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Characterization of a chlorate-hypersensitive, high nitrate reductase *Arabidopsis thaliana* mutant

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Summary. A population of *A. thaliana,* produced by self-fertilization of ethylmethane sulfonate treated plants, was exposed to chlorate in the watering solution, and plants showing early susceptibility symptoms were rescued. Among the progeny lines of these plants five were shown to be repeatably chlorate-hypersusceptible. One of these lines (designated C-4) possessed elevated activity of nitrate reductase (NR). The NR activity of mutant C-4 was higher than that of normal plants throughout the life cycle. Nitrite reductase and glutamine synthetase activities of C-4 were normal, as were chlorate uptake rate and tissue nitrate content. The elevated NR activity apparently was responsible for the chlorate hypersusceptibility of C-4. Inheritance studies of NR indicated that the elevated activity of C-4 was probably controlled by a single recessive allele.

Key words: Inheritance – Nitrate reductase – *Arabidopsis*

Introduction

Defined genetic mutants have proven invaluable in the study of nitrogen metabolism in microorganisms. A number of useful mutants affecting nitrate uptake and utilization (nitrate assimilation) have also been isolated in some species of higher plants.

Chlorate screening of cultured cells in *N. tabacum* and *N. plumbaginifolia* has produced low nitrate reductase (NR) and NR-less mutant cell lines which cannot utilize nitrate as a nitrogen source (Muller and Grafe 1978; Marton et al. 1982). A NR-less mutant which cannot utilize nitrate has also been isolated by resistance to chlorate in barley seedlings (Bright et al. 1983); Negrutiu et al. (1983) have regenerated NR-less plants from cell lines of *N. plumbaginifolia.* The study of such mutants has shown that separate genes may be responsible for the NR apoprotein and molybdenum cofactor (Mendel and Muller 1978), and that several genes affect NR activity (NRA) (Braaksma and Feenstra 1982).

Nitrate reductase mutants have been isolated in soybean, pea and *Arabidopsis* by exposing mutagen-treated whole plants to chlorate (Nelson et al. 1983; Oostindier-Braaksma and Feenstra 1973; Feenstra and Jacobsen 1980). In pea and barley NR mutants have been isolated by a rapid in vivo NR assay procedure conducted on M_2 seedlings (Kleinhofs et al. 1978; Warner et al. 1977, 1982). These mutants tend to be leaky in that they can survive on NO_3 as the sole N source. Nonetheless, they have been effectively employed to study the biochemistry, physiology and genetic control of nitrate assimilation of these plant species (Kleinhofs et al. 1982).

The collection of chlorate-resistant *A. thaliana* mutants affecting nitrate reductase and nitrate uptake has been characterized physiologically and genetically (Braaksma and Feenstra 1982). These lines were all isolated by screening M_2 populations for surviving plants after supplying chlorate to the roots. Numerous mutants, low in or almost lacking NR, as well as mutants exhibiting reduced uptake of chlorate and nitrate, were recovered. The *A. thaliana* mutants therefore represent the most diverse and largest such collection for a single flowering plant species.

The present study was undertaken to develop new uses of chlorate for obtaining novel mutants affecting nitrate assimilation in *A. thaliana* and to characterize the mutants. Specifically, M_2 populations from ethylmethane sulfonate-treated *A. thaliana* seeds were exposed to chlorate, and the plants that exhibited early toxicity symptoms (i.e., chlorate hypersusceptible) were rescued and studied. Through comparison of mutant and parental lines, the effects of the mutation on NRA, other enzyme activities, and nitrate uptake by plants were examined. The results of the initial screening experiment and characterization of a high nitrate reductase, chlorate-hypersensitive mutant are presented in this report.

Materials and methods

1 Plant materials and mutagen treatment

The race WS (collected in Wassilewskija, Russia), of *Arabidopsis thaliana* (L.) Heynh. was used for mutagenic treatment and for comparison to resultant mutant lines. The race has been maintained by self-fertilization and is highly homozygous.

Chemical mutagenesis of WS was carried out according to the method of Feldmann (1985). Seeds (300 per batch) were pre-soaked on filter paper, saturated with distilled water, in 5 cm plastic petri dishes at 2 °C for 12 h. After imbition, excess distilled water was removed from the filter paper and 1.5 ml of a buffered solution (100 mM potassium phosphate, pH 7.5) of ethyl methane sulfonate (EMS) was added. The petri dishes containing seeds and EMS were incubated in a fume hood for varying lengths of time (dim light, 25 °C). The mutagen was removed by rinsing the seeds three times with distilled water. The used EMS, as well as all glassware exposed to EMS, were treated with 0.5% (w/v) glycolic acid in 1N NaOH. After rinsing, the seeds were plated on the surface of vermiculite (pre-saturated with nutrient watering solution) in plastic pots, 300 plants per pot. The resulting plants were designated as the M_1 generation. The progeny resulting from self-fertilization of M_1 plants were designated as the M_2 generation. M_2 seeds from each single pot of M_1 plants were harvested in bulk, stored, and screened for mutants separately.

2 Culture of plants

Whole plants were cultured on three different growth media, depending on the experiment. The media included vermiculite, agar, and submerged cultures. The protocols for these three types of cultures are described below.

a) Vermiculite culture. Seeds were sown in rows on the surface of the vermiculite subirrigated with nutrient solutions in 10×10×9 cm plastic pots. Approximately 25–30 seeds were planted for each row. After a two-day cold pretreatment (1–3 °C) the planted pots were transferred to growth chambers with temperature 25 ± 1 °C and relative humidity 60%. The pots were irrigated with nutrient solution every two days and plants were continuously illuminated. The pots were covered with perforated plastic film during the first five days of growth.

Solutions utilizing NH_4^+, NO_3^-, or $NH_4^+ + NO_3^-$ as nitrogen sources were employed for watering (Tables 1 and 2).

b) Agar culture. The agar culture is a modification of the method developed by Langridge (1957). Test tubes (18×150mm) containing 8 ml of agar-solidified nutrient media were sterilized by autoclaving. Seeds were surface-sterilized for 8 min in a mixture of 0.15% detergent (Tween 20) and sodium hypochlorite. Seeds were then rinsed with sterile H_2O and transferred to test tubes. The planted seeds were cold treated for two days prior to placement in a growth chamber. The temperature, humidity, and light intensity were the same as for vermiculite culture.

The alternate nitrogen sources NH_4^+, NO_3^-, and $NH_4^+ + NO_3^-$ were also used (Tables 2 and 3).

c) Submerged culture. Seeds to be germinated in liquid culture were surface sterilized by the same procedure employed for agar culture. Erlenmeyer flasks (250 ml) containing 40 ml of nutrient solution or flasks (500 ml) with 200 ml of nutrient solution were autoclaved. Approximately 30 surface-sterilized seeds were added to 250 ml flasks or 80 seeds to 500 ml flasks. The flasks were then sealed with aluminum foil and plastic

Table 1. Concentration of the major salts used for vermiculite culture

Components	Medium		
	NH_4^+	NO_3^-	$NH_4^+ + NO_3^-$
	Concentration (mM)		
$K_2HPO_4 \cdot 3H_2O$	0.5	0.5	0.5
KH_2PO_4	2.0	2.0	2.0
$MgSO_4 \cdot 7H_2O$	1.0	1.0	1.0
$CaCO_3$	0.85	0.85	–
$(NH_4)_2SO_4$	2.5	–	1.25
KNO_3	–	5.0	5.0
$Ca(NO_3)_2 \cdot 4H_2O$	–	1.0	1.0
NH_4NO_3	–	–	1.25
Chelated iron[a]	15.0 mg/L	15.0 mg/L	15.0 mg/L
pH	5.8	5.8	5.8

[a] Suppled as "Sequestrene 138-Fe" obtained from CIBA-Geigy Corp, Ardsley, NY

Table 2. Concentration of minor salts employed in *A. thaliana* culture

Components	Concentration (μg/L)
H_3BO_3	43.4
$MnCl_2 \cdot 4H_2O$	27.8
$ZnSO_4 \cdot 7H_2O$	2.9
$CuSO_4$	0.8
$Na_2MoO_4 \cdot 2H_2O$	0.48
EDTA	170.2
$FeSO_4 \cdot 7H_2O$	140.0

Table 3. Concentration of major components of aseptic cultures of *A. thaliana*

Components	Agar cultures			Submerged cultures
	NH_4^+	NO_3^-	$NH_4^+ + NO_3^-$	
	Concentration (mM)			
KH_2PO_4	2.0	2.0	2.0	–
$K_2HPO_4 \cdot 3H_2O$	0.5	0.5	0.5	0.5
$MgSO_4 \cdot 7H_2O$	1.0	1.0	2.0	2.0
$CaCO_3$	2.0	–	–	–
$(NH_4)_2SO_4$	1.0	–	–	–
NH_4HCO_3	1.0	–	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	2.0	2.0	1.0
KNO_3	–	2.0	1.0	–
$Mg(NO_3)_2 \cdot 6H_2O$	–	1.0	–	–
NH_4NO_3	–	–	3.0	3.0
$Ca(H_2PO_4)_2$	–	–	–	0.5
Sucrose	5.0 gm/L	5.0 gm/L	5.0 gm/L	5.0 gm/L
Agar	8.0 gm/L	8.0 gm/L	8.0 gm/L	–
pH	6.5	6.5	6.5	6.5

film and placed on a reciprocating shaker (100 cycles/min). The plants were continuously illuminated by fluorescent light (2,000 lux/cm²) and temperature was 25 °C (room). Only an $NH_4^+ + NO_3^-$ solution was used in these cultures (Tables 2 and 3).

3 Biochemical assays

Freshly harvested tissues (0.1–0.2 g) of whole plants, for submerged culture, or tissues above ground, for vermiculite and agar cultures, were homogenized in a Ten-Broeck homogenizer in ten volumes of an aqueous extraction buffer. This extraction buffer consisted of 10 mM EDTA, 10 mM cysteine, and 50 mM K_2HPO_4–3 H_2O, adjusted to a final pH of 7.5 with KOH (Amos and Scholl 1977). The extract was centrifuged at 0 °C for 15 min at 30,000 × g and the supernatant was used for all biochemical assays.

a) Enzyme assays. NR activity (NRA) was assayed by the method of Amos and Scholl (1977), a modification of the Hageman and Hucklesby (1971) assay. One unit of specific NRA is defined as the amount of enzyme catalyzing the formation of 1 nmole of NO_2^-/mg of protein per min at 28 °C.

Nitrite reductase activity was determined by a modification of the method of Joy and Hageman (1966). The reaction mixture contained 100 µmole K-phosphate buffer (pH 7.0), 1 µmole methyl viologen, and 1 µmole KNO_2 in a volume of 2.4 ml. A 0.1 ml aliquot of the crude extract was added and the reaction started by the addition of 10 µmoles of freshly prepared sodium hydrosulfite ($Na_2S_2O_4$) in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.5), bringing the total reaction volume to 3.0 ml. The reaction tubes were immediately incubated in vacuum at 30 °C for 15 min (in a vacuum jar). The enzyme reaction was terminated by opening of the jar and votexing the reaction tubes vigorously until the viologen dye was completely oxidized (colorless). A 0.1 ml aliquot of the reaction medium was drawn and added to 2 ml of a 1 : 1 mixture of 1% sulfanilamide in 3N HCl and 0.2% N-N-naphthylethylenediamine for nitrite determination.

Glutamine synthetase was determined by the method of Amos and Scholl (1977). Activity was measured by the production of γ-glutamylhydroxamate since the enzyme also catalyzes a γ-glutamyl transfer reaction (Elliot 1953). Cytochrome c reductase was assayed on extracts prepared as described above using the method of Mendel and Muller (1979).

b) Other assays. Nitrate content of the aqueous tissue extracts was determined by the brucine-H_2SO_4 method of Ranney and Bartlett (1972). Brucine reagent (0.15 ml of 2.0 g brucine in 50 ml methanol) was added to 0.1 ml extract, followed by 2 ml of concentrated sulfuric acid. The solution was vortexed, followed by addition of 2 ml of distilled water, and then a second vortexing. After incubation in the dark for 30 min, absorbance at 410 nm was recorded. Chlorate content in the aqueous extract was measured according to Doddema et al. (1978). Protein content of aqueous extracts was determined by Bio-Rad method according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA).

Results and discussion

1 Selection for and evaluation of chlorate susceptibility

The total M_2 population screened consisted of an average of 10 progeny, each, from a total of 5,000 M_1 plants. The M_2 plants were grown on vermiculite (150 per pot) on ammonium medium. Potassium chlorate (0.3 mM) was added to the nutrient solution when the plants were six days old. Plants that exhibited early chlorosis could be identified visually and were assumed to be potentially chlorate susceptible. These plants were immediately transferred to chlorate-free medium. However, such plants frequently did not survive the transplanting. Among the survivors, 38 apparently sensitive plants were successfully rescued in this fashion, and subsequently produced progeny families by self-fertilization. The lines established from these plants were compared to the parental race for visual symptoms of chlorate sensitivity. Five of these were clearly chlorate-hypersusceptible. The description of symptoms of these are presented in Table 4. When young plants (6-days-old at time of chlorate treatment) of these lines were compared, the mutants designated C-1 and C-4 both had somewhat higher NR activity than the parental line (WS). Since the goal of the screening was to identify potential nitrate metabolism variants, these two lines were further characterized. The attributes of the C-4 mutant are discussed in the present report. The characteristics of mutant

Table 4. Results of preliminary examination of growth, NRA, and responses to chlorate of the five selected variant lines and the wildtype plants

Line	NRA (nmol NO_2^-/ g fr wt/min)	Fresh weight (mg/15 plants)	Effect of chlorate treatment[a]
WS	60.82 ± 4.0[b]	53.0 ± 16.7	Chlorosis of leaves in 72 h
C1	78.8 ± 5.4	38.7 ± 5.1	Some expanded leaves wither, plants die rapidly
C4	86.8 ± 6.0	44.8 ± 9.5	Chlorosis in 40–50 h
C2	44.8 ± 2.4	33.7 ± 9.6	Somewhat earlier bleaching of expanded leaves
C6	49.2 ± 5.4	41.0 ± 1.7	Bleaching of leaves along veins
C7	56.4 ± 4.4	53.0 ± 10.6	Somewhat earlier chlorosis

[a] Plants grown on vermiculite with ammonium (5.0 mM), subjected to 0.3 mM potassium chlorate 6 days after planting

[b] Mean ± SE, 3 observations for each mean, $NH_4^+ + NO_3^-$ medium

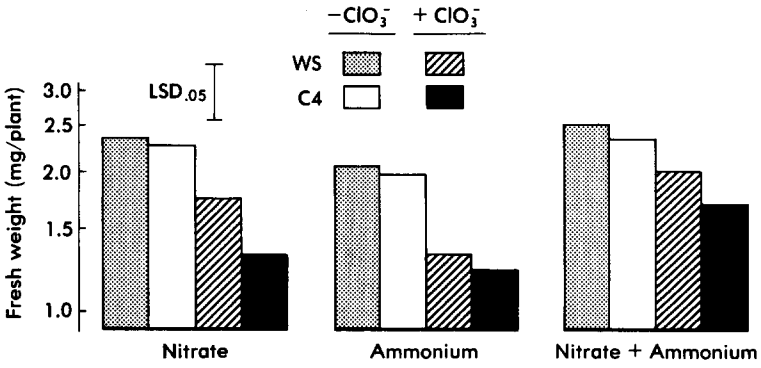

Fig. 1. Effect of 4 days of chlorate treatment on plants of normal and chlorate-susceptible genotypes

C-1 were substantially different and will be reported separately.

Quantitative verification of the chlorate hyper-susceptibility of C-4 was sought by comparison of the fresh weight of normal and C-4 plants after short treatments with chlorate. Potassium chlorate (12 mM for NO_3^- and NH_4NO_3, 0.3 mM for NH_4^- media) was added to the watering solution of vermiculite-grown plants of WS and C-4 8 days after germination. At age 12 days, the plants were weighed fresh. Since growth of *A. thaliana* follows an exponential function at this age, the fresh weights were transformed to the logarithmic scale prior to analysis, and comparisons were conducted on that scale (Fig. 1). For plants grown both on nitrate and ammonium, a significantly greater relative reduction of fresh weight occurred for C-4 in comparison to WS. Likewise, the mean reduction of fresh weight averaged over all the media was greater for C-4. Thus, the hypersusceptibility of C-4 relative to WS is reflected in reduced growth as well as in accelerated leaf chlorosis.

The chlorate-susceptible mutants identified in this research were fairly rare (total of five). In comparison, over 50 chlorate resistant lines were isolated by similar procedures from an M_2 population derived from 24,000 *A. thaliana* M_1 plants (Feldmann 1985). Thus, these susceptible mutations appear to occur less frequently than do those for chlorate resistance. However, the latter are among the most easily isolated biochemical mutants in plants. The present screening method has the weakness that it is necessary to identify plants showing early toxicity symptoms in a large population in which the wildtype also develops symptoms. Consequently, these plants must be rescued to establish genetic lines for further study. The latter step poses some difficulty since disturbance of a plant can accelerate the chlorate-induced chlorosis and cause death. This phenomenon can occur even when a plant is of a resistant genotype (Feldmann 1985). However, the present technique was reasonably successful and it should be possible to isolate other, possibly different chlorate susceptible mutants with this procedure.

2 Nitrate reductase activity

For many of the plant mutants having increased resistance to chlorate, decreased nitrate reductase activity represents the causal lesion (Braaksma and Feenstra 1982). Initially, leaf NRA and nitrate content were studied as functions of plant age for plants grown on nitrate plus ammonium in vermiculite. Leaf nitrate contents of C-4 and WS were extremely similar throughout the term of the experiment (Fig. 2), and nitrate content was relatively constant for all plant ages. However, the NRA of mutant C-4 was significantly higher than that of WS for all plant ages. The NRA of race WS was relatively high on the first day on which plants were large enough to assay (day 9), and gradually decreased to a relative low level from day 13 onward. The NRA of C-4 remained high through to 23 days of age which is approximately ten days after the first flowers pollinate and one week prior to the maturation of the resultant seeds. Hence, this mutant maintains maximal NRA much longer than is normal and throughout most of its life cycle.

Nitrate reductase activity of plants grown on different nitrogen sources was compared in 12-day-old plants (Table 5). The NRA was high in both media in which nitrate was included. This presumably represents the expected substrate induction effect (Tang and Wu 1957). The NRA of the ammonium-containing medium was nevertheless measurable and significantly greater than zero. This apparently indicates the presence of a constitutive activity, possibly similar to what has been shown to exist in other species (Nelson et al. 1983).

A comparison of enzyme activities of plants grown on nitrate and ammonium plus nitrate media indicated that the latter medium gave a somewhat higher NRA. Since ammonium repression effects on NR have only occasionally been reported in plants (Orebamjo and Stewart 1975; Smith and Thompson 1971; Radin 1975), this relationship was unexpected. However, it was observed that nitrate content of shoot tissue was similar in the two media. Consequently, NR was probably

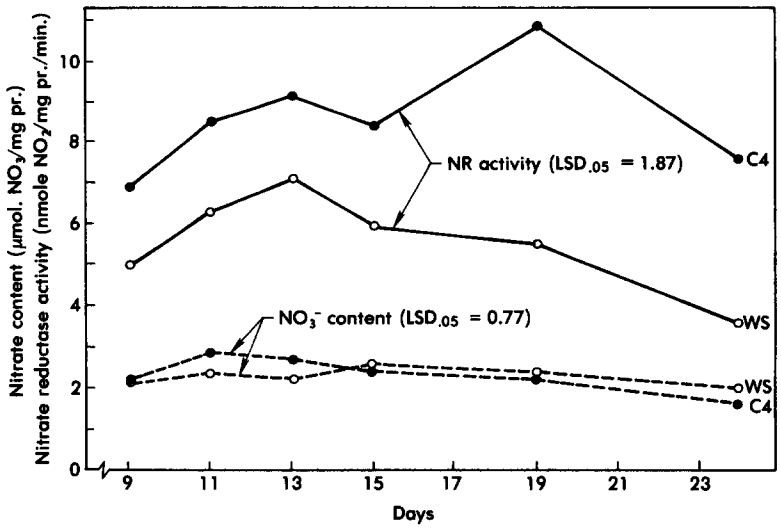

Fig. 2. Nitrate reductase activity and tissue nitrate content of mutant and normal genotypes as affected by plant age

Table 5. NRA and NO_3^- content of shoots of mutants and wild-type plants grown in aseptic agar culture

Nitrogen source	Genotype	NRA (n mol NO_2/ mg pr/min)	Tissue NO_3^- (μmol NO_3/ mg pr)
NO_3^-	WS	9.68 c[a]	3.78 a
	C4	12.41 b	4.18 a
NH_4NO_3	WS	12.21 b	3.70 a
	C4	14.67 a	3.15 a
NH_4^+	WS	0.69 e	b
	C4	1.28 d	

[a] Means followed by the same letter are not significantly different at the 5% level as evaluated by the Newman-Keuls test
[b] Measured nitrate content was very low for ammonium medium

fully induced in both treatments, and the slightly higher activity observed when both nitrate and ammonium are supplied may simply result from generally higher metabolic rates in these plants. Evidence for this arises from the facts that they appeared greener and possessed higher activities of other enzymes than did nitrate-grown plants (Table 6).

The NRA of the C-4 mutant was significantly greater than that of WS for plants grown on all three media (Table 5). Although the differences in activity for the two genotypes were not large at the age sampled, they were consistent across N sources. It thus appears from these results that this NR mutant does not differentially affect the constitutive and inducible components of NR, as has been shown for a soybean locus (Nelson et al. 1983). The chlorate resistant – low NR mutants of *A. thaliana* also tend to exhibit a proportionality of NR activity across these three types of media (Braaksma and Feenstra 1982; Feldmann 1985). Hence, although numerous mutants of *A. thaliana* affecting NR exist, clear differential genetic effects on constitutive versus inducible NR are rare.

3 Enzymatic comparisons

If the increase in nitrate reductase of mutant C-4 is responsible for its increased chlorate susceptibility, it is likely to be the only enzyme for which the mutant possesses substantially altered activity. This is strongly suggested by the fact that the low-NR mutants of *A. thaliana* and other species possess either normal or slightly elevated nitrite reductase activity. Enzymes less closely related to NR would presumably be still less likely to be affected if NR is responsible for chlorate susceptibility.

The enzymes nitrite reductase (NiR), and glutamine synthetase were chosen form comparative activity determinations. Cytochrome C reductase (CcR), an activity utilizing a portion of the NR enzyme was also examined. Enzyme activities were assayed on leaves of 12-day-old plants grown on vermiculite, irrigated with solutions containing three different nitrogen sources. Nitrite reductase activities varied substantially among nitrogen sources (Table 6). However, no significant differences in NiR activity between C-4 and WS were observed on any medium. Glutamine synthetase specific activity was also similar for the two genotypes. Hence, the overall trend of the results of comparative enzymatic analyses suggest that the mutation is relatively specific to NR.

The CcR activity of mutant C-4 was significantly higher than that of WS, when averaged across the three nitrogen sources. This pattern is to be expected if either: 1) the structural gene of the NR apoenzyme were unaltered by the present mutation and quanti-

Table 6. Nitrite reductase (NiR), glutamine synthetase (GS) and cytochrome c reductase (CcR) activities of mutants and wildtype plants grown on vermiculite

Nitrogen source	Genotype	NiR (nmol NO_2^-/mg pr/min)	GS (nmol glutamyl hydroxamate/mg pr/min)	CcR (nmol cytoc/mg pr/min)
Combined	WS	98.29a[a]	5.67a	70.0b
mean	C4	98.40a	5.40a	83.6a
NO_3^-	WS	90.55b	4.98c	92.9b
	C4	94.62b	4.72c	116.3a
$NH_4^+ + NO_3^-$	WS	127.49a	6.52a	79.5c
	C4	127.21a	6.02a	88.5bc
NH_4^+	WS	76.84c	5.52b	37.4d
	C4	73.38c	5.46b	46.0d

[a] Means followed by the same letters are not significantly different at the 5% level as evaluated by the Newman-Keuls test

tative regulatory factors were responsible for the increased activity of C4 or 2) a structural-gene mutation occurred but had equal effects on the NR and CcR functions. The mutant, therefore, probably results from one of these two effects.

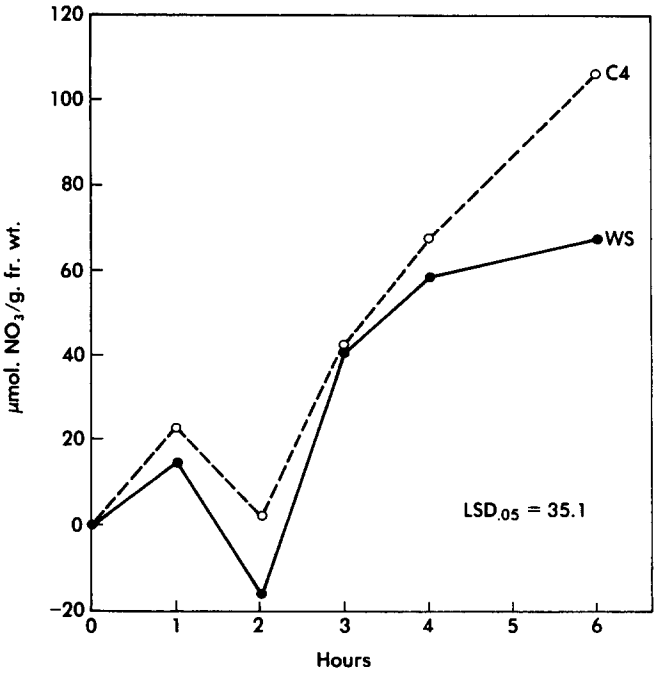

Fig. 3. Cumulative uptake of NO_3^- on nitrate medium across time as measured by disappearance in ambient solution

4 Uptake of nitrate and chlorate

The chlorate-resistant mutants of *A. thaliana* can all be placed in one of two categories with respect to their nitrate metabolism (Feldmann 1985; Braaksma and Feenstra 1982). The first class consists of lines all possessing low NRA but which take up nitrate at normal rates. The second group has near-normal NRA but has reduced rates of nitrate and chlorate uptake. These different sets of symptoms reflect physiologically distinct chlorate-resistance mechanisms that could both also be involved in causing chlorate hyper-susceptibility. Hence the nitrate and chlorate uptake rates of the current mutant were examined. For this purpose plants were grown from seeds in submerged aerated cultures. At age 7 days all nitrogen was removed from the medium. After 48 h of this N starvation, N was reintroduced as 2 mM nitrate.

The kinetics of nitrate uptake (Fig. 3) were similar to those observed in other such studies of *A. thaliana* (Doddema and Telkamp 1979). Specifically, an initial rapid absorption of nitrate was followed by an apparent release of nitrate into the ambient solution during the second hour. Steady nitrate uptake was observed between hours 2 and 6. The basis of this effect is uncertain, (Doddema et al. 1978).

The net cumulative nitrate uptake of C-4 and WS, for the first 6 h after the reintroduction of N (Fig. 3), was calculated from the determination of nitrate removed from the ambient solution, as assayed from aliquots of the solution taken at hourly time intervals. The uptake experiment was repeated twice, and points represent mean values averaged over two runs. Preliminary analysis of variance of the results (not shown) indicated the existence of a small, significant overall difference in nitrate uptake between the genotypes. However, cumulative uptake for C-4 was not significantly greater than WS until the last sampling time was reached. The mutant may consequently remove nitrate from the medium slightly more rapidly than does WS; the extent of any difference is small if real. Previously studied nitrate uptake mutants of *A. thaliana* usually differ from the wildtype by multiples of the lower rate (Feldmann 1985; Wang 1984). On this basis the uptake results do not strongly implicate nitrate uptake as a potential cause of the chlorate hyper-susceptibility of C-4.

To augment the data derived from the above nitrate analysis, direct tissue content of plants was determined at the time when nitrate was added and after 6 h of exposure to nitrate in one nitrate uptake experiment (Table 7). Nitrate contents (and therefore nitrate uptake) of tissue were similar for the genotypes both prior to and after nitrate addition. Significant increases in nitrate content occurred for both genotypes confirming that detectible nitrate uptake occurred during the experiment. These data suggest that genetic effects on the nitrate uptake system were probably minimal.

Table 7. Tissue content of nitrate for *A. thaliana* genotypes prior to and 6 h following addition of nitrate to the ambient solution

Genotype	Nitrate content of tissues (μmol NO_3^-/mg pr)		
	After 48 h of nitrogen starvation (A)	6 h after nitrate addition (B)	B/A × 100%
WS	2.478 b[a]	4.10 a	166
C4	2.577 b	3.79 a	147

[a] Means followed by the same letter are not significantly different at the 5% level as evaluated by the Newman-Keuls test

Table 8. Genetic analysis of mutants in reciprocal crosses between mutant C-4 and the parental line

Parents and cross	No. of observations	NRA (nmol NO_2^-/ mg pr/min)
I Parents		
WS	12	9.62 b[a]
C4	12	11.74 a
II F_1 hybrids		
C4 × WS	5	10.40 b
WS × C4	8	10.25 b

[a] Means followed by the same letter are not significantly different at the 5% level, as evaluated by the Newman-Keuls test

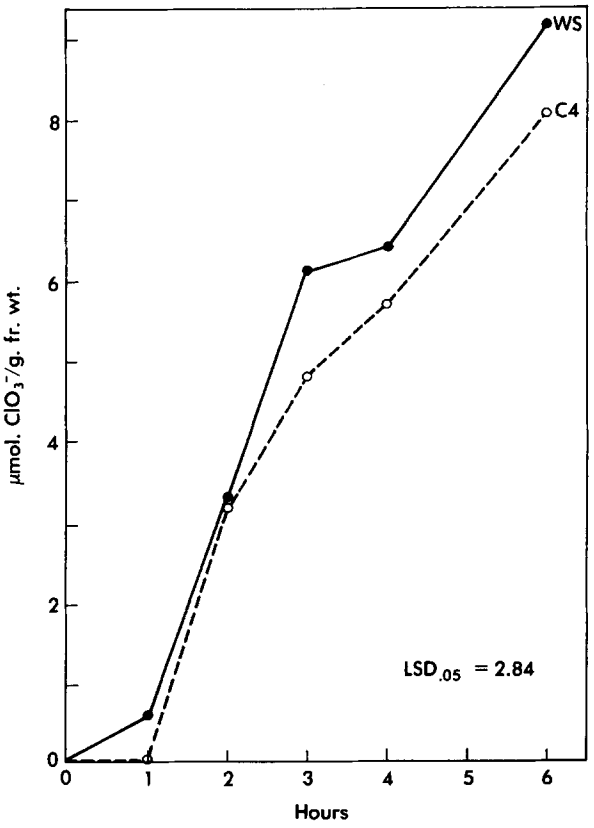

Fig. 4. Cumulative ClO_3^- uptake across time on basic mineral medium in submerged cultures measured by disappearance in the ambient solution

Chlorate uptake of plants occurs by a mechanism similar to that for nitrate uptake. In fact, radioactive chlorate uptake has been utilized as a means to study nitrate uptake kinetics (Deane-Drummond 1982). However, nitrate and chlorate uptake rates apparently are not always equivalent in *Arabidopsis* (Feldmann 1985). Consequently, nitrate uptake was assessed directly as described above, and chlorate uptake was studied independently. In the chlorate uptake study, 2 mM potassium chlorate was introduced to 12-day-old submerged plants that had been starved from N for 48 h. Disappearance of chlorate from the ambient solution was followed for six hours subsequent to its introduction to determine uptake rate.

Cumulative chlorate removed from the medium during the course of the 6 h experimental period increased in a linear fashion both for race WS and mutant C-4, after an initial lag of one hour (Fig. 4). The rates for the two genotypes were extremely similar and not significantly different. Given this strong similarity of chlorate uptake, it is unlikely that any genetic alteration in the nitrate-chlorate uptake system is responsible for the chlorate sensitivity of mutant C-4. If an alteration in the nitrate-uptake system is responsible for the chlorate susceptibility of C-4, chlorate uptake should be altered. Although some changes in nitrate uptake rates may have occurred, these are unlikely to be related in a causal way to relative chlorate susceptibility. Consequently, the primary biochemical effect of the mutation still appears to be concentrated on NR and it remains the most likely cause of the chlorate sensitivity. This is particularly true since NR possesses a clear logical connection to the chlorate phenotype.

5 *Inheritance of increased activity*

Inheritance studies of nitrate reductase activity in the mutant involving F_1 and F_2 progeny were conducted. NR was assayed in the experiments because it proved heritable enough to be determined accurately in single plants whereas chlorate susceptibility was not.

Reciprocal crosses betwen WS and C-4 were carried out, and the NRA of the resulting young F_1 plants was compared (Table 8). The parental lines performed as expected in this experiment: C-4 possessed a higher NRA than did WS. The mean difference between WS

and C-4 was similar to that seen in similar-aged plants in previous experiments (Fig. 2). The reciprocal F_1 hybrids did not differ significantly for NRA. This indicates that cytoplasmic inheritance and maternal effects probably do not influence the trait. The F_1 hybrid enzyme activities were likewise very similar to that of WS and significantly lower than that of C-4 (Table 8). Consequently, the wild-type NR activity is genetically dominant (Table 8).

Eighty-five F_2 hybrid plants were assayed individually for in vitro NRA. These were compared to smaller samples of individual plants from the two parental genotypes. This assay was conducted on older plants to allow the maximum separation of plants with high and low activity. Nitrate reductase activity varied only slightly among plants within parental lines (mean = 1.44, $\sigma^2 = 0.448$ for WS; mean = 6.51, $\sigma^2 = 1.40$ for C-4). The variance among F_2 plants for NRA was significantly (0.01 level) greater than the variance within either parental line (mean = 3.77, $\sigma^2 = 8.38$), indicating that genetic segregation was substantial. The range of NR activities observed in the F_2 generation spanned the ranges of both parental genotypes (Fig. 5). No distinguishable classes of individuals occurred with activity greater than that of C-4 or lower than WS. A tendency toward bimodality of the F_2 population was apparent. However, some individuals with a NRA intermediate to WS and C-4 were observed, and clear natural divisions for separation of the population into qualitatively different phenotypic groups were not available. A major mode clearly coincided with the WS phenotype and a minor mode occurred at NR levels in the C-4 range. If the population is divided into two groups at any NRA value near the midpoint between the these modes, approximately three-fourths occur in the lower group and one-fourth in the upper group. Hence the data suggest that a single locus with dominance of the wildtype activity controls the F_2 variability. This can be further verified by the examination of F_3 progeny families produced from individual F_2 plants. These studies are currently being initiated.

The isolation of this apparently simply inherited mutant by the present procedure suggests that other such mutants can be obtained. Such mutants will be sought in *A. thaliana* in our laboratory, and it should be possible to duplicate this procedure in other species. Hybridization of the mutant C-4 with existing low NR lines can be conducted to determine if it represents a new allele of a known NR locus or occurs at a new locus. This mutant represents an important addition to the *A. thaliana* collection for two reasons. First, it provides a unique opportunity to study the effect of a defined genetic alteration on nitrogen assimilation. Specifically, comparison of nitrate assimilation in the wildtype, which is similar to the mutant for all loci except the NR locus, to C-4 can be made. This would provide a more valid test of the existence of a genetic association between NRA and nitrogen assimilation rates than those obtained by comparison of crop cultivars differing by quantitatively inherited differences in NRA, since the latter type of comparison allows for the existence of genetic effects in addition to those of NR genes. Hence observed correlations in such cases may not be causally based. The second important use for this high NR mutant is in comparative molecular biology. The NR of *A. thaliana* now represents one enzyme for which normal low, and high activity variants now exist, and comparative biochemical and molecular study of these should prove fruitful.

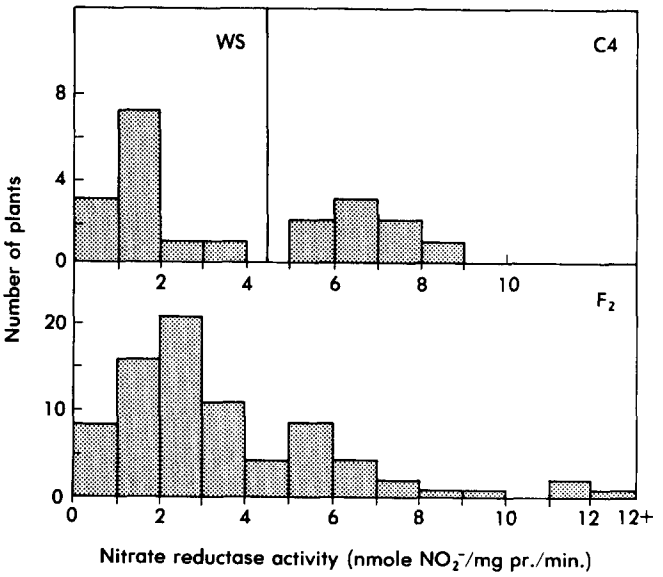

Fig. 5. Frequency distribution of NRA in parental and F_2 (C4 × WS) plants (25-day-old)

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