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# Temporal and spatial regulation of nitrate reductase and nitrite reductase in greening maize leaves

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**Abstract**

The temporal and spatial regulation of nitrate reductase (NR), a cytosolic enzyme, and nitrite reductase (NIR), a chloroplastic enzyme, was examined in first leaf of maize seedlings. The induction of NR and NIR activity showed a biphasic response with reference to exogenous concentration of nitrate, which probably resulted from the biphasic uptake of nitrate in seedlings. The time course of accumulation of NR and NIR activities in maize leaf followed a pattern that was loosely co-ordinated, with NR activity peaking on the sixth day after sowing and NIR activity peaking on the seventh day. Although the magnitudes of induction in intact and detached leaves were different, the profiles of NR and NIR induction were basically similar. A study of NR and NIR distribution along the length of maize leaf showed opposite profiles of distribution, with NR activity being maximal at the base of the leaf, and NIR activity being maximal at the tip. These results suggest that the temporal and spatial regulation of NR and NIR are not tightly co-regulated in maize leaves. 

*Keywords:* Nitrate reductase; Nitrite reductase; Enzyme induction; Distribution; Maize; Leaf development

## 1. Introduction

Nitrate reductase (NR) and nitrite reductase (NIR) are the key metabolic enzymes in plants regulating reduction of nitrate to ammonia. In the cell, these enzymes are located at different cellular compartments, where NR is located in the cytosol and NIR is located in the chloroplasts. Both of these enzymes are inducible enzymes and supply of substrate-nitrate increases the activity of both enzymes [1]. Examination of NR regulation in several plant species revealed that in addition to nitrate, the NR activity in vivo is regulated by multiple control mechanisms involving factors such as light, plastids, sugars and reversible phosphorylation [2–4]. Similarly, studies on NIR regulation showed that NIR activity is regulated by nitrate, light and plastids [2,3,5,6]. Since both enzymes are induced by same substrate-nitrate, it is assumed that the nitrate and nitrite reductase genes are co-regulated by the nitrate, plastid and environmental stimuli such as light [2,7]. For example, in tobacco, mRNA levels of NR and NIR fluctuate in a circadian rhythmic fashion, with similar timings of maximal and minimal transcript accumulation [7], indicating a co-regulation of NR and NIR gene expression.

Only little information is available on co-regulation of activity of NR and NIR enzymes at the cellular level in plant systems. While it is known that these two enzymes are located in distinct cellular compartments, the relative level of these enzymes in the same cell is not known. One of the elegant model systems to study the co-regulation

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of these enzymes is monocot leaves. The monocot leaf offers an ideal system to study spatio-temporal pattern of development of enzymes because in monocots, the leaf growth takes place from continued cell divisions at the basal meristem. Therefore, a developing monocot leaf possesses the most immature cells at the base of the leaf, and the fully mature and differentiated cells at the tip [8]. Several studies have used this feature of monocot leaves to examine the spatio-temporal pattern of biogenesis of chloroplast and associated proteins in these leaves, which show an increase in level from the base to the tip of the leaf [9,10].

Several studies have examined regulation of NR and NIR activity in maize leaves [11–18]. In etiolated maize leaves, the light effect on NR activity is mediated through phytochrome [14] and by stimulating gene expression and reversible phosphorylation [16,17]. Similar to NR, NIR activity is also stimulated by phytochrome in etiolated maize leaves [13], and involves nitrate induced NIR transcript accumulation [18]. However, in all of these studies, the whole leaves from the seedlings have been used [11–14], therefore the information on relative distribution of these two enzymes along the length of leaf is not available. Only distribution of NR and NIR in maize leaf sections has been examined by immunohistochemical analysis, which showed that both NR and NIR enzymes are located solely in the mesophyll cells but not in the bundle sheath cells [15].

In view of limited information on spatial distribution of NR and NIR activity in maize, we examined the temporal and spatial regulation of these enzymes in leaves. We report that in maize leaves, the regulation of NR and NIR activity is not closely co-regulated and the distribution of these enzymes along the length of leaf is in opposition to each other.

## 2. Materials and methods

### 2.1. Plant materials

Maize (*Zea mays*) var Ganga-5 seeds were obtained from AP State Seed Corporation (Hyderabad, India). Seeds were sown on germination papers moistened with distilled water and the seedlings were grown at 25°C under continuous red-light (RL) ($\lambda_{max}$ 650 nm, 0.35 μmole $m^{-2}$ $s^{-1}$) or in darkness. Seedlings were harvested daily from 5 to 9 days after sowing or at the time points indicated, and after removing the coleoptile, the first leaf (outermost leaf) was excised from the seedling at the mesocotyl junction. The dark-grown leaves were excised under dim, green safe light. The enzyme activities were determined after homogenizing the whole leaf [19,20]. For analysis of NR and NIR distribution, the first leaf was dissected into 1 cm long segments successively from the base to the tip of the leaf. The segments were numbered from the base to the tip of the leaf. The enzyme activity was determined in each segment individually. Each experiment was repeated at least three times independently. The standard errors of the experimental data were calculated and were less than 5%.

### 2.2. Nitrate reductase assay

Nitrate reductase assay was performed according to the procedure of Whitelam et al. [19]. One gram of leaf tissue was homogenized in 2 ml of 50 mM potassium phosphate (pH 8.8) buffer consisting of 1 mM EDTA, 25 mM cystein and 3% (w/v) bovine serum albumin (BSA) (fraction V). The assay mixture consisted of 200 μl enzyme extract, 100 μl $KNO_3$ (100 mM) and 600 μl of 100 mM potassium phosphate buffer (pH 7). The assay was started by adding 100 μl of 2 mg/ml freshly prepared NADH solution. The assay was carried out at 30°C for 30 min. Thereafter, the reaction was terminated by transferring the assay mixture into 0.5 ml of boiling 0.3 M $ZnSO_4$ solution. The mixture was boiled further for 1 min and cooled to room temperature. Two hundred microliters of 1 N NaOH was added to the mixture and centrifuged at $5000 \times g$ for 5 min at room temperature. The supernatant was used to estimate the amount of nitrite released. The amount of nitrite released was estimated by adding 1 ml of sulfanilamide (1% (w/v) in 3 N HCl) and 1 ml of 0.05% (w/v) *N*-(1-naphthyl)ethylene diamine dichloride (NED) solution. The nitrite released was estimated by incubating the solution at 30°C for 30 min, and the absorbance was read at 540 nm. The amount of nitrite released was read from a standard curve of nitrite. The entire assay was completed within 1 h after homogenization of the sample.

### 2.3. Nitrite reductase assay

The nitrite reductase assay was performed according to Ramirez et al. [20]. One gram of leaf tissue was homogenized in 2 ml of 50 mM potassium phosphate (pH 8.8) buffer consisting of 1 mM EDTA, 25 mM cystein and 3% (w/v) BSA. The assay mixture consisted of 1.4 ml of 100 mM potassium phosphate buffer (pH 7.5), 100 μl of 5 mM $KNO_2$, 100 μl of enzyme extract and 100 μl of methyl viologen (2 mg/ml). The volume was made up to 1.8 ml with distilled water. To start the assay, 200 μl of sodium dithionite (25 mg/ml in 290 mM $NaHCO_3$ solution) was added and incubated for 30 min at 30°C. At the end of the incubation period, 100 μl of the assay mixture was added to 1.9 ml of water and vortexed immediately to oxidize the dithionite. The amount of nitrite used up by nitrite reductase was estimated by adding 1 ml of sulfanilamide (1% (w/v) in 3 N HCl) and 1 ml of 0.05% (w/v) NED solution. The solution was incubated at 30°C for 30 min, and the absorbance was read at 540 nm. The amount of nitrite used up by nitrite reductase was estimated from a standard curve of nitrite. The nitrate reductase and nitrite activities were expressed in katals.

### 2.4. Protein estimation

Protein was estimated according to the procedure of Lowry et al. [21]. The protein in the crude extracts was estimated after precipitation with an equal volume of 10% (v/v) trichloroacetic acid. The mixture was incubated for 30 min at $-20$°C and then centrifuged at $10\,000 \times g$ for 10 min. The precipitate was dissolved in 0.5 ml of 1 N NaOH. A standard curve for the protein estimation was prepared by using BSA fraction V. The absorbance of the protein sample was measured at 500 nm after 30 min incubation with the reagent mixture.

## 3. Results

NR and NIR activity was analyzed in leaves of 7-day-old maize seedlings grown in various concentrations of nitrate. Fig. 1A shows that NR activity presents a biphasic profile of induction, with the first peak at 40 mM followed by a decline in NR activity, then a gradual increase at higher concentrations. Dark-grown seedlings also showed a similar pattern, albeit the level of NR activity being much lower (Fig. 1A). A similar biphasic response to nitrate was also observed for NIR activity; with increasing nitrate concentrations, NIR activity reached a peak at 40 mM, followed by a decline and then a gradual increase (Fig. 1B).

Fig. 2 shows the time course of NR activity in leaves of maize seedlings grown in RL and darkness. In RL-grown leaf, NR activity gradually increased and reached a peak 6 days after sowing and then declined. Dark-grown leaf showed a pattern similar to light-grown seedlings and from 7 days onwards, both leaves had nearly equal activity (Fig. 2A). Similarly, NIR activity reached a peak level at 7 days and then declined. While NR activity of dark- and RL-grown leaf were nearly similar from 7 days onwards, NIR activity was higher in the RL-grown seedlings. Moreover, NIR activity attained its peak 1 day later than NR, at a time when NR activity had already declined considerably (Fig. 2B).

When 6-day-old light-grown seedlings were transferred to darkness, a decline in the NR activity was observed which could be offset by supplementing with nitrate (Fig. 3A). The

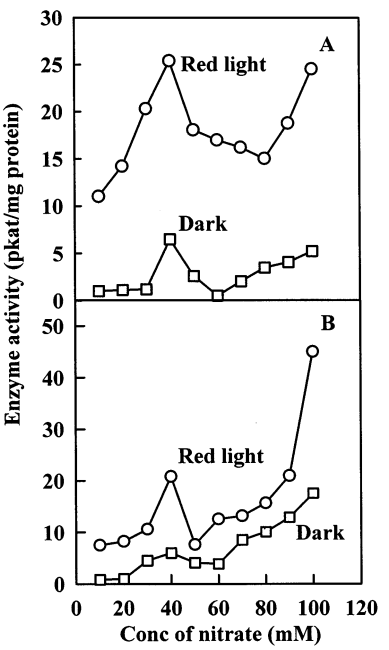


Fig. 1. Effect of different concentrations of nitrate on NR (A) and NIR (B) activity. Maize seedlings were grown in different concentrations of nitrate solution in red light or darkness for 7 days and the enzyme activity was assayed in the first leaf.

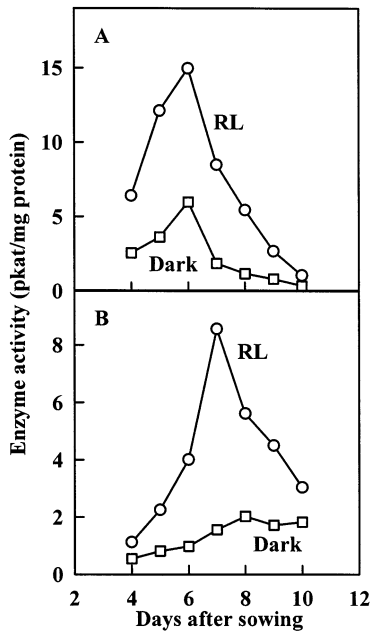


Fig. 2. Time course of NR (A) and NIR (B) activity in the first leaf. Maize seedlings were grown in distilled water in red light (RL) or in darkness (Dark), and the enzyme activity was determined at the time points indicated.

supplementation of 20 mM nitrate at the time of transfer to the darkness arrested the decline of NR, and in fact the presence of 60 mM nitrate stimulated NR activity. In the case of NIR, a similar pattern was observed where transfer to darkness reduced the increase in NIR activity compared with RL control (Fig. 3C). The reduction in NIR activity on transfer to darkness was prevented by 20 mM nitrate, and supplementing with 60 mM nitrate stimulated the NIR level 1.7-fold (Fig. 3C). The reverse experiment involving transfer of dark-grown seedling to light showed that transfer to RL stimulated NR activity 1.7-fold. Addition of 20 mM nitrate further stimulated NR activity 3.2-fold and 60 mM nitrate led to 4.8-fold stimulation (Fig. 3B). In the case of NIR, transfer to RL increased NIR activity 1.6-fold compared with dark control. The supplementation with 20 and 60 mM nitrate stimulated NIR activity 2.9- and 4.5-fold, respectively (Fig. 3D).

The detachment of leaves from seedlings lead to a decline in both NR and NIR activity, but the qualitative response of detached leaves to RL and nitrate treatment was similar to the seedlings (Fig. 4). Incubation of RL-grown detached leaves in darkness triggered decline in NR activity, but addition of 20 and 60 mM nitrate not only blocked the decline, but also stimulated NR activity (Fig. 4A). Similar to NR, transfer to darkness also decreased NIR activity, which was arrested only by 20 mM nitrate, which stimulated NIR activity. However, addition of 60 mM nitrate caused a reduction in NIR induction (Fig. 4C). The transfer of dark-grown detached leaves to RL stimulated NR activity, which was further stimulated by 20 and 60 mM nitrate (Fig. 4B). Similarly, in the case of NIR, transfer of detached leaves from darkness to RL stimulated NIR activity, and addition of 20 and 60 mM nitrate induced higher stimulation of NIR activity (Fig. 4D).

The distribution of NR and NIR activity along the length of maize leaves was examined by excising leaves into 1 cm long segments from the base to the tip, and determining distribution of NR and NIR activity in excised segments. The above analysis in 5-, 7- and 9-day-old seedlings showed that the NR activity was highest at the base of the leaf and it gradually declined towards the leaf tip (Fig.

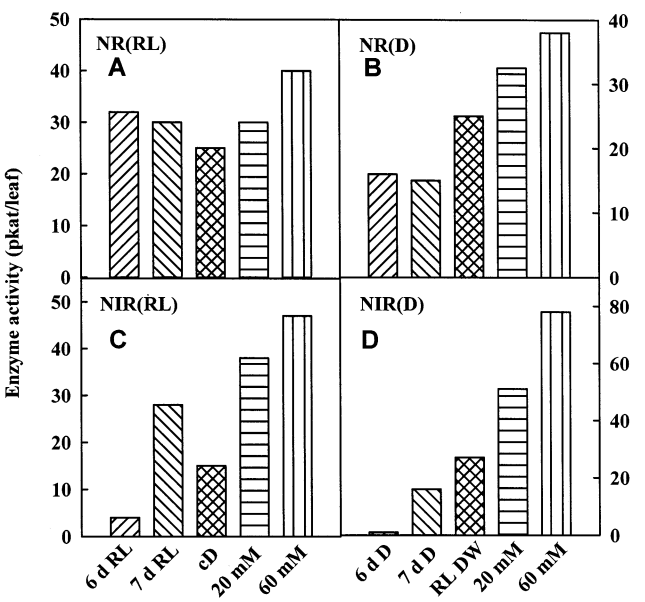


Fig. 3. Effect of light and nitrate on NR (A, B) and NIR (C, D) activity in intact first leaves. (A, C) Maize seedlings were grown in distilled water under red light (RL) for 6 days. Thereafter, seedlings were transferred to darkness and supplemented with distilled water (cD), or 20 or 60 mM nitrate solution. The enzyme activity was determined after 24 h of transfer. The enzyme activity was also estimated in 6-day-old (6 d RL) and 7-day-old (7 d RL) control seedlings. (B, D) Maize seedlings were grown in distilled water under darkness (D) for 6 days. Thereafter, seedlings were transferred to RL and supplemented with distilled water (RL DW), or 20 or 60 mM nitrate solution. The enzyme activity was determined after 24 h of transfer. The enzyme activity was also estimated in 6-day-old (6 d D) and 7-day-old (7 d D) control seedlings.

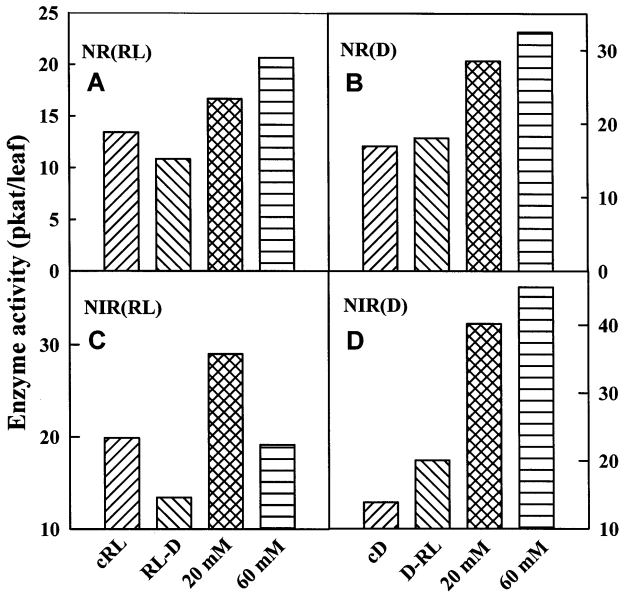


Fig. 4. Effect of light and nitrate on NR (A, B) and NIR (C, D) activity in detached leaves of maize. (A, C) Maize seedlings were grown in distilled water under red light (RL) for 6 days; thereafter, the first leaf was excised at mesocotyl node. The detached leaves were transferred to darkness for 24 h after supplementing with either 20 or 60 mM nitrate solution. The control leaves were kept in distilled water in darkness (RL-D) or red light (cRL). The enzyme activity was determined after 24 h of transfer. (B, D) Maize seedlings were grown in distilled water under darkness (D) for 6 days; thereafter, the first leaf was excised at mesocotyl node. The detached leaves were transferred to red light for 24 h after supplementing with either 20 or 60 mM nitrate solution. The control leaves were kept in distilled water in darkness (cD) or red light (D-RL). The enzyme activity was determined after 24 h of transfer.

5A). This pattern was constitutively observed for NR distribution, irrespective of the age of the seedlings. However, the distribution of NIR activity showed a diametrically opposite pattern. The highest activity of NIR was found at the tip of the leaf and the activity gradually declined towards the leaf base (Fig. 5B). The profile of distribution of NIR activity along the length of leaf remained similar irrespective of the age of the seedlings.

## 4. Discussion

Since NR and NIR both are the substrate inducible enzymes, their induction are closely dependent on the availability of nitrate. The results obtained showed biphasic induction of NR and NIR in relation to the varying concentration of nitrate and, comparatively, both light- and dark-grown seedlings show a similar profile, albeit the NR and NIR levels being higher in light-grown seedlings (Fig. 1). By contrast, in another study using detached wheat leaves, a steady increase in NR activity was observed with an increase in nitrate concentration [22]. Since NR and NIR both show biphasic activity profile in relation to increasing substrate concentration, it indicates the likely operation of a common mechanism causing this phenomenon. Most likely, the observed biphasic induction of enzymes may result from a biphasic uptake of nitrate by leaf cell, signifying the operation of two carriers at the membrane level operating at high and low affinity for nitrate [23,24]. The first phase of induction may be caused by the high affinity carrier and the second phase of induction by the low affinity carrier. However, other alternative explanations based on a dual affinity cytosolic inducer protein are equally plausible and cannot be excluded.

In maize leaves, induction of NR and NIR activity appears to be strictly regulated by development on a temporal scale. The overall profile of enzyme induction in both dark- and light-grown

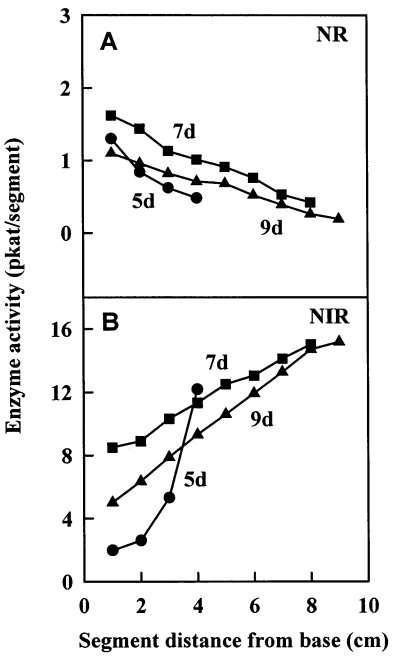


Fig. 5. Distribution of NR (A) and NIR (B) activity along the length of maize leaf. Maize seedlings were grown in distilled water under red light. The first leaf was excised at the mesocotyl node of seedlings 5, 7 and 9 days after sowing. The excised leaf was cut into 1 cm long segments from the base to the tip and enzyme activity was assayed in each segment. The segments are numbered from the base (1) to the tip.

leaves is similar. The exposure to light only elevates the level of NR and NIR but does not modify the pattern of temporal induction of enzymes. Apparently, while NR/NIR are dependent on same substrate for induction, their developmental programs are not strictly linked. This is evident from the observation that the maximal activity of NR and NIR are observed on different days. While NR activity peaked on the sixth day after sowing, NIR activity peaked on the seventh day (Fig. 2). Moreover, the profile of decline is also different. It is evident from the present study that while substrate induced co-regulation is evident in NR and NIR, the developmental programming of their gene expression is not that strictly co-regulated, leading to a difference in the peaking of maximal activity in NR and NIR. Since NR activity peaks earlier compared with NIR activity, it may also be associated with the age of the seedlings. For example, in detached corn leaves, treatment with nitrate led to a higher induction in younger seedlings compared with older seedlings [25]. Although the transcript levels in younger and older tissue are similar, the efficiency of translation in older tissue is much lower, which accounts for the decrease in NR activity and protein found in older leaves [25]. In this study, however, we examined only NR and NIR activities; given the complexities of NR and NIR level, it would be of interest to study whether the observed activity profiles of NR and NIR is also reflected in their respective transcript or protein level.

In maize leaves, light can independently modulate NR and NIR activity without nitrate, as evident by the fact that the light-grown seedlings grown on distilled water have higher activities of both the enzymes compared with dark. It is likely that NR and NIR genes in maize are under leaky regulation and light can stimulate enzyme formation even in the absence of the inducer nitrate [26]. The fact that the light is needed for sustenance of NR and NIR level is evident from the observation that the transfer of both light-grown seedlings or detached leaves to darkness reduces both NR and NIR activity, which can be offset by supplementing with nitrate. By contrast, the transfer of dark-grown detached leaves or seedlings to light significantly stimulated NR and NIR activity.

Several studies have examined NR and NIR induction in detached maize leaves [13,14,17]; these studies suffer from the criticism that the leaf detachment might have caused a stress response, which may alter or affect the NR and NIR induction. It is known that the NR activity is strongly affected by stress such as drought [11]. However, comparison of NR and NIR induction in seedlings with the detached leaves indicates that detachment of leaves does not alter the pattern of induction, which is qualitatively similar in seedlings and detached leaves. At the same time, the results for intact leaves are in conformity with general trends, where enzyme inductions are more effective in intact leaves than detached leaves. It is therefore evident that the detachment of the leaves caused a loss of responsiveness of the leaf to light-mediated NR and NIR induction.

In maize leaf, NR and NIR enzymes are located in mesophyll cells in the cytosol and plastid, respectively, but are absent in bundle sheath cells [15]; however, information on their distribution along the length of leaf is not available. The analysis of the distribution of NR and NIR along the length of the maize leaf showed diametrically opposite patterns. While the activity of NR was a maximum at the base of the maize leaf and gradually declined towards the tip of the leaf, the opposite was true in the case of NIR (Fig. 5). Since in the maize leaf, the basal cells are the youngest, and the cell age increases towards the tip, this finding agrees well with previous studies showing that younger tissues have higher NR activities [25,27]. It is evident that the distribution of NIR is similar to a typical chloroplastic enzyme such as LHCP and RUBISCO [9,10] in maize leaf, with a gradient of increasing activity towards the tip of the leaf. Thus, the expression of NIR is linked to chloroplast biogenesis and it is regulated in a manner similar to other photosynthetic genes such as the SSU of RUBISCO [3].

The observed diametrically opposite gradients of NR and NIR in maize leaf support the notion that these two enzymes are regulated by different developmental programming. Nitrate reductase is apparently regulated by cell age, and NIR by chloroplast development. However, NR activity is also dependent on chloroplasts and the loss of chloroplasts causes the loss of NR activity [2]. The gradual decline of NR activity along the leaf length in maize is somewhat similar to mitochondrial differentiation in wheat leaves [28], which is maximal at the leaf base and lowest at the leaf tip. It is also plausible that an increasing gradient of

cytosolic protease along the length of leaf may have determined the observed gradient of NR. Nevertheless, it is clearly evident that while a cell age gradient regulates the NR distribution, with NR activity being maximal in younger cells, the distribution of NIR is determined by a gradient of plastid maturity, similar to that observed for other plastidic proteins [9,10].

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