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Intact Plastids Are Required for Nitrate- and Light-Induced Accumulation of Nitrate Reductase Activity and mRNA in Squash Cotyledons[1]

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ABSTRACT

Induction of nitrate reductase activity and mRNA by nitrate and light is prevented if chloroplasts are destroyed by photooxidation in norflurazon-treated squash (*Cucurbita maxima* L.) cotyledons. The enzyme activity and mRNA can be induced if norflurazon-treated squash seedlings are kept in low-intensity red light, which minimizes photodamage to the plastids. It is concluded that induction of nitrate reductase activity and nitrate reductase mRNA requires intact plastids. If squash seedlings grown in low-intensity red light are transferred to photooxidative white light, nitrate reductase activity accumulates during the first 12 hours after the shift and declines thereafter. Thus photodamage to the plastids and the disappearance of nitrate reductase activity and mRNA are events separable in time, and disappearance of the enzyme activity is a consequence of the damage to the plastids.

In photosynthetic tissue, reduction of nitrate is catalyzed by two enzymes, NR,[3] which reduces nitrate to nitrite, and NiR, which reduces the toxic nitrite to ammonium. Both proteins are nuclear encoded, but the enzymes are located in different compartments. NR is a cytoplasmic enzyme (*e.g.* 3, 10, 14, 19, 25). On the other hand, NiR is located in the plastids (15, 22, 26) (see Ref. 27 for recent review), and recent isolation and sequencing of cDNA clones for spinach and maize NiR confirmed that the protein is synthesized as a precursor polypeptide with an amino acid extension at the N-terminal end which might serve as a signal sequence to direct the protein into the chloroplasts (1, 16).

Regulation of NR has been investigated intensively. It is generally accepted that nitrate is required for the induction. Only in the presence of nitrate can light (operating through phytochrome or through an additional photoreceptor absorbing in the blue/UV region of the visible spectrum) modulate the level of NR (see Ref. 21 for review). This concept has been confirmed at the level of steady-state NR-mRNA. The NR transcript level increases dramatically if nitrate is applied to the seedlings (5, 7, 9) and light also is involved in controlling NR transcript levels (13, 20).

In addition to nitrate and light, the status of the plastids plays an essential role in controlling the enzyme activity of the cytoplasmic NR. If plastid development is prevented either by mutation (3) or by treatment of the seedlings with the herbicide norflurazon [4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2H)-pyridazinone] in strong white light (11, 18, 19, 21, 22 and references therein) nitrate- and light-mediated induction of NR activity is completely prevented. Norflurazon inhibits colored carotenoid biosynthesis (2). If carotenoid-free seedlings are grown under high light intensities, plastids are destroyed by photooxidation. The damage is restricted exclusively to the plastids and even the outer chloroplast membrane seems to be unimpaired (17). We have recently characterized the dependency of NR activity on the status of the plastid. If carotenoid-free mustard seedlings (norflurazon-treated) are transferred from nonphotooxidative light to photooxidative high light intensities, the plastidic compartment is destroyed completely within 2 h, and plastidic protein levels including NiR decline rapidly during this treatment (18, 19). However, NR activity continued to accumulate for at least 10 h after complete destruction of the chloroplasts (19). It was concluded that the disappearance of cytoplasmic NR is not caused by direct photodamage to the plastids, but that NR activity disappears as a later consequence of the destruction of the chloroplast.

In the present paper, we address the question as to whether the observed dependency of NR activity on the state of the plastids can be detected at the level of its mRNA.

MATERIALS AND METHODS

Squash seeds (*Cucurbita maxima* L., cv Butternut) were imbibed for 2 to 3 h in one of the following: (a) water, (b) 10 mM KNO_3, (c) 10^{-4} M norflurazon (Sandoz 9789), or (d) a solution of 10 mM KNO_3 and 10^{-4} M norflurazon. They were then planted in vermiculite that was soaked with the same solutions as those in which they were imbibed. Seedlings were grown at 25 to 27°C under continuous WL (100 μmol m^{-2} s^{-1}, obtained from a cool white fluorescent lamp) or RL (0.3 μmol m^{-2} s^{-1}, obtained from Sylvania GTE red fluorescent bulbs, F401R 40 W, covered with red acrylic Shinkolite 102, Argo Plastics Co., Los Angeles, CA) as specified in "Results." Light fluence rates were measured with a LiCor Quantum

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[3] Abbreviations: NR, nitrate reductase (EC 1.6.6.1); GPD, glyceraldehyde-3-phosphate dehydrogenase; NiR, nitrite reductase (EC 1.7.7.1); RL, low intensity red light (0.3 μmol m^{-2} s^{-1}); WL, high intensity white light (100 μmol m^{-2} s^{-1}).

Photometer (LI 185-A, Lambda Instruments Corp., Lincoln, NE). For all pigment and enzyme assays, three pairs of cotyledons were harvested and ground on ice with a mortar and pestle in 10 mL of the corresponding extraction medium, and the supernatant was clarified by centrifugation (20 min at 40,000*g*). Aliquots of the supernatant were then used for pigment or enzyme measurement.

For RNA extraction, 12 to 20 pairs of cotyledons were harvested and immediately frozen in liquid nitrogen. Only about 40 to 50% of the norflurazon-treated seedlings were completely bleached, while the cotyledons of the others contain greenish tips and spots. For the results shown here, only those cotyledons that had no visible Chl were harvested. Under our conditions, the seedlings grown in the presence of KNO_3 were somehow bigger than the corresponding water control, while the size of the cotyledons of the KNO_3/norflurazon-treated seedlings were about the same size as the water control.

Total RNA was isolated as described previously (18). Poly(A^+)-containing RNA was obtained by poly-(U)-Sephadex chromatography (Bethesda Research Laboratories) according to manufacturers' instructions. Equal amounts of RNA were resolved on a 1.5% agarose gel under denaturating conditions (6) and transferred to nitrocellulose paper (24). Filters were prehybridized at 42°C in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl, 3 mM Na citrate), 5× Denhardt's solution (100× Denhardt's solution is 2% each of BSA, Ficoll, and polyvinylpyrrolidone), 25 mM potassium phosphate (pH 6.5), 0.5 mg/ml denatured salmon sperm DNA, 0.1% SDS. Hybridization for 40 h was performed using the same buffer system containing the insert of NR probe pCmc2 (8) after labeling using random primer (12) (0.15 μg DNA, specific activity 5×10^7 Cerenkov counts/μg DNA). The DNA was hydrolyzed to 400 to 800 base pair pieces prior to hybridization by boiling in 0.5 M NaOH for 3 min and was neutralized before use. The filters were washed with 2× SSC, 0.1% SDS for 1 h at 42°C, and 30 min at 55°C, 30 min at 65°C and 2 min at room temperature with 0.2× SSC. The insert was purified from an agarose gel after digestion with *Eco*RI. DNA fragments were bound to a DEAE membrane NA45 (Schleicher & Schüll), the membrane was washed with low salt buffer (150 mM NaCl, 0.1 mM EDTA, 20 mM Tris-HCl [pH 8]) at room temperature, and the DNA fragments were eluted at 65°C with high salt buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl [pH 8]), phenol-extracted, and ethanol-precipitated.

The enzyme activity measurements were described previously (18). The enzyme activity measurements were done under conditions where the enzyme reactions were linear with regard to the incubation time and the amount of crude extract used. One katal (kat) of NR activity is 1 mol of nitrite formed s^{-1} at 25°C. Protein determination was performed according to Bradford (4).

Chl and carotenoid determinations were made by HPLC. Three pairs of cotyledons were homogenized with mortar and pestle in the presence of 6 mL of methanol/chloroform v/v plus a few mg solid Na_2CO_3 to remove acid. Three mL of water and 3 mL of chloroform were added, the extract was vortexed for 2 min, and the phases were separated by centrifugation (2 min, 30,000*g*). An equal volume of the organic phase (not shown) or an equal amount of Chl in the organic phase (Fig. 2) was used for the separation. The gradient and the separation system have been described elsewhere (23).

Norflurazon was a gift from Zoecon Corporation, Palo Alto, CA.

RESULTS

Comparison of Squash Seedlings Grown on H_2O, KNO_3, and KNO_3/Norflurazon

We grew squash seedlings on vermiculite, H_2O, 10 mM KNO_3, or a solution containing 10 mM KNO_3 and 10^{-4} M norflurazon in high intensity WL for 5 days. Norflurazon inhibits colored carotenoid formation. As a consequence of the carotenoid deficiency, the unprotected Chl is photooxidized in WL, as has been reported for many other systems (17). In addition, we have measured the plastidic NADP-dependent and the cytoplasmic NAD-dependent GPD activities in crude extracts prepared from squash cotyledons. While the plastidic NADP-dependent enzyme activity is reduced to about 1/50 of the level found in control seedlings in the extract prepared from norflurazon-treated seedlings, no such a reduction can be detected in the level of the cytoplasmic NAD-dependent enzyme activity (Table I). Thus—as with many other seedlings investigated to date (17)—growth in the presence of the herbicide norflurazon in WL destroys the plastidic compartment. Levels of extraplastidic enzymes that are not related to functions in the plastids remain relatively unaffected. In addition, growth of the seedlings in the presence of 10 mM KNO_3 leads to a stimulation of seedling development: the amounts of pigments, both GPD activities, and extractable total protein are about 20 to 25% higher in KNO_3 *versus* H_2O-grown seedlings and this inductive effect of KNO_3 is missing in KNO_3/norflurazon-treated seedlings (compare NAD-GPD activities and total protein in Table I).

Table I. *Amount of Carotenoids and Chl, Plastidic NADP- and Cytoplasmic NAD-GPD Activities, Extractable Soluble Protein, and NR Activity in Squash Cotyledons that Were Grown for 5 d in WL either on Water, 10 mM KNO_3, or 10 mM KNO_3 + 10^{-4} M Norflurazon*

The data are based on amounts per pair of cotyledons; the highest amounts were taken as 100%. As described in "Materials and Methods," seedlings grown in the presence of the herbicide norflurazon were selected to be Chl free. Average of three determinations. Variations are between 0.3 and 9%; 100% NADP-GPD (37.4 nkat pair of cotyledons^{-1}); 100% NAD-GPD (54.2 nkat pair of cotyledons^{-1}), total protein (9.9 mg pair of cotyledons^{-1}), 100% NR (1.4 nkat pair of cotyledons^{-1}).

Assay	WL Growth Conditions		
	H_2O	KNO_3	KNO_3/norflurazon
Carotenoids	80	100	<1
Chl	78	100	1
NADP-GPD (plastid)	73	100	2
NAD-GPD (cytosol)	79	100	73
Total protein	84	100	77
NR activity	1.4	100	3.6

Effect of KNO_3 and KNO_3/Norflurazon on NR Activity and NR mRNA Levels

When squash seedlings are grown for 5 d in WL on water, scarcely any NR activity can be detected, whereas when they are grown for the same time in WL in the presence of 10 mM KNO_3, activity is high (Table I). The effect of KNO_3 on NR activity accumulation is almost completely prevented after photooxidative destruction of the chloroplasts. Therefore, we conclude that, as in other systems, nitrate-mediated accumulation of NR activity in squash cotyledons requires intact plastids.

Total RNA was isolated from plants grown as for Table I, and an equal amount of RNA (5 μg) separated on a denaturating agarose (Fig. 1, left panel). The 25S and 18S RNA bands of the cytoplasmic ribosomes are detectable in H_2O, KNO_3 and KNO_3/norflurazon-treated seedlings, while the plastidic ribosomal bands (23S and 16S [arrow heads] as well as their breakdown products formed during the isolation procedure) are missing in the KNO_3/norflurazon-treated seedlings. This result also indicates the organelle specificity of the photooxidative events in squash.

We transferred an equal amount of poly(A^+)-enriched RNA (2 μg, Fig. 1, right panel) to nitrocellulose and hybridized to a ^{32}P-labeled NR-specific DNA fragment. Hybridization to a transcript about 3200 bases long can only be detected in RNA preparations from seedlings grown in the presence of 10 mM KNO_3 (lane 2). Scarcely any hybridization signal is obtained with RNA from water-grown seedlings (lane 1) or from seedlings whose chloroplasts had been destroyed by photooxidation in the presence of KNO_3 (lane 3). With total RNA (not shown), nonspecific hybridization to ribosomal RNA could not be eliminated, but as with the poly(A^+)-enriched RNA, hybridization of an RNA 3200 base pairs long could only be detected from seedlings grown in the presence of KNO_3 but in the absence of norflurazon. If the autoradiogram (shown in the right panel) is overexposed, slight hybridization bands can also be detected in seedlings grown on water and in the presence of KNO_3/norflurazon. Spectrophotometric scanning of an overexposed autoradiogram indicates that nitrate induction of NR mRNA is at least 50- and 100-fold compared to the water control (as previously shown [7] for the same seedlings), while the transcript level detectable in KNO_3/norflurazon-treated seedlings is only 3 to 6 times higher than the level detectable in the water control. This result shows that the absence of NR activity in chloroplast-deficient seedlings is likely the consequence of the absence of NR message.

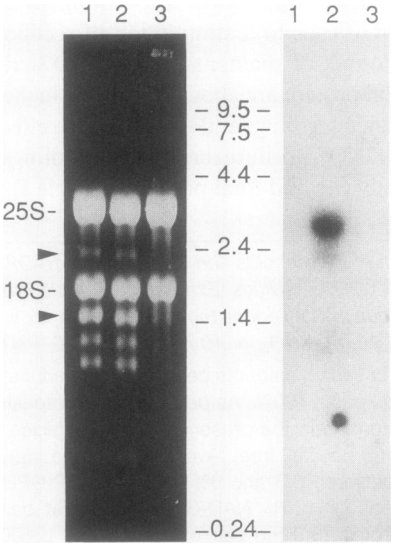

Figure 1. Left panel: Five μg of total RNA extracted from the cotyledons of squash seedlings which were grown for 5 d in WL either on water (lane 1), on 10 mM KNO_3 (lane 2), or 10 mM KNO_3/10^{-4} M norflurazon (lane 3) were separated on an 1.5% agarose gel and stained with ethidium bromide. Right panel: Poly(A^+)-enriched RNA was isolated from RNA preparations 1 to 3, 2 μg of RNA separated on each lane and hybridized to a labeled DNA fragment encoding NR. Numbers (in kilobases) refer to the position of RNA size markers. 25S, 18S = cytoplasmatic ribosomal RNA. Black arrowheads indicate position of plastidic ribosomal RNA.

Induction of NR Activity and NR mRNA by Application of Nitrate (100 mM) for 12 h

We grew squash seedlings with H_2O or norflurazon in WL or RL for 4.5 d, added nitrate (100 mM) at that time, and grew them for an additional 12 h under the same light conditions. NR activity and mRNA was measured immediately before and 12 h after nitrate application. Growth of the seedlings in RL minimizes photodamage to carotenoid-free plastids (17). This phenomenon is demonstrated by the results shown in Figures 2 and 3. Separation of pigment extracts from RL/H_2O-grown and RL/norflurazon-treated 5-d-old squash seedlings by HPLC confirm that most of the carotenoids are absent in the herbicide seedlings, and only a small amount of lutein accumulates (*cf.* Fig. 2, top and bottom). Even though the total amount of Chl in the norflurazon-treated seedlings was reduced by about 30%, the Chl *a*/*b* ratio was not affected. In addition, analysis of total RNA from these seedlings also confirms that plastidic ribosomal RNA accumulates in norflurazon-treated seedlings grown under low fluence rate RL (arrows, Fig. 3). However, the abundance of the 16S ribosomal RNA seems to be somehow reduced in norflurazon-treated seedlings, indicating that some photodamage occurs even in RL. Comparison of the abundance of the plastidic 16S RNA relative to the cytoplasmic 18S RNA in RL grown (Fig. 3) and WL-grown (Fig. 1, left panel, lanes 1 and 2) seedlings shows that there is less plastidic ribosomal RNA, relative to cytoplasmic ribosomal RNA, when the seedlings develop in low fluence-rate RL rather than high fluence-rate WL in the absence of NF. Thus, plastid development seems to be limited by the RL.

Table II shows that 12 h after nitrate application a roughly 100-fold increase in the NR activity can be detected in H_2O-grown seedlings kept in WL, while essentially no induction is observed in norflurazon-treated seedlings. NR activity can also be induced in H_2O- and norflurazon-treated seedlings kept in RL. Thus, it is the photodamage to the plastids rather than the norflurazon treatment *per se* or the absence of carotenoids that is responsible for the lack of the induction of NR activity in WL. However, inducibility of NR activity in RL is low and less than 30% of the activity detectable in WL-grown seedlings (see below).

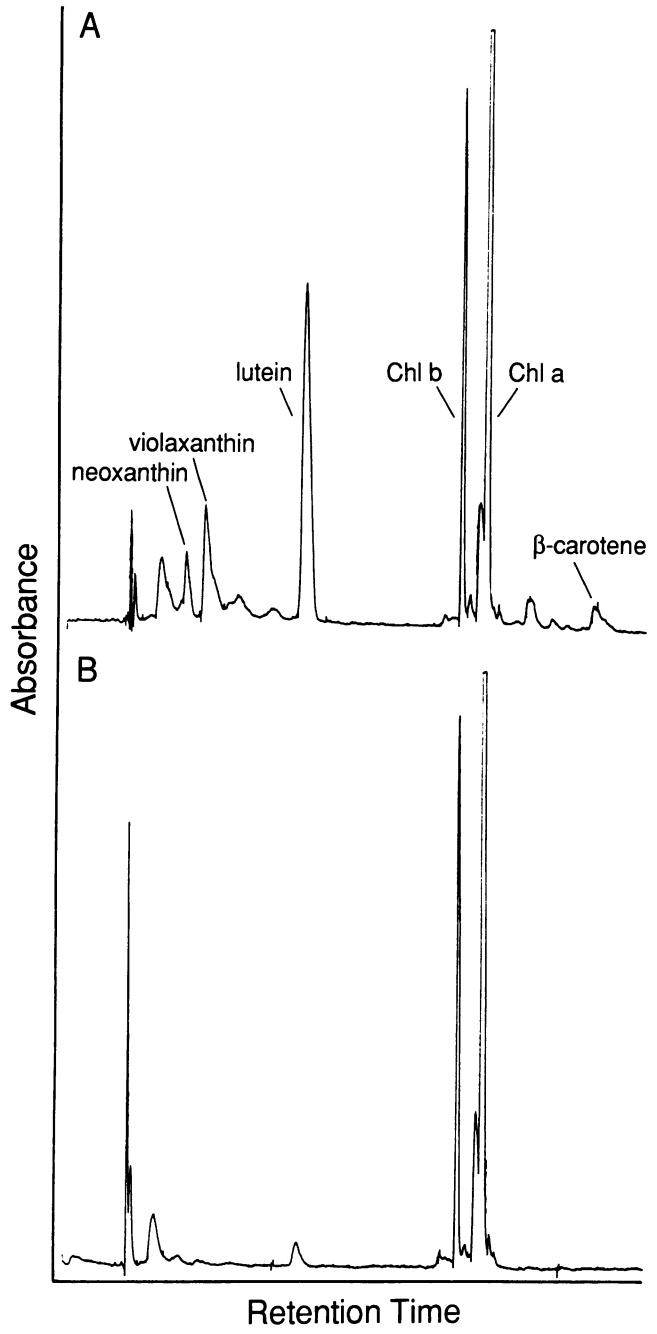

Figure 2. Separation by HPLC of a methanol/chloroform extract prepared from cotyledons of squash seedlings which were grown in the absence (A) or presence (B) of norflurazon in RL. See legend for Figure 1. An equal amount of Chl in the organic phase was used for the separation. The Chl content in the norflurazon-treated seedlings was about 70% of the content of untreated seedlings on a per cotyledon basis.

Isolation of poly(A^+)-enriched RNA from squash seedlings grown under the same conditions and hybridization to the NR-specific DNA fragment shows that the differences in the enzyme activity seen in Table II are reflected by differences in the level of NR mRNA (Fig. 4). Essentially no NR mRNA can be detected in cotyledons of squash seedlings before

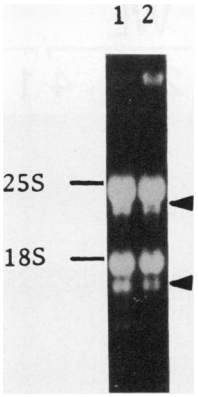

Figure 3. Ethidium bromide-stained agarose gel with total RNA (2.5 μg) from squash cotyledons that were grown on H_2O (lane 1) or norflurazon (lane 2) in RL. 25S, 18S = cytoplasmic ribosomal RNA. Black arrowheads indicate position of plastidic ribosomal RNA.

Table II. *Inducibility of NR Activity in Cotyledons of Squash Seedlings by 12 h of KNO_3 (100 mM)*

Seedlings were either grown on H_2O or norflurazon. KNO_3 was applied to the seedlings at 4.5 d after sowing and NR activity determined immediately before and 12 h after nitrate application (0 *versus* 12 h). Seedlings were kept continually under WL or RL from germination through nitrate treatment. The highest enzyme level was taken as 100% (0.43 nkat pair of cotyledons^{-1}). Average of three experiments.

Light Treatment	Hour after Nitrate Application	NR Activity	
		H_2O	Norflurazon
WL	0	0.8	0.6
	12	100.0	1.2
RL	0	0.3	0.7
	12	26.6	22.7

nitrate application (lanes 1 and 2). The NR mRNA level is high if nitrate is applied to WL-grown seedlings (lane 3, WL), and the inductive effect of nitrate on NR mRNA is prevented if the plastids are destroyed by photooxidation in the presence of norflurazon (lane 4, WL). If squash seedlings are kept in RL, NR mRNA is induced in both water- and norflurazon-pretreated seedlings (lanes 3 and 4, RL). In this case, also, the inductive effect of nitrate is less in RL-grown than WL-grown seedlings (compare lanes 3, WL and RL). If the autoradiogram is overexposed, slight hybridization signals can be detected in all RNA samples. Estimations of the relative amounts of NR mRNA levels (based on different exposure times) revealed that NR mRNA is induced at least 70-fold in WL/H_2O seedlings and about 4-fold in WL/norflurazon seedlings. In addition, the NR-mRNA level in WL/H_2O-grown plants is about 3.5-fold higher than in RL/H_2O-grown plants. These estimations are not dissimilar to the results of the NR activity measurements shown in Table II.

The reduced inducibility of NR activity in RL (compared to WL) is not limited by the lack of light during nitrate induction. Comparison of inducibility of NR activity in seedlings grown in WL, in RL, and after transfer from RL to WL

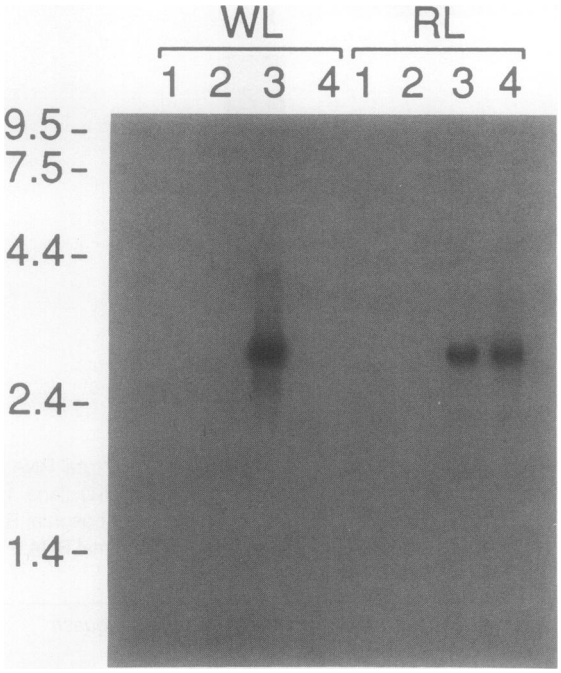

Figure 4. Inducibility of NR mRNA by KNO_3 (100 mM). Squash seedlings were grown in either WL or RL and were harvested for RNA extraction either before (lanes 1 and 2) or 12 h after nitrate application (lanes 3 and 4). Lanes 1 and 3: Seedlings grown on water; lanes 2 and 4: seedlings grown on norflurazon. Five μg of RNA was separated on each lane. Position of size markers are given to the left. When this experiment was repeated, the hybridization signal in lane 4 (RL) was about 30 to 25% less than the signal in lane 3 (RL).

Table III. *Inducibility of NR Activity by Application of Nitrate (100 mM) in Cotyledons of WL- and RL-Grown Squash Seedlings, and Seedlings which Were Transferred from RL to WL 4.5 d after Sowing*

Nitrate was applied 4.5 d after sowing and the cotyledons harvested 12 h later. The highest value was taken as 100% (0.43 nkat pair of cotyledons^{-1}). Average of three independent experiments.

Light Treatment	NR Activity
	%
WL, 5 d	100
RL, 5 d	24
RL, 4.5 d–WL, 12 h	34

at the time of nitrate application (Table III) indicates that other factors than light limit inducibility of NR activity. We suggest that the poorly developed status of the plastids in RL-grown squash seedlings may be responsible for the reduced inducibility of NR activity.

Photodamage to Plastids and Disappearance of NR Activity Can Be Separated in Time

We have grown squash seedlings in RL in the presence and absence of norflurazon. After 4 d, 100 mM KNO_3 was applied to the seedlings and the time course for change in NR activity and for Chl accumulation measured in seedlings which were either kept in RL or transferred from RL to WL at the time of nitrate application (time 0 in Fig. 5). In agreement with the results shown in Table II, NR activity is induced about 25-fold in RL within 24 h and this induction occurs in the presence or absence of norflurazon (Fig. 5, lower panel). However, if RL-grown seedlings are transferred to photooxidative WL at the time of nitrate application, NR activity can still be induced during the first 12 h, while no further accumulation occurs between 12 and 24 h after the shift to WL. On the other hand, a strong decrease in the amount of Chl was detected already during the first 12 h in WL indicating an initiation of photodamage to the plastids after the light shift (Fig. 5, upper panel, inset). Thus photodamage to the plastids and inducibility of NR activity by nitrate are separable. Although NR activity seems to disappear as a consequence of photodamage to the plastids, it disappears considerably later than the Chl.

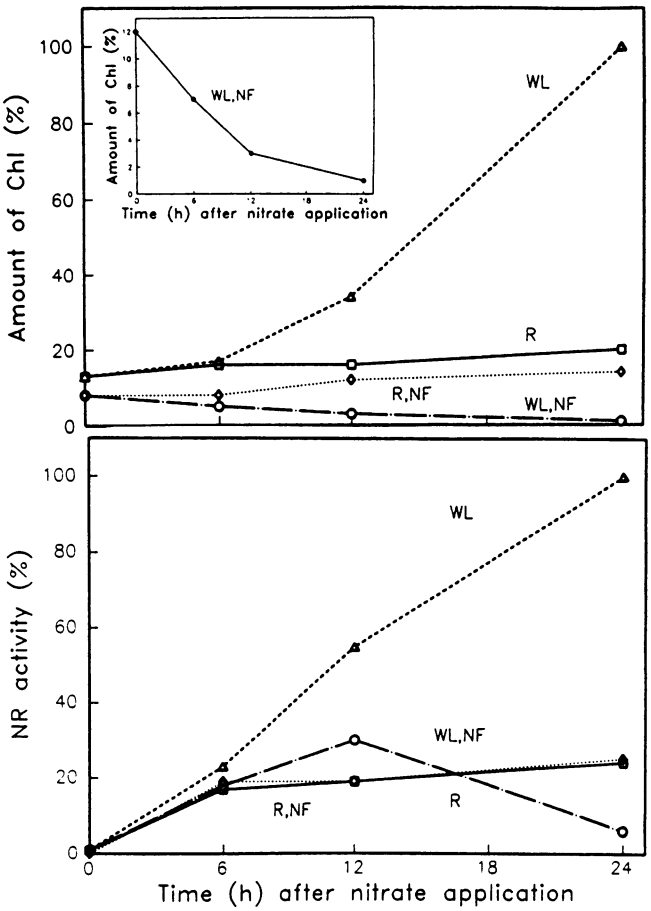

Figure 5. Comparison of the relative amount of Chl (upper panel) and the accumulation of NR activity (lower panel) in the cotyledons of squash seedlings. Seedlings were grown on H_2O or norflurazon (NF) for 4 d in RL. After application of 100 mM KNO_3, Chl and NR activity accumulation were followed for 24 h in seedlings which were either kept in RL or were transferred to WL at the time of nitrate application. For better comparison, the highest value of each graph was taken as 100% (corresponds to 1.16 nkat NR activity pair of cotyledons^{-1}). Average of three measurements, variation of the data between 0.9 and 14.3%. Inset: Expanded time course for loss of Chl from norflurazon-treated plants in white light.

DISCUSSION

It is well established that photooxidative destruction of the chloroplasts leads to the specific disappearance of cytoplasmic mRNAs which code for proteins located in the plastids (see ref. 17 for review). Here we report for the first time that a cytoplasmic mRNA level coding for a cytoplasmic enzyme is also affected by the damage to the chloroplast. Thus not only nuclear-encoded mRNAs which code for proteins located inside the plastids are affected by the photodamage to the plastids, but presumably also those mRNAs which code for cytoplasmic proteins with functions related to intact plastids. It is not yet known whether the plastidic control over NR mRNA accumulation is at the level of transcription or is posttranscriptional.

We also do not know at present whether the disappearance of NR mRNA is the consequence of the absence of a signal originating from the plastids (as proposed to be required for cytoplasmic mRNAs coding for proteins located in the plastids) or brought about by the accumulation of a product such as nitrite in the cytoplasm after photooxidative destruction of the plastids, a product that might inhibit NR mRNA accumulation via some feedback mechanism. The dependency of NR activity and mRNA accumulation on intact plastids might simply be caused by the inability of the chloroplast-free cell to detoxify nitrite. Thus the eukaryotic cell might have developed a mechanism which monitors nitrite in the cell and prevents its accumulation by the down-regulation of NR gene expression. Analysis of NR mRNA regulation in NiR mutants or transgenic plants which are capable of expressing an antisense DNA sequence coding for NiR will be helpful in exploring the mechanism(s) involved.

Nitrate and light are both involved in controlling NR accumulation in the cytoplasm. We have grown seedlings in the presence of nitrate in WL (which activates both phytochrome and a photoreceptor absorbing in the blue/UV region of the visible spectrum). Because NR-mRNA accumulation is inhibited completely after photodamage to the plastids, it seems that nitrate- and light-mediated induction of NR mRNA both require intact plastids.

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