NR activity in the dark, with the exception of cytokinins, kinetin, benzylaminopurine and zeatin, which enhanced NR activity significantly (Table 1; Fig. 1). All three cytokinins enhanced NR activity to a level nearer to the 5 min red light treatment. However, the kinetics of enhancement obtained by these cytokinins differed from phytochrome-mediated stimulation of NR [24], except in the lag phase. The rate of enhancement was much slower and the pattern of kinetics was different (Fig. 2). The time taken to reach the optimum NR level with cytokinins was *ca* 16 hr, whereas in the case of phytochrome-mediated NR it was 4 hr.

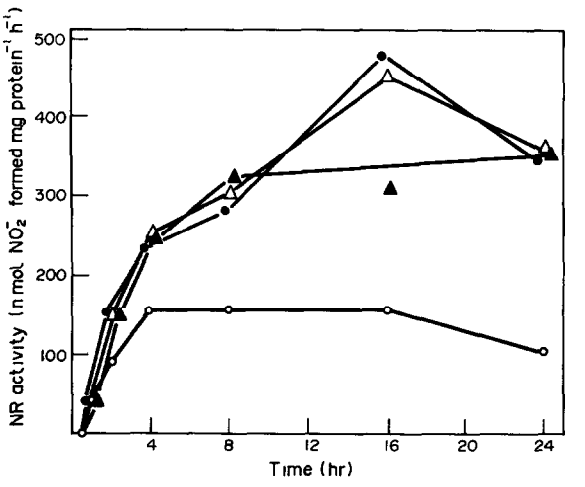

Fig. 2. Kinetics of stimulation of nitrate (60 mM) induced nitrate reductase activity by cytokinins (○) KNO_3 alone; (●) nitrate + 10^{-5} M; BAP, (▲) nitrate + 10^{-6} M zeatin; (△) nitrate + 10^{-4} M kinetin.

Cytokinin induction of NR activity has been reported in many systems [13–16, 26–28]. Kinetin markedly enhanced NR activity in *Agrostemma githago* embryos in the absence of nitrate [13, 28]. However, we did not find induction of NR by kinetin alone (Table 3). Recently [29], an additive effect of kinetin and nitrate in inducing NR in bean roots has been found.

With kinetin, 5 min of red light had an enhancing effect on NR activity (Table 3). Far-red light did not reverse the effect of kinetin. Under continuous far-red light treatment, however, the enhancement of NR activity was inhibited slightly. Hormone-mediated repression of photoresponse has been reported earlier in few cases, e.g. in pea [30, 31] and in maize [32]. In the present study, it seems that under prolonged illumination of far-red light some changes occur in the system which prevent the application of kinetin from increasing the NR activity further.

The experiments on the interaction of short irradiation of red light and kinetin rule out the possibility of a role of cytokinins in phytochrome-mediated enhancement of NR activity. It was shown earlier that NR reached an optimum level after 4 hr when leaves were exposed to 5 min of red light [24]. At this juncture, when kinetin was supplied to leaves, NR activity further enhanced significantly. However, if leaves were re-illuminated with red light for 5 min at this stage, there was no significant effect on the enzyme activity. A similar trend was noticed at 16 hr. Therefore red light and kinetin given together, or even if given after a darkness interruption, enhanced NR activity more than either of them alone. This suggests that kinetin and red light act independently and have a different mode of action in stimulating nitrate-induced NR. These results then support the two-factor hypothesis proposed earlier [10, 11] and the view that hormones may not mediate the phytochrome regulation of NR activity in maize. Sharma *et al.* [32] also showed that phytochrome and hormones act independently in the enhancement of peroxidase activity in maize. Similarly, Pfaff and Schopfer [12] have also pointed out that hormones do not form a link in phytochrome-mediated adventious root formation in mustard seedlings, and Knypl [16] too had reported earlier that the control of NR activity in lettuce seeds is independent of hormonal action. However, the fact that a pretreatment with kinetin made it more sensitive to red light treatment (Table 5) opens up other kinds of interactions which need further study as proposed earlier [11].

Stimulation of NR by kinetin was inhibited significantly by 6-methylpurine and cycloheximide (Table 6) suggesting a requirement of RNA and protein synthesis. Both these inhibitors have been shown to affect RNA and protein synthesis respectively in maize [24]. It has been suggested by other workers [13, 27, 28] also, either by using heavy water or by using inhibitors of RNA and protein synthesis, that there is a *de novo* synthesis of NR mediated by kinetin. The complete inhibition of cytokinin stimulation of NR by tungstate further supports the view that cytokinin induction of NR is controlled by its *de novo* synthesis.

EXPERIMENTAL

Seeds of *Zea mays* var. C 51–54 were obtained from the Indian Agricultural Research Institute, New Delhi. The procedures for the growth of the plants and extraction of NR have been described in detail [24]. Activity of NR was measured as described in ref. [33]. Protein was estimated as in ref. [34] using bovine serum albumin as standard.

The treatments of various chemicals were given by transferring the etiolated, excised maize leaves (6-day-old) to different Petri dishes supplied with the respective solns, 30 min prior to light treatments. Unless specified otherwise, all the chemicals were dissolved in 60 mM nitrate soln. In all cases, controls were supplied with nitrate medium. The green safe light (1 $\mu W/cm^2$), red light (500 $\mu W/cm^2$), and far-red light (140 $\mu W/cm^2$) were obtained as described earlier [35]. The values given in the tables and figures are mean values of at least three experiments and wherever essential, standard deviations have been recorded.

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