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Consequence of Absence of Nitrate Reductase Activity on Photosynthesis in *Nicotiana plumbaginifolia* Plants[1]

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C. SAUX, Y. LEMOINE, A. MARION-POLL, M. H. VALADIER, M. DENG, AND J. F. MOROT-GAUDRY*
Laboratoire du Metabolisme et de la Nutrition des Plantes (C.S., M.H.V., M.D., J.F.M-G.), *Laboratoire de Biologie Cellulaire* (A.M-P.), *I.N.R.A., route de St-Cyr, 78000 Versailles, France; and Ecole Normale Supérieure, rue Lhomond, Paris, France* (Y.L.)

ABSTRACT

Chlorate-resistant *Nicotiana plumbaginifolia* (cv Viviani) mutants were found to be deficient in the nitrate reductase apoprotein (NR⁻ *nia*). Because they could not grow with nitrate as sole nitrogen source, they were cultivated as graftings on wild-type *Nicotiana tabacum* plants. The grafts of mutant plants were chlorotic compared to the grafts of wild type. Mutant leaves did not accumulate nitrogen and nitrate but contained less malate and more glutamine than wild leaves. They exhibited a slight increase of the proportion of the light-harvesting chlorophyll *a*/*b* protein complexes and a lowering of the efficiency of energy transfer between these complexes and the active centers. After a 3 second $^{14}CO_2$ pulse, the total ^{14}C incorporation of the mutant leaves was approximately 20% of that of the control. The ^{14}C was essentially recovered in ribulose bisphosphate in these plants. It was consistent with a decline of ribulose bisphosphate carboxylase activity observed in the mutant. After a 3 second $^{14}CO_2$ pulse followed by a 60 second chase with normal CO_2, ^{14}C was mainly accumulated in starch which was labeled more in the mutant than in the wild type. These results confirm the observation that in the nitrate reductase deficient leaves, chloroplasts were loaded with large starch inclusions preceding disorganization of the photosynthetic apparatus.

In higher plants, there is great interest in the isolation of NR⁻[2] mutants for studying genetic and biochemical aspects of plant nutrition. Recently NR⁻ deficient mutants have been reported in *Arabidopsis thaliana* (16), *Hordeum* (8), *Pisum sativum* (4), *Nicotiana tabacum* (14), and *Nicotiana plumbaginifolia* (13). *N. plumbaginifolia* mutants were found to be deficient either in the NR apoprotein (type nia) or in the synthesis of the molybdenum cofactor (type cnx) leading to simultaneous loss of nitrate reductase and xanthine dehydrogenase activities (14). *Nia* and *cnx* mutants proved to be incapable of growth with nitrate as sole nitrogen source. They were cultivated as graftings on *N. tabacum* plants. Grafts from mutants were chlorotic (19). They are important tools for studying the regulation of the nitrate and carbon assimilatory pathways. The objective of this study is to characterize some physiological and biochemical aspects of these mutants and in particular to determine whether the nitrate reduction mutation (NR⁻) involves changes in the photosynthetic machinery.

MATERIALS AND METHODS

Plant Material. Nitrate reductase deficient clones of *Nicotiana plumbaginifolia* were isolated for protoplast cultures by selecting for chlorate-resistant colonies. Seedlings were regenerated from these colonies and grown *in vitro* on a medium containing 5 mM diammonium succinate as sole nitrogen source (5). These seedlings died when transferred on a medium containing 10 mM KNO_3 as sole nitrogen source. Seedlings incubated for 1 week on this medium were assayed for *in vitro* nitrate reductase activity and found to express levels of nitrate reductase activity lower than 0.5% of the controls.

Various attempts were made to grow these seedlings in the greenhouse, but no growth was observed when seedlings were watered with a nutrient solution containing NH_4NO_3. This was assumed to result from ammonium poisoning of plants unable to utilize nitrate. Addition of organic acid to the nutrient solution resulted in the development of bacterial infestation. Consequently the growth of nitrate reductase deficient *N. plumbaginifolia* plants in the growth chamber was obtained by grafting onto a wild-type scion. More than 200 grafts were performed with nitrate reductase deficient mutants. Grafted mutants could develop, flower, and set seeds. Invariably, these grafted mutants displayed a chlorotic leaf phenotype, whereas various grafts performed with wild-type *N. plumbaginifolia* resulted in the development of green, healthy plants (5).

In our experiments, the NR⁻ *N. plumbaginifolia* clone (cv Viviani) *nia* F58 was used. This NR⁻ clone was classified as deficient in the apoenzyme of the nitrate reductase and had an absolute requirement for reduced nitrogen. This selected clone was propagated *in vitro* as a shoot culture on medium B with ammonium succinate as sole nitrogen source (5). Wild-type and mutant seedlings were cultivated during one, two, three, or four weeks in a growth chamber as grafts on *Nicotiana tabacum* plants (cv Wisconsin). The growth conditions were as follows: 27°C during a 16 h light period (580 $\mu E/m^2 \cdot s$) and 19°C during an 8 h subsequent dark period with a relative humidity maintained at 85%. Plants were watered and fertilized daily with nutrient solution containing ammonium nitrate as a nitrogen source (15).

Nitrogen and Organic Compounds Determination. Nitrogen, nitrate, malate, glutamine, and starch levels were determined as previously described (19).

Pigment Analysis and Electrophoretic Separation of the Chl Protein Complexes. The HPLC separation and determination of photosynthetic pigments were carried out by a nonaqueous re-

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[2] Abbreviations: NR⁻, nitrate reductase deficient plant; CPI, photosystem I chlorophyll protein complex; CPa, photosystem II chlorophyll *a* protein complex; FP, free pigments; F685, fluorescence emission band with maximum at 685 nm; LHCP, light-harvesting chlorophyll *a*/*b*-protein complex; NR⁺, wild type; PGA, glyceric acid 3-phosphate; RuBP, ribulose 1,5-bisphosphate.

versed-phase method (gradient of 3 to 40% dichloromethane in a mixture acetonitrile:methanol, 70:30 v/v), using a Du Pont Zorbax octadecyl silica (ODS) column.

The isolation of chloroplasts and the purification of thylakoid membrane fractions from NR^+ and NR^- leaves were made according to Machold *et al.* (12). The purified membranes suspended in 6.2 mM Tris-glycine (pH 8.8) containing 13% glycerol were solubilized with SDS at a SDS/Chl (w/w) ratio of 15/1. Electrophoretic patterns of unstained gels were recorded with a Vernon PHI 6 photometer.

Electron Microscopy. The leaves were prefixed during a period of 2 h with 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) and 0.4 M sucrose, and postfixed during 16 h with 1% OsO_4 in the same buffer. After dehydration they were embedded in araldite. Sections were double stained with $KMnO_4$ and lead citrate. The observations were carried out with a Philips EM 300 electron microscope.

Fluorescence Measurements. The fluorescence emission properties at 77°K of isolated chloroplasts were recorded with a homemade device already described (10). To avoid distortions of the spectra due to Chl concentration, chloroplasts equivalent to 0.3 μg Chl were spread on an AP 20 Millipore prefilter and then dived in liquid N_2.

Chl fluorescence kinetics of intact leaves at room temperature were recorded either with a homemade device (10) or with a portable fluorometer realized according to Schreiber *et al.* (20). The excitation light was emitted by a Monsanto MV 5020 light-emitting diode and fluorescence intensity was measured by a RTC BPY 13 photodiode protected by 89 B and 44 Kodak Wratten filters and connected with a current-voltage converter realized with a CA 3140 operational amplifier. The fluorescence induction curves were recorded on a Tektronix storage oscilloscope.

$^{14}CO_2$ pulse-$^{12}CO_2$ Chase Experiments. Leaf fragments (0.2–0.3 g fresh weight) of 2 week old *N. plumbaginifolia* grafts were enclosed in a thermostated (22°C) copper and perspex assimilation chamber as described previously (15). The leaves under steady state photosynthesis were exposed to $^{14}CO_2$ for 3 s (pulse experiment) and chased over a time course of 60 s in air (chase experiment).

Analysis of ^{14}C Labeled Products after a 3 s $^{14}CO_2$ Pulse. Samples frozen in liquid N_2 were ground in melting isopentane and lyophilized. Analysis of the distribution of ^{14}C among primary photosynthetic products was performed on an aliquot of the powdered extract in formamide. It was subjected to analytical thin-layer electrophoresis in the first dimension and then chromatography in the second dimension. After autoradiography, the various spots were counted in a scintillation counter (15).

Analysis of ^{14}C Labeled Products after a 3 s $^{14}CO_2$ Pulse Followed by a 60 s Chase in Air. The liquid N_2 frozen samples were lyophilized and powdered with a "Cyclotec 1092 Sample Mill." The dry powder was suspended in 95% ethanol (v/v) and homogenized for 2 h. The homogenates were extracted successively in 80/60% (v/v) ethanol and finally in water. The amino acids were separated on a column of cationic resin and the sugars on a column of anionic resin in the borate form. The eluates were passed through an anthracene filled teflon cell. The remaining insoluble material was treated by α- and β-amylases at 37°C for 12 h to estimate the radioactivity incorporated in starch (18).

Preparation and Assay of the Ribulose Bisphosphate Carboxylase. Leaf material (0.5–1 g) was fixed in liquid N_2 and ground in a chilled mortar with 2 ml of homogenizing medium: 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM ethylene diamine tetracetic acid, 10% (v/v) glycerol, BSA (1 mg/ml), 0.1 mM sodium fluoride, PVP (1 g/g dry weight leaf), and about 1 g of acid-washed sand. The homogenate was centrifuged at 38,000*g* for 10 min. RuBP carboxylase activity was determined in the supernatant by incorporation of $NaH^{14}CO_3$ into acid-stable products in the presence of RuBP and after activation with $MgCl_2$ and $NaHCO_3$ (17).

RESULTS

Growth Characteristics of Mutant Grafts. As opposed to grafted wild-type *Nicotiana plumbaginifolia* which developed green leaves, grafted mutants quickly became chlorotic, their leaves turning yellow and crimped (19). The measurement of the nitrogen and nitrate content of leaves after 2 weeks of growth showed no significant discrepancy between wild-type and mutant plants (Table I). However, mutant leaves contained significantly less malate than the grafted wild leaves. NR^- leaves also had large amounts of glutamine and starch (Table I).

Along yellowing of NR^- leaves, their pigment content which, at first, was similar to that of NR^+ leaves, decreased from 790 ± 110 μg to 53 ± 27 μg Chl/g fresh weight. The pigment deficiency was accompanied by modifications of the proportions of the various pigment classes. After 2 weeks of growth, the Chl *a*/Chl *b* ratio fell from 3.3 to 2.2 in yellow leaves in which the most prominent feature was their huge pheophytin *a* content (Chl *a*/pheophytin *a* = 1.2). In the meanwhile, the relative carotenoid content decreased: the molecular ratio (Chl + pheophytins)/carotenoids rose from 3.6 to 5.6. The xanthophylls deficiency was more important (xanthophylls/β-carotene = 1.7 in yellow leaves instead of 3.2). The main xanthophyll was lutein and some monohydroxy-α-carotene accumulated which was nearly absent in young leaves (Fig. 1).

Chlorophyll-Protein Complexes Content. The Chl-protein complexes electrophoretic pattern of thylakoid membranes were weakly modified in yellow NR^- leaves as shown in Figure 2. The main difference noticed in chlorotic leaves was a slight increase of their light-harvesting complexes LHCPs content, especially the monomer LHCP3, to the detriment of the PSI and PSII complexes CP1 and CPa (Table II).

Ultrastructure of the Chloroplasts. The chloroplasts of 1 and 2 week old leaves from the mutant grafts exhibited the classical features of higher plant plastids including a well developed lamellar system with numerous grana stacks joined with isolated thylakoids. Their only peculiarity was their huge starch content (Fig. 3a). Upon a slight decrease, with growth, of the pigment content of the leaves, the plastids remained puffed out with big starch grains but their lamellar system began to disorganize: the isolated thylakoids as well as the grana thylakoids became swelled (Fig. 3b). In 3 week old leaves, the plastids of the remaining green parts, nearby the veins, exhibited the same weakly modified lamellar system but starch grains were totally lacking (Fig. 3c). The main part of 4 week old leaves became yellow and the level of disorganization of its plastids was more dramatic: the extension of the lamellar system was greatly reduced and the thylakoids were swelled into vesicles. Nevertheless, the proportion of appressed membranes remained high. If the stroma was devoid of starch grains it contained numerous osmiophilic lipid globules (Fig. 3d).

Fluorescence Properties. The room-temperature time-courses of fluorescence emission by NR^- whole leaves are presented in Figure 4. The relative amplitude of the constant fluorescence (Fo level) was higher in yellow leaves (Fig. 4b) than in young green leaves (Fig. 4a): their variable fluorescence yield was so dramatically lowered that the Fo/Fvar ratio became 3 times higher.

The low-temperature fluorescence emission spectra of isolated plastids from 2 week old NR^- green leaves were rather similar to that of the control NR^+ plastids (Table III). On the contrary, yellow leaf plastids exhibited an increased F685 fluorescence emission as compared to the far-red F730 or to the F695 emissions (Table III).

^{14}C Distribution after $^{14}CO_2$ Pulse. Photosynthetic rates were

Table I. *Nitrogen, Nitrate, Malate, Glutamine, and Starch Content of Leaves of N. plumbaginifolia NR⁺ and NR⁻ after 2 Weeks of Growth as Graftings on N. tabacum Scion*

	Total Nitrogen	NO_3^-	Malate	Glutamine	Starch
	mg/g dry wt		*μmol/g dry wt*		*mg/g dry wt*
Control NR⁺	72 ± 5	2000 ± 180	68 ± 5	40 ± 4	18 ± 3
Mutant NR⁻	69 ± 4	1900 ± 170	15 ± 3	52 ± 4	79 ± 9

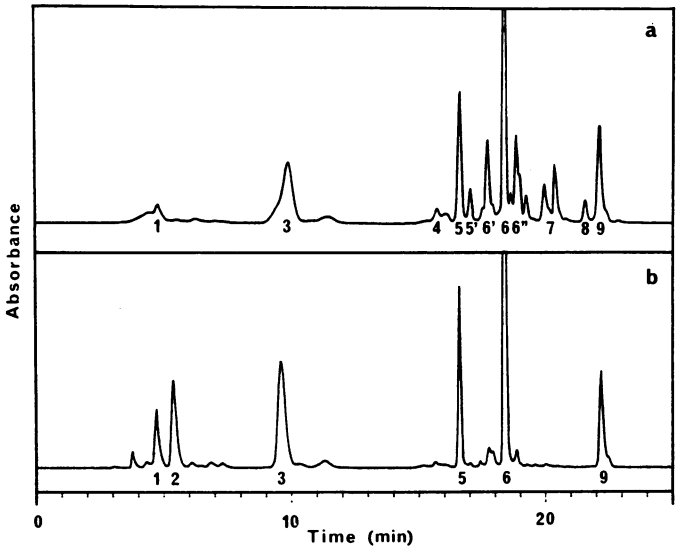

FIG. 1. HPLC elution profiles at 437 nm of the pigment contents from 3 to 4 week old NR⁻ yellow leaves (a) and 1 to 2 week old NR⁻ green leaves (b). 1, Neoxanthin; 2, violaxanthin; 3, lutein; 4, monohydroxy-α-carotene; 5 + 5′, Chl *b*; 6 + 6′ + 6″, Chl *a*; 8, pheophytin *a*; 9, β-carotene.

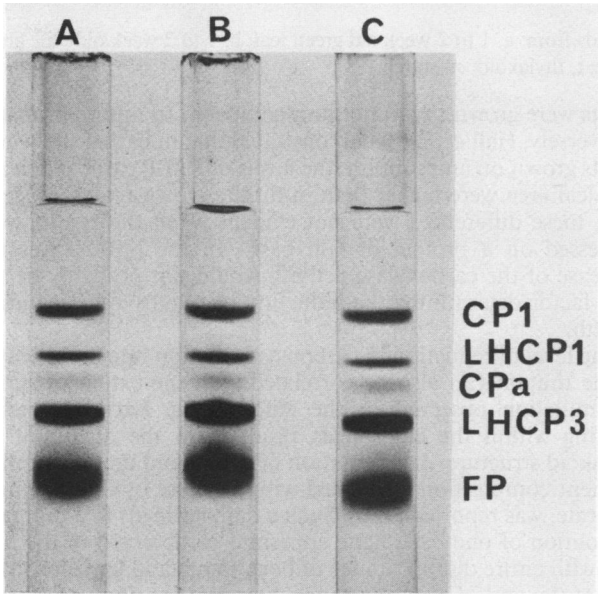

FIG. 2. Unstained SDS-polyacrylamide gels after electrophoresis of chloroplast thylakoids isolated from 1 to 2 week old green (A) and yellow (B) NR⁻ and from the control (C) NR⁺ leaves.

determined during steady state photosynthesis either as rate of CO_2 uptake or total radioactivity incorporated in stable products after pulse and chase experiments. Table IV shows a dramatic decrease of the photosynthetic rate (−80%) in the 2 week old leaves of the NR⁻ grafts compared to the 2 week old leaves of the NR⁺ grafts.

After a 3 s $^{14}CO_2$ pulse, the ^{14}C was mainly recovered in PGA, RuBP and other phosphorylated intermediates of the Benson Calvin cycle. In the NR⁺ leaves, [^{14}C]PGA and ^{14}C monophosphorylated intermediates were the main labeled products. Conversely, in the NR⁻ leaves, [^{14}C]RuBP was the main labeled intermediate (Table IV). These latter leaves exhibited a lower RuBP carboxylase activity (9 ± 4 nmol/s·g fresh weight than the former 37 ± 8 nmol/s·g fresh weight).

^{14}C Distribution after a 60 s Chase by $^{12}CO_2$ following a 3 s $^{14}CO_2$ Pulse. After pulse-chase experiments, ^{14}C was recovered in organic and amino acids, free sugars and starch (Table V). The amount of ^{14}C in sucrose was higher in the NR⁺ leaves than in the mutant ones. Conversely, starch was more labeled in the NR⁻ leaves than in the NR⁺ ones. The percentage of radioactivity recovered in amino acids linked to the glycolate pathway was similar in the NR⁺ and NR⁻ leaves. The radioactivity found in α-alanine was negligible in the leaves of the control but accounted for 3 to 4% of the total radioactivity in the leaves of the mutant (Table V).

DISCUSSION AND CONCLUSION

As a consequence of nitrate reductase deficiency, grafted mutants display a chlorotic leaf phenotype as opposed to grafted wild-type plants. This deficiency does not seem to result from nitrate poisoning or nitrogen starvation. The mutants do not accumulate nitrate, but contain as much total nitrogen and more reduced nitrogen than the wild type, as described previously (19). Grafted mutant leaves also exhibit significant changes in pigment composition, chloroplast structure, and operation of the photosynthetic carbon assimilation pathway.

The first observed modification is a transformation and a loss of pigments with a bleaching of the leaves. The increase of their β-carotene content is probably related more to the protective function of this pigment against photodestruction (9) than to its intervention as an accessory pigment mainly of PSI (23). The considerable accumulation of pheophytins in NR⁻ plastids should be a consequence of Chl degradations during early senescence stage of the NR⁻ chloroplasts.

In spite of their considerable pigment deficiency, the plastids of yellow NR⁻ leaves exhibit a rather faint modification of their Chl-protein complex composition. As the LHCPs are mainly restricted to granal regions (21), the relative enrichment of LHCP1 and LHCP3 of NR⁻ thylakoid membranes is in good agreement with the high proportion of appressed membranes observed in disorganized plastids. Conversely, the large increase of F685 fluorescence emission at low temperature can be opposed to the faint enrichment in these light-harvesting complexes. This increase of the short red fluorescence band may be attributable to a reduced spillover of energy between PSII and PSI, to a disconnection of the LHCPs from PSII centers (10), and to an

Table II. *Relative Percentage Distribution of Chl in the Chl-proteins of Thylakoid Membranes from NR⁺ and NR⁻ Plastids and Comparison of the Different Complexes Contents*

Material	Distribution of Chl							
	CP1	LHCP1	CPa	LHCP3	FP	LHCPs/CP1	LHCP3/LHCP1	LHCPs/CP1+CPa
			%				*ratio*	
Control NR⁺	19.4	12.1	10.2	36.7	21.6	2.5	3.0	1.6
Green NR⁻	18.8	13.7	8.1	35.2	24.2	2.6	2.6	1.8
Yellow NR⁻	16.0	13.1	7.9	36.0	27.0	3.1	2.7	2.0

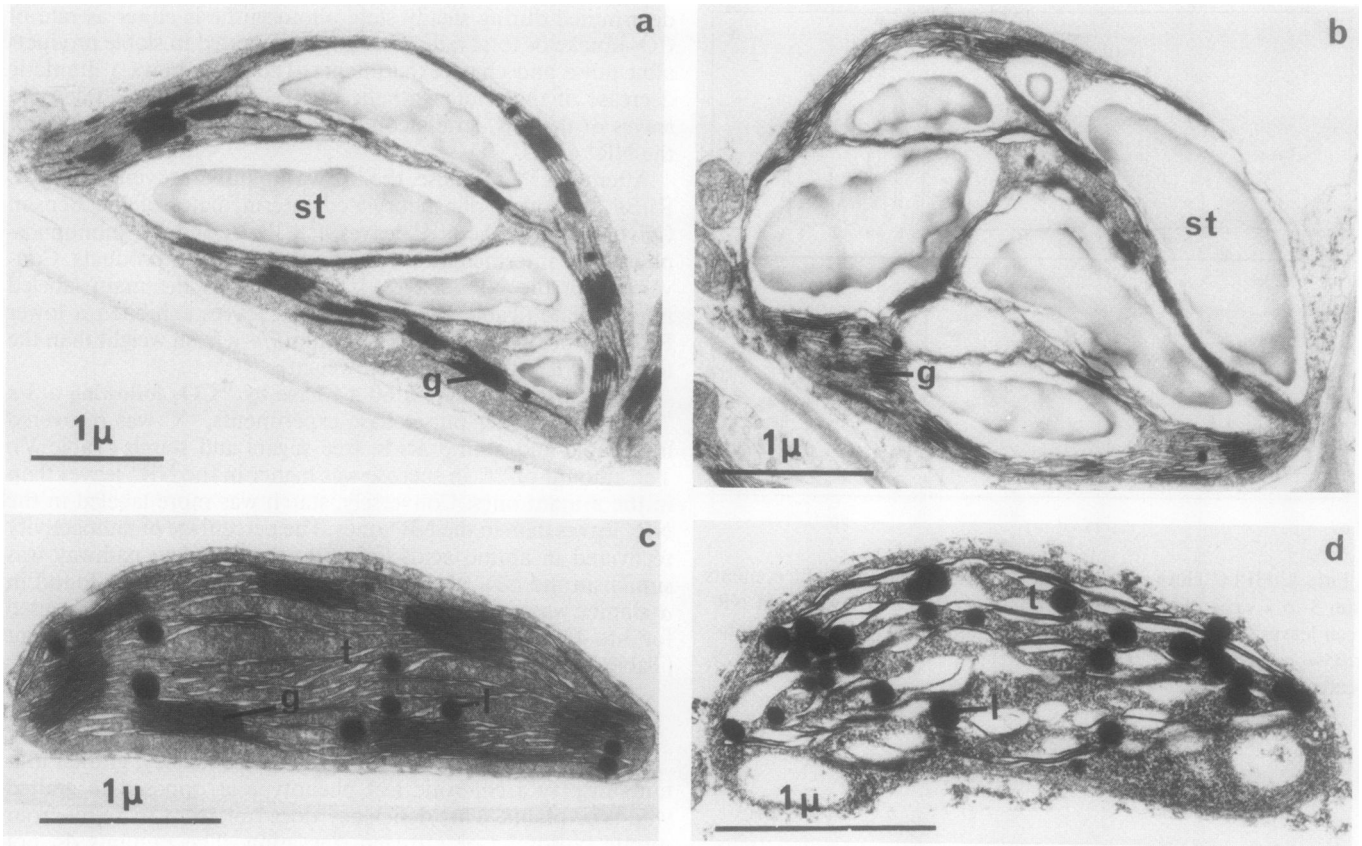

FIG. 3. Cross-section electron micrographs of the ultrastructure of NR⁻ plastids from: a, 1 to 2 week old green leaf; b, 1 to 2 week old paie green leaf; c, green part of a 4 week old leaf; d, yellow leaf. g, Granum; L, lipid globule; t, thylakoid; st, starch.

increased concentration of Chl *a* not energetically connected to the photosystems (2). The room temperature fluorescence kinetics of NR⁻ leaves exhibit a decrease of the variable fluorescence which reveals a lowering of the PSII electron transfer rates (2, 10).

In the leaves of the mutant compared to those of the control, the decrease of carbon fixation rate and the accumulation of radioactivity in RuBP after a very short term pulse in $^{14}CO_2$ are consistent with the decline of RuBP carboxylase activity. Changes in RuBP carboxylase activity owing to variation in the amount of actived enzyme were already observed in different physiological conditions. The activity of carboxylase was particularly known to be changed with leaf age and to be altered by the nitrogen growth regime. During the senescence of terminal wheat leaves, changes of carboxylase activity was shown to occur in parallel to the decreased photosynthetic fixation of CO_2 (22). Lurie *et al.* (11) measured a decrease of the carboxylase/oxygenase ratio with the age of tobacco leaves. Vaklinova *et al.* (24) observed a stimulation of the activity of RuBP carboxylase in greening pea and maize seedlings when fed on nitrate nitrogen compared to ammonium. Fair *et al.* (3) reported a higher glycolate oxidase to RuBP carboxylase activity ratio when barley plants were grown in ammonium compared to nitrate nitrogen. Conversely, Hall *et al.* (7) demonstrated that in barley and wheat plants grown on ammonium, the levels of RuBP carboxylase per unit leaf area were higher than in nitrate grown material. However, these differences were not evident when the results were expressed on a protein or Chl basis. In the NR⁻ leaves the decrease of the carboxylase activity would not probably be the only factor determining the reduction of photosynthesis during growth.

The level of reduction in carbon assimilation rate in the leaves of the mutant can also be correlated with the extent of starch accumulation observed in the chloroplasts. Excessive starch packing within the chloroplast resulting in the disruption of thylakoid structure, disorientation of grana, and modification of pigment composition associated with decrease of CO_2 assimilation rate, was reported (1, 6). Such a damage leads to a dramatic demolition of photosynthetic apparatus as observed in the mutant with entire disappearance of both starch and lamellar structure at the end of the senescence. Starch is the form of storage of photoassimilated carbon within the chloroplast and sucrose the cytosolic form in which carbohydrates are exported to the rest of the plant. Partitioning of carbon between starch and

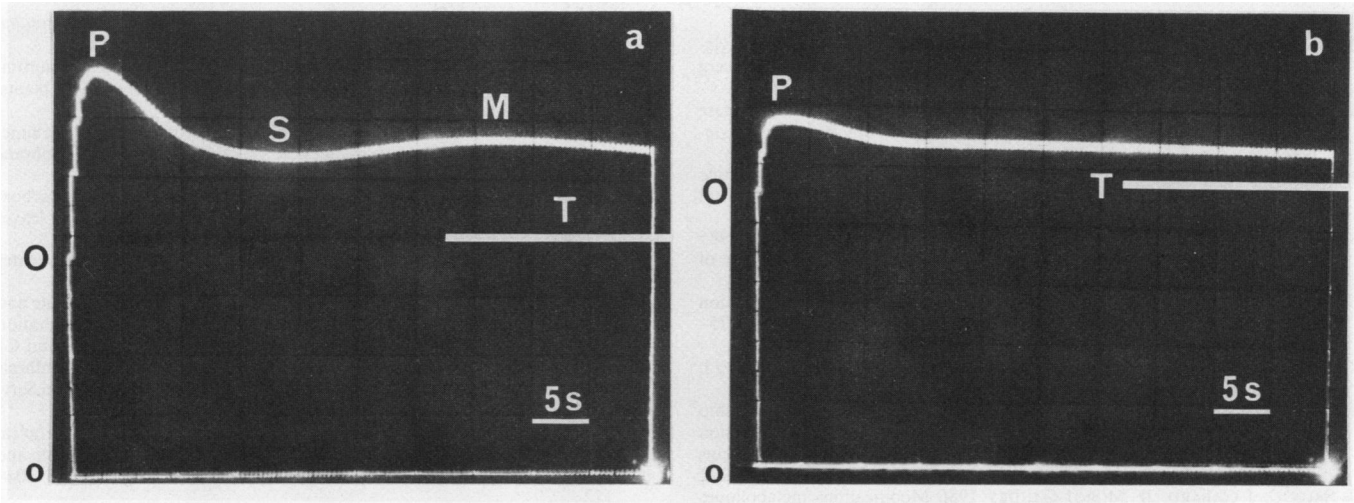

FIG. 4. Fluorescence induction transients of chloroplasts from 1 to 2 week old NR⁻ green leaves (a) and 4 week old NR⁻ yellow leaves (b). The stationary level T has been recorded after a 4 min illumination. The OPS transient is known as the first wave, the SMT transient as the second wave of fluorescence induction (10).

Table III. *Ratio of the F685, F695, and F735 Fluorescence Emission Yields of Chloroplasts Isolated from the Control NR⁺ and from Green and Yellow NR⁻ Leaves*

Material	F685/F735	F685/F695	F695/F735
		ratio	
Control NR⁺	0.60	1.16	0.52
Green NR⁻	0.64	1.17	0.55
Yellow NR⁻	0.88	1.25	0.70

Table IV. *Net Photosynthesis and ^{14}C Distribution (in Percentage of ^{14}C Recovered) in Leaves of NR⁺ and NR⁻ N. plumbaginifolia Grafts after a 3 s $^{14}CO_2$ pulse*

Data represent means of 10 replications.

CO_2	NR⁺	NR⁻
nmol/s·g fresh wt	34 ± 12	7 ± 1
% ^{14}C in PGA	26 ± 4	17 ± 5
% ^{14}C in RuBP	10 ± 3	43 ± 6
% ^{14}C in other phosphorylated compounds	60 ± 9	26 ± 4

Table V. *^{14}C in Leaves of NR⁺ and NR⁻ N. plumbaginifolia Grafts after a 60 s Chase by $^{14}CO_2$ following a 3 s $^{14}CO_2$ Pulse*

Data represent means of four replications.

Radioactive Compound	NR⁺	NR⁻
	% ^{14}C	
Sucrose	18 ± 4	5 ± 3
Starch	26 ± 6	37 ± 7
Glycine	19 ± 5	16 ± 4
Serine	13 ± 4	11 ± 4
α-Alanine	Trace	4 ± 2

sucrose which is an important branch point in photosynthetic metabolism is demonstrated to be adjusted to different nitrogen regime. In chloroplasts of leaves of nitrogen-deficient plants, starch has been demonstrated to accumulate and to alter the chloroplastic structure and the photosynthetic processes (6, 25). The type of nitrogen was also observed to modify the utilization of photosynthates. Nitrogen in nitrate form is believed to stimulate the translocation of assimilates from the chloroplast to the cytosol (1). Conversely, NH_4^+ form changes the carbon metabolism in photosynthesis toward the synthesis of amino acids and starch at the expense of reduced sucrose synthesis (1, 15).

Our results suggest that the mutant compared to the wild type very early undergoes many of the changes normally observed during senescence, such as a decline of RuBP carboxylase activity, demolition of the photosynthetic apparatus. The associated degradation of pigments is manifested in yellowing of the leaves. Accumulation of photoassimilated carbon as starch in the chloroplasts of the NR⁻ grafts is the first metabolic deviation preceding the ultimate deterioration of the photosynthetic machinery. Further work on the elucidation of the mechanism by which triose-translocation out of the chloroplast is prevented in the NR⁻ plants is being undertaken.

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