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Chloroplast iron-sulfur cluster protein maturation requires the essential cysteine desulfurase CpNifS


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NifS-like proteins provide the sulfur (S) for the formation of iron-sulfur (Fe-S) clusters, an ancient and essential type of cofactor found in all three domains of life. Plants are known to contain two distinct NifS-like proteins, localized in the mitochondria (MtNifS) and the chloroplast (CpNifS). In the chloroplast, five different Fe-S cluster types are required in various proteins. These plastid Fe-S proteins are involved in a variety of biochemical pathways including photosynthetic electron transport and nitrogen and sulfur assimilation. In vitro, the chloroplast cysteine desulfurase CpNifS can release elemental sulfur from cysteine for Fe-S cluster biogenesis in ferredoxin. However, because of the lack of a suitable mutant allele, the role of CpNifS has not been studied thus far in planta. To study the role of CpNifS in Fe-S cluster biogenesis in vivo, the gene was silenced by using an inducible RNAi (interference) approach. Plants with reduced CpNifS expression exhibited chlorosis, a disorganized chloroplast structure, and stunted growth and eventually became necrotic and died before seed set. Photosynthetic electron transport and carbon dioxide assimilation were severely impaired in the silenced plant lines. The silencing of CpNifS decreased the abundance of all chloroplastic Fe-S proteins tested, representing all five Fe-S cluster types. Mitochondrial Fe-S proteins and respiration were not affected, suggesting that mitochondrial and chloroplastic Fe-S assembly operate independently. These findings indicate that CpNifS is necessary for the maturation of all plastidic Fe-S proteins and, thus, essential for plant growth.

Results

CpNifS Is Essential for Plant Growth and Maintenance of Chloroplast Structure. Initially, CpNifS was silenced constitutively by RNAi using the cauliflower mosaic virus 35S promoter. Lines in which CpNifS expression was significantly reduced displayed severely chlorotic cotyledons and died as seedlings. Although plants with milder phenotypes could be propagated, we found these constitutively driven CpNifS RNAi lines to be unstable for the trait. To avoid these problems, and to be able to study the effects of complete CpNifS silencing at a later developmental stage, an ethanol-inducible RNAi construct was used to silence CpNifS in Arabidopsis plants. Eleven transgenic inducible CpNifS RNAi lines were obtained and bred to homozygosity. Two of these lines, CpNifS-6 and CpNifS-9, were selected for further studies. To determine the efficacy of the inducible construct, wild-type (WT) and the two selected transgenic RNAi lines were grown for 2 weeks and then induced by ethanol treatment; control plants were not treated with ethanol. WT and transgenic plants that were not induced with ethanol typically did not show any signs of stress (Fig. 1A) and had normal development and seed production. Indeed, cysteolic ascorbate peroxidase I, a marker of oxidative stress in plants (13), was not induced in ethanol-treated plants. Similar to bacterial IscS, the MtNifS homologue in yeast is required for Fe-S cluster formation in the mitochondria and cytosol, and MtNifS may function similarly in plants (7). CpNifS is a group II NifS-like protein (9). In vitro, CpNifS can provide the S for Fe-S cluster insertion into apo-Fd to form functional holo-Fd (10). Similar to the bacterial SufS, CpNifS cysteine desulfurase specific activity is low but greatly stimulated by a SufE protein (11, 12).

It may be hypothesized that CpNifS provides the sulfur for all five types of Fe-S proteins that occur in plastids. However, the specific function of CpNifS in chloroplastic Fe-S cluster assembly and its significance for plant development and survival has not been established in planta because of the lack of a suitable mutant. To determine the role of CpNifS in the synthesis of chloroplastic Fe-S clusters, we silenced the Arabidopsis CpNifS gene by using an ethanol-inducible RNAi construct and investigated the effects on levels of Fe-S proteins and photosynthesis.

Abbreviations: Fd, ferredoxin; MtNifS, mitochondrial NifS-like protein; CpNifS, chloroplastic NifS-like protein; PSI/II, photosystem I/II; ETR, electron-transport rate; RRF, ribosome-recycling factor; Rubisco, ribulose biphosphate carboxylase/oxygenase; SIR, sulfite reductase; NRI, nitrite reductase; GOGAT, 2-oxoglutarate aminotransferase.

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treated plants [see supporting information (SI) Fig. 6]. Only in rare cases was slight chlorosis observed in uninduced transgenics, possibly because of leakiness of the ethanol-inducible promoter. After 3 weeks of ethanol treatment, the CpNifS-6 and CpNifS-9 transgensics showed severely stunted growth, chlorosis, and leaf necrosis. Ethanol-treated WT plants showed no such symptoms, albeit that ethanol-treated WT plants were sometimes slightly smaller after 3 weeks of treatment (Fig. 1A). At this stage, the leaves of ethanol-treated CpNifS-6 and CpNifS-9 plants had at least 5-fold lower chlorophyll content compared with WT and untreated control plants (Fig. 1B). When ethanol treatment was stopped at this stage, the CpNifS plants recovered and were able to set seed. However, continued ethanol treatment ultimately resulted in irreversible damage and death of the RNAi transgenics, whereas it had no visible or only a marginal effect on the WT.

To determine whether the stunted growth of ethanol-treated transgenic plants coincided with the loss of CpNifS mRNA, leaf samples were taken from treated and untreated plants at week 3 of plant growth (1 week after the start of ethanol treatment) for RT-PCR analysis. The CpNifS transcript was not detected in leaves of chlorotic CpNifS-6 and CpNifS-9 plants that were treated with ethanol (Fig. 1C). The mRNAs for Actin2 and MtNifS (NFS1) were not affected by ethanol treatment, showing that the RNAi ethanol-inducible construct is specific for the CpNifS gene product.

The severe chlorosis displayed by the ethanol-induced CpNifS-silenced plants prompted us to analyze their ultrastructure by transmission electron microscopy to determine the effects of CpNifS deficiency on leaf cell and plastid structure. The overall cell shape and the morphology of the nucleus appeared the same in ethanol-treated WT, CpNifS-6, and CpNifS-9 plants; it is also noteworthy that the structure of the mitochondria was unchanged in CpNifS plants, suggesting that MtNifS and mitochondrial processes were not affected (data not shown). However, chloroplast structure was drastically changed in plants in which CpNifS was silenced (Fig. 2). Compared with the discrete and stacked thylakoid membrane grana displayed by WT, the grana in silenced CpNifS-6 and CpNifS-9 plants were hypertrophied and dissociated from each other. Also of interest was the absence of starch granules in CpNifS-6 and CpNifS-9, likely caused by a disruption of photosynthesis.

Taken together, these results strongly suggest that CpNifS is an essential protein in Arabidopsis. CpNifS loss of function causes pleiotropic phenotypes and eventually plant death. To analyze the primary cause of these phenotypes and to insight into the direct function of CpNifS in plants before pleiotropic phenotypes were apparent, further experiments with the inducible RNAi lines were performed by using plants in which ethanol treatment started 3 weeks after germination, analyzing the plants 10 days after induction.

**CpNifS Mutants Have Impaired Photosynthesis but Unaltered Respiration.** We hypothesized that CpNifS silencing would affect photosynthesis, because photosynthetic electron transport requires many Fe-S proteins, particularly in photosystem I (PSI). To determine how loss of CpNifS affects photosynthetic electron transport, we measured chlorophyll content in leaves of WT and CpNifS-6 and CpNifS-9 plants after 1 week of ethanol treatment (Fig. 1C). The mRNAs for Actin2 and MtNifS (NFS1) were not affected by ethanol treatment, showing that the RNAi ethanol-inducible construct is specific for the CpNifS gene product.

**Fig. 1.** Phenotypes of CpNifS-silenced plants. (A) Two-week-old WT, CpNifS-6, and CpNifS-9 plants were treated with 2% ethanol (E) every 4 days for 3 weeks. Control plants (C) were treated with water. (B) Chlorophyll content in plants treated with ethanol or control plants. P < 0.05 for ethanol-treated CpNifS-6 and CpNifS-9. (C) CpNifS, MtNifS, and Actin2 transcript detection by RT-PCR 1 week after the start of ethanol treatment. FW, fresh weight.

**Fig. 2.** Chloroplast ultrastructure is altered in CpNifS-silenced plants. Leaf samples were fixed 10 days after ethanol treatment of WT (A and B), CpNifS-6 (C and D), and CpNifS-9 (E and F) plants. Thin sections were examined by transmission electron microscopy. B, D, and F show magnifications of the boxed areas in A, C, and E, respectively. St, starch granules; Gr, grana. (Scale bars, 1 μm.)
transport in intact plants, a chlorophyll fluorescence imaging system was used. For silenced plants and controls, images were captured of two chlorophyll fluorescence parameters: $F_{v}/F_{m}$ (representing the maximum photochemical efficiency of PSII, $\Phi_{PSII}$) and $\Delta F/\Delta F_{0}$, which is indicative of the P700 activity of PSI, was analyzed in leaf disks by measuring the light-induced absorbance change at 820 nm ($\Delta A_{820}$), which occurs with photooxidation of the P700 reaction center of PSI. Ethanol-treated and Psi9-silenced plants had less than one third of the $\Delta A_{820}$ exhibited by ethanol-treated WT or untreated plants (Fig. 4 D), which indicates a substantial loss of photochemically active P700. The rate of dark reduction of P700 after the oxidizing flash was also reduced in the CpNifS-silenced plants, indicating that the flow of electrons into PSI from upstream donors was slower (Fig. 4 B). To test whether the leaf phosphate transport activity limited plant productivity, we measured photosynthetic CO$_2$ fixation. Indeed, the CO$_2$ fixation rate in the light was reduced by 50% in the ethanol-treated CpNifS-silenced plants compared with WT and untreated plants (Fig. 4 C). However, oxygen consumption in the dark, which is indicative of mitochondrial respiration, was not affected (Fig. 4 D). Thus, CpNifS silencing does not appear to disrupt mitochondrial function, while severely affecting chloroplast function.

**Levels of Chloroplastic Fe-S Proteins Are Reduced by CpNifS Silencing.**

We next investigated the direct effect of CpNifS silencing on the abundance of chloroplast proteins with or without Fe-S clusters (Fig. 5 A). Consistent with an absence of CpNifS mRNA, very little CpNifS protein was detected in transgenic plants treated

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**Fig. 3.** Chlorophyll fluorescence analysis. (A) Chlorophyll fluorescence imaging. Shown are a regular-color photograph (Top) and false-color images for $F_{v}/F_{m}$ (Middle) and $\Phi_{PSII}$ (Bottom) of the same plants. False-color scales for fluorescence parameters are shown to the right (red color represents the highest values and blue represents the lowest values). (B) Upper) Relative ETR at varying light intensities. Squares, diamonds, and circles represent WT, $CpNifS$-6, and $CpNifS$-9, respectively. Open symbols correspond to untreated controls, and closed symbols signify ethanol treatment. (B Lower) Relative ETR at varying light intensities. Squares, diamonds, and circles represent WT, $CpNifS$-6, and $CpNifS$-9, respectively. Open symbols correspond to untreated controls, and closed symbols signify ethanol treatment. (B Lower) Relative ETR at varying light intensities. Squares, diamonds, and circles represent WT, $CpNifS$-6, and $CpNifS$-9, respectively. Open symbols correspond to untreated controls, and closed symbols signify ethanol treatment. (B Lower) Relative ETR at varying light intensities. Squares, diamonds, and circles represent WT, $CpNifS$-6, and $CpNifS$-9, respectively. Open symbols correspond to untreated controls, and closed symbols signify ethanol treatment.
with ethanol. The abundance of the CpNiFS activator, CpSufE, was decreased in the absence of CpNiFS, compared with WT and untreated controls. In contrast, the relative abundance of CpIscA, a putative scaffold protein for chloroplastic Fe-S cluster formation (17), was hardly affected by CpNiFS silencing.

To identify which specific steps of photosynthesis may be affected by the silencing of CpNiFS, we examined the abundance of a variety of proteins involved in photosynthesis (Fig. 5B). CpNiFS silencing did not affect the D1 protein, a component of the PSII reaction center in which no Fe-S clusters are present (18), suggesting that most PSII was intact. In contrast, components of the cytochrome b_{6}f complex were affected by CpNiFS silencing. Most noteworthy is the near absence of the 2Fe-2S Rieske protein. Although the abundance of cytochrome b_{6} was not affected, cytochrome b_{6} was more abundant in CpNiFS-silenced plants. Both cytochrome f and cytochrome b_{6} are heme (Fe) proteins and chloroplast-encoded. A very profound effect of CpNiFS silencing was observed in PSI. PsA, PsB, and PsaC, all 4Fe-4S proteins, were strongly reduced or absent in CpNiFS-silenced plants. The PsaD protein, which is not an Fe-S cluster protein but thought to be involved in Fd docking (19), was reduced also. This reduction in PsaD abundance may reflect the overall lack of integrity of PSI in CpNiFS-silenced plants. Perhaps to compensate for the very low abundance of PSI reaction-center proteins in CpNiFS-silenced plants, there was an increased abundance of subunits of the light-harvesting complex proteins of PSI (LhCl).

Surprisingly, no effect was seen on the abundance of the 2Fe-2S protein Fd in soil-grown CpNiFS-silenced plants. One possible explanation is that the Fd already present in the 3-week-old plants before ethanol induction was very stable, so that Fd levels did not go down once further production of holo-Fd ceased. To test this possibility, we induced CpNiFS silencing during germination on media plates. After 1 week, ethanol-treated transgenic plants had extreme chlorosis and stunted growth compared with WT and untreated plants. Western blotting showed that CpNiFS and Fd were absent in these ethanol-treated transgenics, indicating that Fd levels do decrease if CpNiFS is silenced at an early stage in plant development (Fig. 5E). Therefore, holo-Fd may be very stable and remain abundant even when the production of its cofactor is impeded. D1, a plastid-encoded subunit of PSI, and ribosome-recycling factor (RRF), which is a nuclear-encoded plastid protein, were not affected by CpNiFS silencing from germination.

In the soil-grown plants that were ethanol-induced at the 3-week-old stage, the ATP-A subunit of chloroplastic ATP synthase and the large subunit of ribulose biphosphate carboxylase oxygenase (Rubisco), involved in photosynthetic ATP production and CO_{2} fixation, respectively, were not affected; neither of these are Fe-S proteins (Fig. 5B). There was also no major effect on plastocyanin, the copper protein that is active in electron transport between cytochrome b_{6}f and PSI (Fig. 5B). Two other copper-containing proteins, Cu/ZnSOD1 and Cu/ZnSOD2, involved in free radical scavenging, were also not affected by CpNiFS silencing (Fig. 5D).

In addition to the photosynthetic proteins described above, other plastidic Fe-S cluster proteins are expected to depend on CpNiFS for their maturation. Indeed, the 3Fe-4S cluster protein Fd-glutamine-2-oxoglutarate aminotransferase (GOGAT) was slightly less abundant compared with WT and untreated controls. Moreover, sulfite reductase (Sir) and, to a lesser extent, nitrite reductase (Nir) were reduced in CpNiFS-silenced plants (Fig. 5C). Sir and Nir contain the stoicheiometric 4Fe-4S cluster unique to plants. The polypeptide level of NiR was reduced only moderately (30%) compared with WT and untreated plants. In contrast, the enzyme activity of NiR was significantly reduced, by 70% in CpNiFS-6 and 45% in CpNiFS-9, compared with untreated transgenic plants (SI Fig. 7). The observation that the activity of NiR was reduced to a greater extent than Nir protein levels suggests that apo-NiR, lacking the Fe-S cofactor, had accumulated in CpNiFS-deficient plants.
The levels of CpNifS and several Fe-S proteins were monitored over a 10-day period after ethanol induction to determine how quickly CpNifS decreased in CpNifS-silenced plants and how quickly it was followed by a decrease in chloroplastic Fe-S proteins. CpNifS began to diminish in transgenic plants 1 day after ethanol induction and had almost completely disappeared by day 10 (SI Fig. 8). Protein abundance of PSI subunits PsA/B began to decrease on day 2 after ethanol induction, whereas decline of PsAC levels started on day 7. By day 10, a slight reduction in GOGAT, NiR, and SiR was seen. Levels of the control protein RRF remained constant over the course of 10 days.

To determine whether the decrease in chloroplastic Fe-S proteins was caused by a decrease in mRNA, a Northern blot was performed to detect the transcript abundance of Rieske, GOGAT, and SiR. A decrease in mRNA was not observed (SI Fig. 6) despite a large reduction in Rieske, GOGAT, and SiR protein levels (Fig. 5 B and C), which suggests that these Fe-S proteins need their appropriate cofactor to be stable and supports the hypothesis that CpNifS is critical for Fe-S cluster formation in the plastid.

In view of the decrease in several abundant Fe-S proteins in the CpNifS-silenced lines, leaf nutrient status was investigated (SI Table 1). We did not see a reduction in total leaf iron content on the basis of dry mass; in fact, a modest increase (+25%, nonsignificant) was seen in the ethanol-induced transgenics, compared with ethanol-induced WT. Sulfur and phosphorous levels were significantly increased (by 25% and 50%, respectively) in these same plants.

To rule out the possibility that CpNifS plants suffered from a defective plastid-import machinery, protein levels of the nuclear encoded protoclorophyllide reductase B and the small subunit of Rubisco were tested to determine whether their mature protein levels are reduced together with accumulation of precursors, as reported for plastid-import mutants (20, 21). Both proteins were present in mature size at equal levels in all plant types and treatments. Protoplastophyllide reductase B and the small subunit of Rubisco precursor-sized proteins did not accumulate, suggesting that protein import was not affected by CpNifS silencing. Finally, the abundance of two mitochondrial Fe-S proteins, Nad9 (a component of the respiratory complex I) and biotin synthase, were not affected by CpNifS silencing (Fig. 5D). In summary, CpNifS silencing seems to specifically affect the maturation of Fe-S proteins in plastids.

Discussion

Silencing of CpNifS severely affected levels of chloroplastic Fe-S proteins and photosynthesis, and prolonged silencing resulted in a pleiotropic-stressed phenotype and eventually plant death. These results suggest that CpNifS is an essential protein that functions in plastid Fe-S cluster assembly and cannot be bypassed or complemented by MtNifS. When silencing was induced after the seedling stage, specific and reversible defects could be observed that yielded information about the functions of CpNifS. Silencing of CpNifS caused a defect in the accumulation of all eight chloroplastic Fe-S cluster proteins that were tested. The Fe-S proteins affected by CpNifS silencing together represent all five types of Fe-S clusters found in plastids, supporting the hypothesis that the cysteine desulfurase activity of CpNifS is required for the maturation of all Fe-S proteins in this organelle. Two mitochondrial Fe-S proteins were not affected, lending evidence that Fe-S cluster assembly in the mitochondria can operate independently of the chloroplast cysteine desulfurase.

Fd did not exhibit any decrease when ethanol was initiated at week 3. However, Fd was absent when CpNifS silencing was induced with ethanol from germination, suggesting that holo-Fd is very stable once formed. In contrast to Fd, several other chloroplastic Fe-S proteins had decreased significantly 10 days after CpNifS silencing at week 3. These different delays in reduction of Fe-S proteins after CpNifS silencing may reflect protein stability or priority of Fe-S cofactor delivery.

The observed defects in photosynthetic electron transport and carbon fixation likely were a consequence of the lack of thylakoid Fe-S proteins, particularly in the cytochrome b6/f complex and PSI. Indeed, PSI function was severely compromised after CpNifS silencing. At the same time, PSII was only marginally affected in comparison, as evidenced by the presence of the D1 protein and functional heat dissipation, measured as nonphotochemical quenching of chlorophyll fluorescence (data not shown). A comparison of the FΨPSII and Fv/Fm images suggests that FΨPSII was affected before Fv/Fm, which may imply that damage to PSI could be a secondary consequence of a downstream defect in photosynhetic electron transport.

The altered chloroplast ultrastructure observed in CpNifS-silenced lines is reminiscent of the ultrastructure reported for an APO1 mutant (22) that affects PSI accumulation, as well as a mutation in Hcf101 and other mutations that affect PSI (23, 24). Therefore, the dilated stromal lamellae and absence of grana may be a consequence of a lack of PSI.

Mitochondria and chloroplasts originated from separate endosymbiotic events during the evolution of eukaryotes, and the two organelles have separate NifS-like proteins. It is likely that these two NifS-like proteins with their different properties each evolved to function optimally in their respective environments. The main function of mitochondria is to carry out the oxygen-consuming process of respiration, whereas chloroplasts perform the oxygen-generating process of photosynthesis. Thus, although both organelles contain an electron-transport chain that depends on Fe-S protein assembly, they contrast in redox conditions. Moreover, photosynthesis is known to produce reactive oxygen species, which can lead to oxidative stress. Fe-S cluster biosynthesis is particularly sensitive to oxygen. Therefore, it is not surprising that the chloroplastic NifS that has to operate under high-oxygen conditions is most similar to the bacterial SufS, which is thought to function under oxidative stress (3). MtNifS is most similar to bacterial IscS, the housekeeping NifS-like protein that is more sensitive to oxygen. Therefore, MtNifS likely would not function properly in an oxygen-producing compartment.

Another difference between the chloroplast and mitochondrion is that the chloroplast is the main site of cysteine synthesis in plant cells. It may be important to tightly control the cysteine desulfurase activity of the chloroplastic NifS, to avoid futile cycling. CpNifS may be particularly suited for the chloroplast because its cysteine desulfurase activity is extremely low in the absence of its activator CpSufE, in contrast to group I NifS-like proteins such as IscS and MtNifS (11). In summary, the two Fe-S cluster biosynthesizing machineries in the chloroplast and mitochondrion likely have different evolutionary origins and display properties that fit their function and environmental conditions.

After these two Fe-S biosynthesis machineries came together in the same plant cell, have they shared or transferred some of their functions? As shown here, CpNifS silencing is lethal in Arabidopsis and affects all five chloroplast Fe-S cluster types. Thus, MtNifS cannot complement the function of CpNifS in the biogenesis of any of these cluster types. At this point, it has not been reported whether MtNifS is essential as well, as was shown to be the case in yeast (25). In our studies, CpNifS silencing had no effects on mitochondrial Fe-S protein levels or respiration, suggesting that the mitochondrial Fe-S biogenesis machinery does not depend on CpNifS. Together, these results indicate that in plants, mitochondria and chloroplasts still have separate, essential cysteine desulfurases and Fe-S cluster assembly machineries.

Materials and Methods

Generation of CpNifS-Silencing Constructs and Induction. Standard cloning techniques were used to make the plant-transformation constructs and to generate transgenic Arabidopsis thaliana. For a detailed description of the cloning steps, see SI Text, Supporting Information on the Cloning and Plant Transformation. The primers

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used in plasmid construction and verification are listed in SI Table 2. Ten constitutive RNAi lines and 11 inducible RNAi lines were obtained, which were selfed and propagated to homozygosity. Two of these lines were used for further functional characterization: CnPnSiS::RNAi and CnPnSiS::RNAi-9 (denoted as CnPnSiS- and CnPnSiS-9, respectively). To induce the RNAi construct, plants grown in soil were sprayed and soil-drenched every 4 days with a 2% ethanol solution, a concentration that was reported not to induce stress (26, 27); untreated control plants were sprayed with water. For RNAi induction on agar medium, plants were germinated on 

RT-PCR and Immunoblotting. The presence of transcripts in plants was detected by using RT-PCR (29). Protein extraction, SDS/PAGE, and immunoblot analysis were performed essentially as described (30). Leaf tissue for protein analysis was collected 10 days after induction. Antibodies for CnPnSiS (9), CnSufE (32), CnFd and CnSiR (31), cytochrome 

Electron Microscopy. Leaves were sampled from soil-grown plants 10 days after the start of ethanol treatment. Fixation and sectioning before analysis by transmission electron microscopy were performed as described (40).

Photosynthesis and Respiration Measurements. Chlorophyll content was assayed as described (41). Chlorophyll fluorescence images of Fv/Fm and Fv/Fm were captured from control and ethanol-treated dark-adapted soil-grown plants by using a Photon System Instruments imaging system (Photon System Instruments, Brno, Czech Republic). Default protocol settings were used with an actinic light intensity of 110 μE. A fluorescence monitoring system chlorophyll fluorometer (Hansatech, Cambridge, U.K.) was used for quantitative chlorophyll fluorescence analysis on detached, fully expanded leaves taken from dark-adapted plants. Fv/Fm, Fv/Fm, and ETR were calculated as described (14).

Photooxidation and dark-reduction kinetics of P700 (PSI) were measured in leaf disks by determining the light-induced absorbance change at 820 nm (ΔA820) (16, 42) and at a saturating light intensity of 1600 μE, determined empirically. Carbon assimilation was assayed in detached leaves at 770 μE by using a Qubit Systems analyzer according to manufacturer instructions (Qubit Systems, Kingston, Ontario, Canada). Oxygen consumption by dark respiration was monitored in leaf tissue with a Hansatech LD2/5 leaf-disk O2 electrode system maintained at 26°C.

Elemental Analysis, Enzyme Activity, and Statistics. Elemental composition was measured as described previously (43). Enzyme activity of NiR was measured according to ref. 44. All statistical analyses (ANOVA, t tests) were performed by using the Jmp-In software package (SAS Institute, Cary, NC).

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