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A mutant of *Nicotiana sylvestris* deficient in serine glyoxylate aminotransferase activity

Callus induction and photorespiratory toxicity in regenerated plants

N.A. McHale, E.A. Havir and I. Zelitch

Department of Biochemistry and Genetics, The Connecticut Agricultural Experiment Station, P.O. Box 1106, New Haven, CT 06504, USA

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Summary. A photorespiration mutant of *Nicotiana sylvestris* lacking serine : glyoxylate aminotransferase activity was isolated in the M_2 generation following EMS mutagenesis. Mutants showing chlorosis in air and normal growth in 1% CO_2 were fed [^{14}C]-2-glycolate to examine the distribution of ^{14}C among photorespiratory intermediates. Mutant strain NS 349 displayed a 9-fold increase in serine accumulation relative to wild-type controls. Enzyme assays revealed an absence of serine: glyoxylate aminotransferase (SGAT) activity in NS 349, whereas other peroxisomal enzymes were recovered at normal levels. Heterozygous siblings of NS 349 segregating air-sensitive M_3 progeny in a 3 : 1 ratio were shown to contain one half the normal level of SGAT activity, indicating that air sensitivity in NS 349 results from a single nuclear recessive mutation eliminating SGAT activity. Since toxicity of the mutation depends on photorespiratory activity, callus cultures of the mutant were initiated and maintained under conditions suppressing the formation of functional plastids. Plantlets regenerated from mutant callus were shown to retain the SGAT deficiency and conditional lethality in air. The utility of photorespiration mutants of tobacco as vehicles for genetic manipulation of ribulose bisphosphate carboxylase/oxygenase at the somatic cell level is discussed.

Key words: *Nicotiana sylvestris* – photorespiration mutants – serine : glyoxylate aminotransferase – plant tissue culture

Introduction

Photorespiration is the light-dependent loss of CO_2 from the leaves of C_3 plants, resulting in a significant reduction in photosynthetic efficiency. The process is initiated by the reaction of oxygen with ribulose bisphosphate carboxylase/oxygenase (Bowes et al. 1971), diverting carbon out of the Calvin cycle and into a complex sequence of reactions comprising the glycolate pathway. Loss of carbon occurs primarily through decarboxylation of glycine in the mitochondria (Kisaki et al. 1971).

Somerville and Ogren (1979, 1980) demonstrated that mutations blocking the glycolate pathway in *Arabidopsis thaliana* are conditionally lethal, permitting normal growth in a CO_2-enriched atmosphere, but converting photorespiration into a lethal process in air. These mutants were employed as vehicles for direct selection of mutations reducing ribulose bisphosphate oxygenase activity by screening mutagenized populations for variants that regained the capacity for survival in air. The only survivors obtained, however, were reversions of the original enzyme deficiency (Somerville and Ogren 1982). This scheme is genetically sound, but its implementation at the whole plant level is complicated by the fact that oxygenase activity resides on the large subunit of the enzyme coded by chloroplast DNA, which is present in multiple copies per cell.

Recent reports indicate, however, that mutations in cytoplasmic DNA can be induced and recovered with high efficiency using the techniques of somatic cell genetics. Protoplast cultures of *Nicotiana plumbaginifolia* have been employed for large scale isolation of maternally inherited mutations conferring resistance to lincomycin (Cseplo and Maliga 1984) and resistance to triazine (Cseplo et al. 1985). Triazine is an herbicide that inhibits photosynthesis by interrupting the flow of electrons through photosystem II (Moreland 1980). The recovery of maternally inherited mutations for triazine

resistance in tobacco provides an encouraging precedent for manipulation of photosynthetic functions at the somatic cell level, presumably through alterations in chloroplast DNA.

Our principal reason for generating photorespiration mutants in tobacco was to obtain appropriate vehicles to undertake genetic manipulation of ribulose bisphosphate carboxylase/oxygenase at the somatic cell level. Here we report the isolation of a recessive nuclear mutation in *Nicotiana sylvestris* eliminating the activity of serine : glyoxylate aminotransferase (SGAT), a peroxisomal enzyme in the glycolate pathway. The mutant is unable to survive in air, but grows normally when photorespiratory activity is suppressed by CO_2-enrichment. We demonstrate the induction and maintenance of somatic cell cultures from the mutant, and regeneration of a population of R_1 plants retaining the SGAT deficiency and conditional lethality in air.

Materials and methods

Mutagenesis and screening

Seeds of *Nicotiana sylvestris* (Spegazzini and Comes) were soaked in water for 12 h at 25 °C and transferred to a 0.4% solution of ethyl methanesulfonate (EMS) in 0.02 M phosphate buffer (pH 7.0) for a 2 h exposure. Treated seeds were washed in 3 changes of distilled water for 24 h prior to germination under continuous illumination (80 $\mu E\ m^{-2}\ s^{-1}$) at 27 °C on RN medium, modified from the R medium of Kasperbauer and Collins (1972) by omission of sucrose and addition of NH_4NO_3 and KNO_3 at 250 mg/l, and solidified with agar (8 g/l). The EMS treatment had no effect on germination frequency or emergence of cotyledon leaves relative to control seedlings, but a large proportion of treated seedlings were delayed in emergence of the first and second true leaves. About 800 M_1 seedlings showing delayed development were transplanted to the field and grown to maturity for self-pollination. M_2 seeds were collected separately from 683 M_1 plants.

The M_2 families were screened for photorespiratory defects by germinating M_2 seeds from each family (n = 400/family) in normal air on the RN medium in closed petri plates under continuous illumination (80 $\mu E\ m^{-2}\ s^{-1}$). After 7 d, chlorotic variants were transferred to plexiglas chambers (31 l) flushed (100 ml/min) with an atmosphere of 1% CO_2/21% O_2 to identify those that recovered under CO_2-enrichment. By this criterion, six of the 683 families were segregating photorespiratory mutants, which were grown in 1% CO_2 for further analysis.

Biochemical characterization

The mutants strain NS 349 and wild-type control plants were raised in 1% CO_2/21% O_2 to provide leaf material for analysis of ^{14}C distribution among photorespiratory intermediates following exposure to [^{14}C]-2-glycolate. Six 1.6 cm discs were placed in Warburg flasks with 1.2 ml 10 mM K [^{14}C]-2-glycolate (4.96×10^6 DPM), and shaken for 60 min in a water bath at 30 °C under a continuous stream of moist air, and incandescent illumination (350 $\mu E\ m^{-2}\ s^{-1}$). The discs were immersed in boiling 20% ethanol, homogenized, adjusted to 25 ml and assayed for total radioactivity by scintillation counting. The homogenate was centrifuged at 38,000 × g for 20 min, and the supernatant was fractionated on columns of Dowex-1 acetate and Dowex-50. The basic fraction eluted from Dowex-50 was dried under an air stream (45 °C), dissolved in 0.1 ml water, spotted on Whatman 3 MM paper (together with carrier glycine or serine), and subjected to high voltage electrophoresis using formic acid : acetic acid : water (12 : 48 : 340) at pH 1.9. After electrophoresis, the paper was dried in air (100 °C) and scanned for serine and glycine (blue fluorescent spots) with a 366 nm light source (Fowden 1951). Sections of paper (1.0 cm) were cut and transferred to scintillation vials to determine ^{14}C content.

Enzyme assays were conducted on leaf extracts from wild-type and NS 349 plants grown in 1% CO_2 using methods described previously for catalase, serine : glyoxylate aminotransferase and glutamate : glyoxylate aminotransferase (Havir and McHale 1987) and glycolate oxidase (Havir 1983). Activity of hydroxypyruvate reductase was assayed in a reaction mixture containing 100 µmol K-phosphate buffer (pH 7.0), 0.35 µmol NADH, 12.5 µmol hydroxypyruvate, and 0.05 to 0.10 units of enzyme. Oxidation of NADH was monitored at 340 nm.

Cell culture

Expanding leaves were excised from NS 349 (grown in 1% CO_2), surface sterilized and divided into strips for induction of callus cultures on a Linsmaier and Skoog (1965) (LS) medium supplemented with 0.10 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3.0 mg/l naphthaleneacetic acid (NAA), 0.04 mg/l 6-benzylaminopurine (BAP), 2% sucrose, and 1% agar. Callus induction was carried out in darkness to avoid toxic accumulation of photorespiratory intermediates in the leaf strips. Since the emerging callus cells are pale in color and lack functional plastids, the callus explants were incubated under continuous illumination (80 $\mu E\ m^{-2}\ s^{-1}$) in all subsequent steps. After several three-week passages on the LS medium, callus was transferred to the same medium with no 2,4-D, 0.3 mg/l NAA and 0.3 mg/l BAP to induce shoot formation. After three weeks, shoots were transferred to a Murashige and Skoog (1962) (MS) medium with no hormones and 1% sucrose (MSO), and incubated in petri plates (lids on but not sealed) in the 1% CO_2 chambers under continuous illumination (80 $\mu E\ m^{-2}\ s^{-1}$). Rooted plantlets were transferred to the same medium lacking sucrose and grown in open plates in 1% CO_2, and leaves were excised for SGAT assays. Callus cultures from wild-type plants were propagated and regenerated in the same manner to provide suitable controls. Regenerated plantlets (R_1) from NS 349 and wild-type callus were tested for air sensitivity by scoring the onset of chlorosis seven days after transfer to chambers flushed with an atmosphere of normal air.

Results

Mutant identification

The M_2 progenies resulting from self-pollination of M_1 plants were screened for photorespiration mutants by a procedure modified from that of Somerville and Ogren (1979). Seed samples were screened initially in normal air to identify chlorotic mutants, which were then screened for restoration of normal growth in 1% CO_2. Of 683 M_2 progenies germinated in air, 34 were seg-

regating albino mutants, and an additional 98 contained chlorotic mutants which were unable to develop beyond the cotyledon stage. Chlorotic mutants in six of these 98 progenies became green and resumed normal growth after transfer to 1% CO_2. In one of the six progenies, some of the chlorotic individuals recovered in 1% CO_2 and some did not, the two classes presumably resulting from segregation of two different mutations. In contrast, chlorosis appeared to result from a single mutation in each of the remaining five progenies, where the mutants recovered uniformly under CO_2-enrichment.

To ensure that the air-sensitive phenotype was not confined to the cotyledon stage, mutants from each of the six progenies were grown to the fourth or fifth true leaf stage in 1% CO_2 and returned to normal air. In all cases this produced necrosis (brown spots) appearing initially in the young (2–4 cm) expanding leaves within a few days, and spreading to the older leaves.

Biochemical characterization

Preliminary characterization of the mutants was carried out by analyzing the distribution of ^{14}C among photorespiratory intermediates following exposure to $^{14}CO_2$ or [^{14}C]-2-glycolate. Results obtained with mutant strain NS 349 are presented in Table 1. Leaf discs of mutant and wildtype plants metabolized glycolate similarly, but the mutant displayed more than a 9-fold increase in serine accumulation, and a corresponding decrease in labelling of the neutral fraction.

Accumulation of serine under photorespiratory conditions suggested that SGAT was the primary lesion in NS 349. Accordingly, NS 349 was grown in 1% CO_2 to examine activities of SGAT and other peroxisomal enzymes (Table 2). Assays indicated that SGAT activity was absent in NS 349, whereas the activities of four other peroxisomal enzymes were similar to those found in wild-type control plants. Mixing extracts from wild-type and NS 349 produced the theoretical intermediate values for SGAT activity, showing that the absence of SGAT activity in NS 349 does not result from enzyme inhibitors.. These observations suggest that air-induced chlorosis in NS 349 results from the absence of SGAT activity.

Genetic analysis

Since the NS 349 strain occurred at a frequency of nearly 25% in the original M_2 family, it appeared to result from segregation of a single nuclear recessive mutation. To test this hypothesis, we sought to identify heterozygotes among the normal M_2 siblings of NS 349, based on segregation of normals and mutants in a 3 : 1 ratio in M_3 progenies. Normal siblings (n = 10) were selected randomly from the original M_2 family, grown to maturity in the greenhouse, and self-pollinated. Five of the 10 M_2 plants produced M_3 progenies segregating air-sensitive mutants. Three of the segregating progenies were scored for frequencies of normal and mutant phenotypes, and computation of the pooled χ^2 indicated a close fit to a 3 : 1 ratio (Table 3). Air-sensitive mutants from one of the M_3 progenies (NS 349-1) were rescued and grown in 1% CO_2 for enzyme assays which confirmed the absence of SGAT activity. In addition, two of the heterozygous plant segregating air-sensitive M_3 progeny were shown to possess one-half the SGAT activity found in homozygous normal plants. On this basis, the gene has been assigned the *sat* designation.

Table 1. Distribution of radioactivity following metabolism of [^{14}C]-2-glycolate by leaf discs from wild-type and NS 349

Fraction	% of ^{14}C recovered	
	Wild type	NS 349
Serine	7.7	63.9
Glycine	5.2	4.7
Neutral	30.1	2.6
Organic acids	56.0	28.6

Table 2. Activities of peroxisomal enzymes in leaf extracts from wild type and NS 349 plants grown in 1% CO_2/21% O_2

	Enzyme activity (μmol/min/g fresh wt)	
	Wild type	NS 349
Catalase	695	838
Hydroxypyruvate reductase	1.80	2.22
Serine-glyoxylate aminotransferase	0.86	0.00
Glutamate-glyoxylate aminotransferase	0.68	0.74
Glycolate oxidase	0.35	0.34

Table 3. Segregation of air-sensitive mutants in M_3 progenies of heterozygous M_2 siblings of mutant NS 349

M_2 heterozygotes	Observed M_3 ratio				
	normal	mutant	χ^2	pooled χ^2	P
NS 349-1	308	99	0.12[a]	1.92	0.59[b]
NS 349-5	176	66	0.74[a]		
NS 349-6	192	55	1.06[a]		

[a] Tested for 3 : 1 ratio
[b] P value for df 3

Cell culture

During all phases of callus induction and maintenance under heterotrophic conditions, cultures from the *sat* mutant displayed characteristics indistinguishable from the wild-type control cultures. Both were pale and friable on the LS medium with 2,4-D, and regenerated shoots readily following transfer to the low auxin medium. The small expanding leaves on shoots from *sat* cultures developed no symptoms under these conditions, perhaps a reflection of low photosynthetic rates on a sucrose-containing medium in sealed plates.

Excised shoots (R_1) were transferred to 1% CO_2 chambers on MSO medium in closed (not sealed) plates to avoid desiccation during root formation. During this phase, several R_1 plantlets from the *sat* cultures developed foliar necrosis (brown spots) typical of that induced by exposure to normal air. This resulted presumably from depletion of CO_2 inside covered plates, in spite of high CO_2 levels in the surrounding atmosphere. When rooted R_1 plantlets were transferred to an MSO medium lacking sucrose and incubated in 1% CO_2 in open plates, there was no further evidence of foliar necrosis in the *sat* mutants or wild-type controls.

A random sample of 22 R_1 plantlets from the *sat* cultures was selected for SGAT assays on excised leaves. All 22 displayed no detectable SGAT activity. Three wild-type R_1 plantlets were included as controls, all of which had normal SGAT levels.

After R_1 plantlets were established in 1% CO_2, they were screened for the appearance of air-induced necrosis. All of the wild-type plantlets ($n = 45$) continued to grow normally after transfer to air chambers, but all plantlets ($n = 225$) from *sat* cultures developed the typical pattern of necrotic spots in the young expanding leaves within seven days.

Discussion

Results presented here show that the air-sensitive phenotype of mutant strain NS 349 results from a single nuclear recessive mutation eliminating the activity of SGAT. Serine accumulation and absence of SGAT activity in NS 349 provided the initial evidence for this interpretation, which was reinforced by the observation that air-sensitivity is governed by a single nuclear recessive mutation, and that M_2 plants heterozygous for the mutation possess one-half the wild-type level of SGAT activity. Finally, our conclusion is supported by prior demonstrations of air-sensitivity in SGAT-deficient mutants of *Arabidopsis* (Somerville and Ogren 1980) and barley (Murray et al. 1987).

Although the photorespiration mutants of *Arabidopsis* and barley were identified by growing M_2 populations in high CO_2 prior to screening for air-induced chlorosis, we chose to impose these atmospheres in reverse order, primarily for convenience. By germinating M_2 progenies in air at the outset, a large portion of the population not segregating chlorotic mutants was quickly eliminated from further consideration, curtailing greatly the number of plants requiring examination in high CO_2. We screened all types of chlorotic mutants for recovery in high CO_2, but it became evident that the photorespiration mutants had certain distinguishing features. Whereas many mutants were chlorotic immediately upon emergence of the cotyledons, the photorespiration mutants were initially green, and developed chlorosis only after prolonged (6–7 d) exposure to air. This may be a function of the low irradiance (80 $\mu E\ m^{-2}\ s^{-1}$) employed in our screening, tending to delay expression of photorespiratory toxicity.

Since toxicity of the *sat* mutation depends on photorespiratory activity, it seemed reasonable to assume that mutant cell cultures could be maintained under conditions suppressing the formation of functional plastids. This was accomplished readily with leaf mesophyll callus from NS 349. Using a hormone balance that suppresses greening, we have maintained mutant cultures for six months. In addition, these cultures were regenerated into a population of R_1 plantlets retaining the SGAT deficiency and air-sensitivity.

Most selection schemes involving somatic cell cultures have been confined to traits expressed under heterotrophic conditions, but the photosynthetic competence of photomixotrophic cultures has recently been exploited to obtain mutations affecting photosynthetic functions (Cseplo et al. 1985). It has also been established that autotrophic cell cultures possess an active photorespiratory pathway (Berlyn et al. 1978), and that the relationship between photosynthesis and photorespiration is similar to that observed in intact leaves (McHale et al. 1987). Expression of photorespiration in cultured cells should provide the opportunity to devise selection schemes employing tools such as the *sat* mutation to impose direct selection for chloroplast mutations diminishing ribulose bisphosphate oxygenase activity. We are currently developing methods for isolation and growth of protoplast cultures from the *sat* mutant to facilitate the screening of large mutagenized populations.

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