Hydrogen Peroxide Mediates the Induction of Chloroplastic Ndh Complex under Photooxidative Stress in Barley

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Chloroplast-encoded NDH polypeptides (components of the plastid Ndh complex) and the NADH dehydrogenase activity of the Ndh complex (NADH-DH) increased under photooxidative stress. The possible involvement of H2O2-mediated signaling in the photooxidative induction of chloroplastic ndh genes was thoroughly studied. We have analyzed the changes in the NADH-DH and steady-state levels of NDH-F polypeptide and ndhB and ndhF transcripts in barley (Hordeum vulgare cv Hassan) leaves. Subapical leaf segments were incubated in growing light (GL), photooxidative light (PhL), GL and H2O2 (GL + H2O2), or PhL and 50 mM paraquat in the incubation medium. Treatments with H2O2 under GL mimicked the photooxidative stimulus, causing a dose-dependent increase of NADH-DH and NDH-F polypeptide. The kinetic of Ndh complex induction was further studied in leaves pre-incubated with or without the H2O2-scavenger dimethylthiourea. NADH-DH and NDH-F polypeptide rapidly increased up to 16 h in PhL, GL, and H2O2, and, at higher rates, in PhL and paraquat. The observed increases of NADH-DH and NDH-F after 4 h in PhL and GL + H2O2 were not accompanied by significant changes in ndhB and ndhF transcripts. However, at 16-h incubations NADH-DH and NDH-F changes closely correlated with higher ndhB and ndhF transcript levels. All these effects were prevented by dimethylthiourea. It is proposed that the induction of chloroplastic ndh genes under photooxidative stress is mediated by H2O2 through mechanisms that involve a rapid translation of pre-existing transcripts and the increase of the ndh transcript levels.

The plastid DNA contains 11 ndh genes (Maier et al., 1995) encoding polypeptides (NDH) that are components of the plastid Ndh complex, analogous to the NADH dehydrogenase or complex I (EC 1.6.5.3) of mitochondrial respiratory chain (Sazanov et al., 1998; Casano et al., 2000). The increases of NDH polypeptides and Ndh dehydrogenase activity of the Ndh complex (NADH-DH) under photooxidative stress (Martín et al., 1996; Casano et al., 1999, 2000) suggest that the Ndh complex is involved in the protection against such stress. In fact, ndh-less mutants show increased sensitivity to photooxidative stress (Endo et al., 1999; Horvath et al., 2000). The purified Ndh complex catalyzes the transfer of electrons from NADH to plastocyanin and, in vivo a thylakoid plastoquinol peroxidase probably oxidizes the reduced plastocyanin with H2O2 (Casano et al., 2000). Ndh complex (providing electrons) plus plastoquinol peroxidase with Mehler reaction and superoxide dismutase (draining electrons) might poise the redox level of the electron carriers. This mechanism (chlororespiration) would most likely ensure the photosynthetic electron transport under a variety of environmental conditions that include rapid changes of light intensity associated with sunflecks and leaf movements. In addition, the chlororespiration may act as system scavenging reactive oxygen species generated under continuous photooxidative stress or by the successions of sunflecks and light gaps (Casano et al., 2000).

The increase in the levels of NDH polypeptides and Ndh complex activity (Martín et al., 1996; Casano et al., 1999, 2000) is the first described case of plastid DNA-encoded proteins that are stimulated by photooxidative stress. Thus, even though assuming an initial control of the plastid-targeted actions at the level of nucleus-cytoplasmic system, it is of interest to investigate whether or not H2O2 generated in the chloroplast could mediate the increase in the level of plastid-encoded proteins.

The photooxidative stress response shares strong similarities with the response of plants to pathogens (Levine, 1999), where a still poorly understood signal transduction pathway includes H2O2 and salicylic acid as components. Increasing evidence (for review, see Levine, 1999) suggests that the high concentrations of superoxide anion radical and H2O2 in the infection focus are high enough to kill not only the pathogen, but also the targeted plant cells (hypersensitive response). In these cells the concentration of H2O2 would trigger a programmed cell death. Meanwhile, the concentration of H2O2 in neighboring cells, at appropriate distance, would reach a lower level, which induces a succession of protecting genes that encode the pathogenesis-related proteins. The response of leaves to increasing photooxidative stress

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is very similar (Casano et al., 1999). At low photooxidative stress the battery of enzymes scavenging or preventing reactive oxygen species accumulation is induced. However, at higher photooxidative stress even these protective enzymes are more rapidly destroyed than induced (Casano et al., 1999). Moreover, H$_2$O$_2$ seems to be involved in the oxidative stress-mediated induction of nuclear-encoded defensive enzymes such as cytosolic ascorbate peroxidase (Karpinski et al., 1999; Morita et al., 1999), glutathione S-transferase, and catalase (Polidoros and Scandalios, 1999). Significant age-dependent differences in the response of protective enzymes to increasing photooxidative stress (Casano et al., 1999) also suggest a close relation among the mechanisms involved in photooxidative stress response, pathogen defense response, leaf cell senescence, and hypersensitive response.

Bearing in mind the above mentioned similarities we have investigated the possible involvement of H$_2$O$_2$ in the increase of plastid NDH polypeptides and NADH-DH under photooxidative stress. We have also investigated the possibility of whether these increases are related to the increases of the levels of the corresponding chloroplastic mRNAs.

RESULTS

Expression of Plastid ndh Genes in Response to Photooxidative Stress

Most of ndh genes are transcribed as policistrons in a fashion similar to a number of chloroplast-encoded genes (del Campo et al., 2000). However, ndhB and ndhF are transcribed monocistronically (Martinez et al., 1997) and their transcripts were detected at their predicted size, 1,650 and 2,400 b (data not shown), respectively, in freshly detached leaves of 7- and 14-d-old barley (Hordeum vulgare cv Hassan) plants (Fig. 1). The steady-state level of ndhB transcripts decreased or did not change after darkness or under growing light (GL) conditions, whereas ndhF transcripts slightly increased under GL in both types of leaves. In contrast, a marked increase of the steady-state level of both transcripts was observed in response to 20-h incubation under relatively excess light (photooxidative light [PhL]). However, the initial as well as the photooxidative-induced levels of both transcripts were significantly higher in 14-d-old leaves than in expanding 7-d-old leaves. This pattern of ndh transcripts correlates with parallel changes in the amount of NDH-F protein and the activity of the Ndh complex (Casano et al., 1999).

Effects of Hydrogen Peroxide on the Expression of ndh Genes

Generation of H$_2$O$_2$ during photooxidative stress has been proposed as a part of the signaling cascade leading to induction of nuclear-encoded protecting enzymes (Morita et al., 1999; Polidoros and Scandalios, 1999). It was interesting to investigate whether or not H$_2$O$_2$ is involved in the induction of Ndh complex, which participates in the protection of chloroplasts against photooxidative stress (Casano et al., 1999, 2000). As a consequence, a study was carried out on 14-d-old leaves due to their increased response to photooxidative treatments as stated above.

Changes in the Activity of the Ndh Complex and in the Level of NDH-F Protein

The NADH-DH of the thylakoid Ndh complex can be determined in crude extracts through zymogram analysis since it can be clearly distinguished from other pyridine nucleotide dehydrogenases (Casano et al., 2000). The incubation of leaf segments in the presence of H$_2$O$_2$ for 20 h under GL caused a dose-dependent increase of NADH-DH, reaching a 2-fold increase at 5 mM over the control incubated with water (Fig. 2). The incubation with H$_2$O$_2$ seemed to mimic photooxidative treatment, and its inductive effect was also observed in leaves maintained under darkness. In addition, H$_2$O$_2$-induced changes in NADH-DH closely correlated with variations in the level of one of the subunits of the Ndh complex, the NDH-F polypeptide (Fig. 2).

To investigate further the involvement of H$_2$O$_2$ in the photooxidative induction of Ndh complex, leaf segments were pre-incubated with dimethylthiourea

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**Figure 1.** Changes in the steady-state transcript levels of the plastid ndhB and ndhF genes in response to different light treatments. Primary leaves from young (7-d-old) and mature-senescent (14-d-old) plants were incubated for 20 h at 23°C in darkness (D), GL (100 μmol photon m$^{-2}$ s$^{-1}$), or PhL (300 μmol photon m$^{-2}$ s$^{-1}$). Total RNA was isolated from barley leaves, separated by agarose electrophoresis (12.5 μg each), blotted on a nylon membrane, and hybridized with ndhB or ndhF probes or stained with methylene blue (for rRNAs) as described in “Materials and Methods.”
(DMTU), a trap for H$_2$O$_2$ (Levine et al., 1994), and then transferred to different treatments. The results shown in Figure 3 indicate that pre-incubation with or without DMTU did not have a direct effect on the NADH-DH. As expected, NADH-DH was not affected by a subsequent incubation for 20 h under GL. However, treatments that presumably increase the endogenous generation of H$_2$O$_2$ such as PhL and PhL with paraquat (PQ) in the incubation medium (PhL + PQ), or the exogenous addition of H$_2$O$_2$ to leaves under GL (GL + H$_2$O$_2$) increased NADH-DH by 2.5-, 5-, and 3-fold of the initial level, respectively. In accordance with this, when H$_2$O$_2$ was quenched by pre-incubation with DMTU, no inductive effect of photooxidative treatments was observed.

The kinetic of photooxidative induction of the Ndh complex was studied in leaves pre-incubated with or without DMTU and then incubated under GL, PhL, GL + H$_2$O$_2$, and PhL + PQ up to 16 h at 23°C. Typical zymograms were shown in Figure 4A. Incubation under GL did not change NADH-DH or NDH-F polypeptide levels in water- and DMTU-pretreated leaves (Fig. 4, B–E). However, NADH-DH rapidly increased up to 16 h and then continued to rise at a lower rate in PhL and GL + H$_2$O$_2$ or it began to decrease in PhL + PQ (Fig. 4B). The pre-incubation with DMTU prevented the photooxidative induction of the enzyme up to 16 h (Fig. 4C). The amount of NDH-F polypeptide followed a pattern similar to that of NADH-DH during the course of incubations up to 16 h in both pretreated leaves (Fig. 4, D and E). Further incubation times produced complex effects. Thus, although PhL or GL + H$_2$O$_2$ but not PhL + PQ reduced NDH-F level, PhL + PQ, but not PhL or GL + H$_2$O$_2$, reduced NADH dehydrogenase. At first glance this differential response seems contradictory and requires further investigation because many factors are likely to be involved, e.g. membrane disassembly, changing barriers to diffusion of externally added H$_2$O$_2$, and/or complex dose-dependent effects of H$_2$O$_2$. In summary, NADH-DH and NDH-F polypeptide of Ndh complex were strongly induced by H$_2$O$_2$ and conditions that increase the generation of active oxygen species.

Changes in the Level of ndhB and ndhF Transcripts

To study whether or not the photooxidative- and H$_2$O$_2$-mediated induction of Ndh complex correlates with variations in the expression of plastid ndh genes we have analyzed the changes in the steady-state levels of ndhB and ndhF transcripts up to the 16-h incubation with H$_2$O$_2$ or under photooxidative conditions in leaves pretreated with water or DMTU. Typical northern blots for ndhB and ndhF are shown in Figures 5A and 6A, respectively. A 4-h incubation under GL, GL + H$_2$O$_2$, or PhL did not modify the levels of both transcripts in water- and DMTU-pretreated leaves (Figs. 5B and 6B, respectively). However, the amount of ndhB and ndhF transcripts was increased 2-fold with respect to the initial level by PhL + PQ in water pretreated leaves. In general, after a 16-h incubation a strong increase in the level of both transcripts was observed, with changes more

![Figure 3. Effects of DMTU, hydrogen peroxide, and photooxidative stress on Ndh activity. NADH-DH of Ndh complex was deduced from zymograms (not shown) of crude extracts (50 μg of protein per lane) from 14-d-old leaves pre-incubated with 0 and 5 mM DMTU for 4 h at 23°C under 100 μmol photon m$^{-2}$ s$^{-1}$. Thereafter, leaves were transferred to GL, GL and 5 mM H$_2$O$_2$ in the incubation medium (GL + H$_2$O$_2$), PhL and PhL and 50 nM PQ in the incubation medium (PhL + PQ) for 20 h at 23°C. Activities were expressed as percentages of the values in freshly detached leaves (7 nmol NADH oxidized min$^{-1}$ mg$^{-1}$ protein). Values are means of four different experiments.](https://www.plantphysiol.org/doi/fig/10.1104/pp.125.4.1452/fig10)
intense in ndhF (Figs. 5C and 6C). Under GL, only ndhF transcript of water-pretreated leaves increased 3-fold with respect to the initial level. Photooxidative conditions (PhL and PhL + PQ) and H₂O₂ had a very strong stimulating effect on the levels of both transcripts in water-pretreated leaves. However, quenching of H₂O₂ through pre-incubation with DMTU prevented partially, but significantly, this photooxidative induction.

Effects of Hydrogen Peroxide on Thylakoid Peroxidase

The thylakoid peroxidase can scavenge H₂O₂ by oxidizing the plastoquinol, which has been reduced by the action of Ndh complex (Casano et al., 2000). Like Ndh complex, plastoquinol peroxidase activity increases under photooxidative stress, especially in mature-senescent leaves (Casano et al., 1999). Therefore, it was interesting to study whether or not H₂O₂ is also involved in the induction of the thylakoid peroxidase activity. Time-dependent changes of peroxidase activity were studied in leaves pre-incubated with or without DMTU and then incubated under GL, PhL, and GL + H₂O₂ up to 30 h at 23°C. Typical peroxidase zymograms are shown in Figure 7A. Incubation under GL did not change peroxidase activity significantly in water- and DMTU-pretreated leaves (Fig. 7B and C, respectively). However, in water-pretreated leaves PhL caused a sharp increase of the enzymatic activity after a 4-h incubation, reaching a 3-fold level of the initial value and it then stabilized (Fig. 7B). The early inductive effect was also triggered by incubation with H₂O₂ (GL + H₂O₂), but after a 16-h incubation peroxidase activity did not differ from that of control. When leaves were pretreated with DMTU, no inductive effect of PhL or GL + H₂O₂ on peroxidase activity was observed (Fig. 7C). In general, the obtained results are in agreement with previous observations (Casano et al., 1999, 2000) and suggest that photooxidative induction of thylakoid peroxidase proceeds in time that of Ndh complex.

DISCUSSION

The ndhB and ndhF genes are transcribed from monocistronic units encoded in the inverted repeated and the small single-copy region of plastid DNA, respectively (Freyer et al., 1995; Maier et al., 1995; Martínez et al., 1997). On the other hand, six (H, A, I, G, E, and D) and three (C, K, and J) ndh genes are respectively grouped within two polycistronic transcriptional units that produce multiple transcripts, probably by a complex processing of primary transcripts (Maier et al., 1995; del Campo et al., 2000). The first unit also includes the psaC gene encoding a polypeptide of the photosystem I between ndhE and ndhD. There are uncertainties about which transcripts of each of the two polycistronic units are translated. Preliminary assays in our laboratory indicate that photooxidative and hormone treatments affect the steady-state levels of the different transcripts of polycistronic units in a complex way, suggesting that post-transcriptional processing may be involved in the control of the NDH polypeptides synthesis encoded in polycistronic units. In contrast, the effects
of leaf treatments on the levels of the single transcripts of ndhB and ndhF genes can be more easily investigated. Figures 1, 5, and 6 show that ndhB and ndhF transcripts increased after treatments producing oxidative stress, especially in mature-senescent leaves. This correlates well with increases of NDH polypeptides and NADH-DH of Ndh complex under photooxidative conditions (Martı ´n et al., 1996; Catala´ et al., 1997; Casano et al., 1999, 2000). All together, the results indicate that the induction of plastid Ndh complex is mediated, at least in part, by increases of mRNA levels.

Even though photooxidative stress is initiated within the chloroplasts, the unscavenged excess of H₂O₂ can rapidly diffuse out of the plastid (directly or indirectly), generating a situation of high risk of oxidative damage for the entire cell. To orchestrate an effective cell protection, a number of nuclear- and chloroplast-encoded genes must be induced coordinately. It is possible that some common signaling intermediate is involved in the antioxidant response at chloroplastic and nuclear levels. Environmental stresses such as suboptimal or extreme temperatures (Prassad et al., 1994; Fadzillah et al., 1996; Dat et al., 1998) and excess light (Karpinski et al., 1997) are known to increase the steady-state levels of H₂O₂.

Kovtun et al. (2000) recently demonstrated that a specific H₂O₂-responsive mitogen-activated protein kinase cascade mediates the H₂O₂ induction of Gst expression. A direct signaling action of H₂O₂ has also been described (Karpinski et al., 1999; Morita et al., 1999) for the nuclear-encoded cytosolic ascorbate peroxidase. In a similar manner, the incubation of barley leaf segments with H₂O₂ increased NADH-DH (Figs. 2 and 3), NDH-F polypeptide (Fig. 2), and ndhB and ndhF transcripts (Figs. 5 and 6). H₂O₂ applied under GL mimicked the effects of PhL and PhL + PQ, and all these effects were suppressed when leaf segments were pre-incubated with the H₂O₂ scavenger DMTU. These results strongly suggest a direct signaling of H₂O₂ in the induction of protective response against photooxidative stress in chloroplasts. Thus, H₂O₂, which is diffusible through membranes and, at least under photooxidative conditions, is mainly generated in the chloroplasts, induces the expression of specific nuclear and plastid genes. One may wonder whether its action on the expression of plastid ndh genes would depend on previous action(s) at the nucleus-cytoplasmic compartments.

Although increases of NADH-DH and NDH-F polypeptide after 10 to 30 h of H₂O₂ or photooxidative treatments (PhL and PhL + PQ; Fig. 4) may be mainly due to increases of transcript levels, the 100% increase of NDH-F and the 50% to 80% increase of

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**Figure 5.** Effects of DMTU, hydrogen peroxide, and photooxidative stress on the steady-state levels of plastid ndhB transcripts. A, Northern blot of total RNA from 14-d-old leaves, including RNA of freshly detached leaves (T0) and RNA of leaves pre-incubated with 0 and 5 mM DMTU for 4 h at 23°C under 100 μmol photon m⁻² s⁻¹, and then transferred to GL and 5 mM H₂O₂ in the incubation medium (GL + H₂O₂), GL and PhL, and PhL and 50 nM PQ in the incubation medium (PhL + PQ) at 23°C for the indicated times. Total RNA was isolated from barley leaves, separated by agarose electrophoresis (12.5 μg each), blotted on a nylon membrane, and hybridized with ndhB probe as described in “Materials and Methods.” B and C, Relative level of ndhB transcripts was deduced from experiments as those of A and normalized to the respective ribosomal RNA. Values were expressed as percentages of those in freshly detached leaves and represent the mean of three experiments.
NADH-DH after 4 h of treatment were not accounted for by increases of ndhB and ndhF transcripts (Figs. 5 and 6), except for PhL + PQ treatment. This suggests that in addition to the effect mediated by increasing mRNAs levels, photooxidative treatment and H2O2 (as its transduction signal) promote the translation of pre-existing transcripts of ndh genes. It is significant that an increase in NDH-F polypeptide and NADH-DH under oxidative treatment (Fig. 4, B and D) showed a two-phase behavior, the first one probably due to an effect on the translatability and the second one to an effect on mRNA level.

On the other hand, the relatively small, but significant increase of transcripts after 4 h of incubation with PhL + PQ (Figs. 5B and 6B) suggests that the intrachloroplastic H2O2 produced under such conditions could modulate transcript levels without the involvement of newly synthesized nucleus-cytoplasmic intermediates. However, the rapid increase of thylakoid peroxidase (Fig. 7, A and B), which is presumably encoded in the nucleus, also suggests a rapid action of H2O2 in the nucleus-cytoplasmic compartments, as reported for other H2O2-scavenging enzymes (Karpinski et al., 1999; Morita et al., 1999; Polidoros and Scandalios, 1999). An alternative explanation is that the thylakoid peroxidase could be activated directly by H2O2.

The enhanced expression of ndh genes reported in this paper is first described for plastid DNA genes under photooxidative stress. Plastid DNA from several species has been completely sequenced and although the control plastid gene expression includes different post-transcriptional steps (del Campo et al., 2000; Mayfield et al., 1995), its low size makes it a good candidate for investigating the induction of gene expression under photooxidative stress. The involvement of H2O2 as a key component of the signal transduction pathway in the responses of nucleus and chloroplast DNAs suggests that some similar steps or mechanisms coordinate the responses of the two genomes against photooxidative stress.

MATERIALS AND METHODS

Plant Material

Barley (Hordeum vulgare cv Hassan) was grown on vermiculite in a controlled growth chamber at 23°C under a 16-h photoperiod of 100 μmol photon m⁻² s⁻¹ white light as described by Casano et al. (1999). In the present work we have used primary leaves of 7- and 14-d-old plants as young expanding and aged-senescent leaves, respectively. Subapical leaf segments (3 cm in length) were cut 4 to 5 h after the beginning of photoperiod and were incubated at 23°C up to 30 h with different concentrations of H2O2 or 50 nM PQ in the incubation medium (PhL + PQ) at 23°C for the indicated times. Total RNA was isolated from barley leaves, separated by agarose electrophoresis (12.5 μg each), blotted on a nylon membrane, and hybridized with ndhF probe as described in “Materials and Methods.” B and C, Relative level of ndhF transcripts was deduced from experiments as those of A and normalized to the respective ribosomal RNA. Values were expressed as percentages of those in freshly detached leaves and represent the mean of three experiments.
segments were treated with 0 or 5 mM DMTU for 4 h in GL at 23°C prior to treatment of leaves as described above.

RNA Isolation and Northern-Blot Analysis

Total RNA of leaf segments (2 g) was extracted by phenol-SDS treatment and selective precipitation with LiCl as described (Jones et al., 1985; Eker and Davies, 1987). It was typical that RNA yields were around 0.25 mg/g leaves. RNA samples (12.5 μg) were denatured in formaldehyde and run on 1.2% (w/v) agarose-18% (v/v) formaldehyde gels (Sambrook et al., 1989). After electrophoresis, denatured RNA was immobilized on nylon membranes (Zeta-Probe, Bio-Rad, Hercules, CA). Ribosomal RNAs and Mr markers (Boehringer Mannheim, Mannheim, Germany) were stained with methylene blue (Sambrook et al., 1989) and then scanned using an UVP Easy digital image analyzer (Ultra-Violet Limited, San Gabriel, CA), with automatic background correction. Thereafter, membranes were hybridized to digoxigenin-labeled PCR probes of \textit{ndhB} and \textit{ndhf} genes. Washings were performed under high stringency conditions. Transcript bands were scanned and mRNA levels were expressed on a total rRNA basis as described (Casano et al., 1994).

Homologous digoxigenin-labeled PCR probes of \textit{ndhB} and \textit{ndhf} genes were prepared (Lo et al., 1990) from barley plastid DNA as template. Barley plastid DNA was isolated as described by Heinhorst et al. (1988). The probes were enriched in the strand complementary to transcripts by using a 50:1 ratio of 3’ terminal:5’ terminal primer for each corresponding mRNA. In this order the used primers were: ATCGATTCAACCTGAT and AGCCTCATTAGCCG-TAG spanning a 396-bp of \textit{ndhB} probe in barley (Freyer et al., 1995) and CCCACAGTAACTACCT and GCGTTTTATATGTTTCGG spanning a 735-bp \textit{ndhf} probe near the 3’ end in rice (Hiratsuka et al., 1989) and probably in barley.

Preparation of Leaf Crude Extracts

For zymographic and western-blot assays, activities and proteins were assayed in whole-leaf extracts obtained as follows: 10 leaf segments were homogenized with a mortar and pestle in 2 mL of 50 mM potassium phosphate, pH 7.0, 1 mM l-ascorbic acid, 1 mM EDTA, and 5% (w/v) polyvinylpyrrolidone, and were centrifuged at 500 g for 10 min. Triton X-100 was added to supernatant to make a final 2% (w/v) solution and gently stirred for 30 min. The suspension was centrifuged at 20,000g for 30 min. Supernatants contained 0.7 to 1.3 mg protein mL\(^{-1}\). The entire procedure was carried out at 4°C.

Gel Electrophoresis, Zymograms, and Immunoassays

Native PAGE was carried out at 5°C (usually with 100 μg of protein samples) in a linear gradient gel of 3% to 10%


Jones JDG, Dunsmuir D, Bedbrook J (1985) High level expression of induced quimeric genes in regenerated transformed plants. EMBO J 4: 2411–2418


protein kinase cascade in plants. Proc Natl Acad Sci USA 97: 2940–2945


