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Nitrate reductase deficient cell lines from diploid cell cultures and lethal mutant M2 plants of *Arabidopsis thaliana*

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Summary. Cell suspensions of diploid *Arabidopsis thaliana* were screened for resistance to chlorate on a medium with ammonium nitrate as the nitrogen source, and after plating on filters to increase the plating efficiency. Thirty-nine lines were selected, four of which were still resistant after two years of subculturing on non-selective medium. Of the latter lines three were nitrate reductase deficient but exhibited some residual nitrate reductase activity; the fourth line showed a high level of enzyme activity. Screening M2-seeds for callus production on selective medium with amino acids as the nitrogen source and chlorate revealed resistant calli in 17 out of 483 M2-groups. Nine well-growing lines, all but one (G3) exhibiting no detectable in vivo nitrate reductase activity, were classified as defective in the cofactor. Two lines (G1 and G3) could be analysed genetically at the plant level. Chlorate resistance was monogenic and recessive. Sucrose gradient fractionation of callus extracts of G1 revealed that a complete enzyme molecule can be assembled. Nitrate reductase activity in G1 could partly be restored by excess molybdenum. It is suggested that G1 is disturbed in the catalytic properties of the cofactor. It appeared that G1 is neither allelic with another molybdenum repairable mutant (B73) nor with another cofactor mutant (B25). Wilting of intact G1 plants could be ascribed to non-closing stomata.

Key words: *Arabidopsis thaliana* – Chlorate resistance – Nitrate reductase deficiency – Suspension cultures – M2-seeds

Introduction

In higher plants, mutants which are disturbed in the uptake or reduction of nitrate can be isolated by selection for chlorate-resistance.

The first chlorate-resistant mutants of *Arabidopsis thaliana,* isolated by Oostindiër-Braaksma and Feenstra (1973), have been followed by a number of resistant plants and cell lines in other species (Kleinhofs et al. 1978; Müller and Grafe 1978; Murphy and Imbrie 1981; Márton et al. 1982; Nelson et al. 1983; Steffen and Schieder 1984; Wakasa et al. 1984). By direct screening for in vivo nitrate reductase (NR) activity, Warner et al. (1977) found NR-mutants in *Hordeum vulgare.* In *Hyoscyamus muticus,* NR deficiency was found using a procedure for selection of auxotrophs (Strauss et al. 1981). Most chlorate-resistant variants fall in three distinct classes: mutants disturbed in the uptake of chlorate, mutants in the apoenzyme of NR (nia-type), and mutants disturbed in the functions of the cofactor of this enzyme (cnx-type; Mendel and Müller 1976). Some of the latter are repairable by addition of excess molybdenum (Braaksma et al. 1979; Mendel et al. 1981).

Uptake mutants and cnx-type mutants were found to be present among the chlorate-resistant mutants of *A. thaliana,* isolated by Oostindiër-Braaksma and Feenstra (1973) and Braaksma and Feenstra (1982a, c), while there were indications that some other mutants might be of the nia-type. The NR deficient (NR^-) mutants all exhibited residual NR activity.

The objective of the present study is to extend the spectrum of chlorate-resistant mutants of *A. thaliana.* Except for a mutant of *H. vulgare* (Bright et al. 1983) the only fully NR^- mutants have been isolated in cell cultures with media containing reduced nitrogen compounds. Since it is difficult to recover recessive mutations in diploid cultures, most NR^- cell lines have been isolated in haploid protoplasts or suspension cultures. Although some embryoid-like structures emerged from anthers, we could not succeed in establishing stable haploid cell lines from anther cultures (Scholten 1985). However, NR^- variants were isolated by Müller and Grafe (1978) in allodiploid suspension cultures of *Nicotiana tabacum.*

Therefore, it was to be expected that variant cell lines could also be recovered in diploid suspension cultures of *A. thaliana.* However, calli from the wildtype strain used in this study could only be obtained when the suspensions were grown with ammonium nitrate as the nitrogen source. Plating on filters (Raveh et al. 1973) was used to increase the plating efficiency. In this way

four stable NR^- cell lines with residual NR activity were isolated and these lines were partially characterized.

Chlorate-resistant plant mutants sometimes proved to the lethal when exposed to normal growth conditions (Kleinhofs et al. 1978; Bright et al. 1983). In addition, chlorate-resistant mutants of *Arabidopsis,* which could not survive at humidities lower than 100%, were found. It was conceivable that this type of mutant was fully NR^-. By using a combined procedure of mutation induction at the plant level and selection for resistance in callus, it proved possible to isolate fully NR^- cell lines from emerging M2-seeds, on selective medium with amino acids. One of these lines was characterized biochemically at the cellular level and genetically at the plant level.

Preliminary reports of this work have already been presented (Steingröver and Feenstra 1981; Scholen et al. 1982; Scholten and Feenstra 1983).

Materials and methods

Plant material

Seed stocks of *A. thaliana* (L.) Heynh. used in this study were of the mutant *erecta* of the ecotype 'Landsberg'. The NR^- mutants B25 and B73, which are mutated in different genes, *rgn* and *cnx,* respectively, were isolated as M2-seedlings by Braaksma and Feenstra (1982a, c). Wildtype suspension cultures were obtained from callus derived from leave explants.

Media and culture conditions

Plants were grown as described by Oostindiër-Braaksma and Feenstra (1973). Callus was cultured at 25 °C with a 14 h photoperiod (8 $W m^{-2}$) on medium PG2, PG5 or PG11. The medium PG11 is a modified medium PG2 (Negrutiu et al. 1975) with the nitrogen source (glutamine, aspartic acid, arginine and glycine), vitamins and growth substances as described by Müller and Grafe (1978). In medium PG5 the nitrogen source in PG11 is replaced by 20 mM NH_4NO_3 and 40 mM KNO_3. Callus on germinating seeds of wildtype and mutants was induced on medium PG11 or on PG11 with 10 mM KNO_3.

Mutagenic treatment and screening for resistance

Suspension cultures of wildtype in PG2 were sieved through a nylon sieve with a pore size of 210 µm. Portions of 12.5 ml were incubated with 0.05 ml ethylmethane sulphonate (EMS) for 1 h at room temperature. Each portion was divided into two equal parts and each part was filtered over a double layer of filter paper (Schleicher and Schüll, no. 595) on a Büchner funnel. The collected cells were washed with 25 ml PG2 medium. Filters with cells were placed on PG2 and incubated at 25 °C. After 2 weeks when small calli became visible, the filters were transferred to PG2 supplemented with 8 mM sodiumchlorate. After 1 month yellow colonies were removed onto PG11 medium.

Wildtype seeds were treated with 40 mM EMS as described by Braaksma and Feenstra (1982a). M2-seedlings were screened for chlorate-resistance in petri dishes on perlite with nutrient solution containing 1.5 mM ammonium sulphate as the sole nitrogen source, at 25 °C under continuous illumination (15 $W m^{-2}$). Sodiumchlorate was added to a final concentration of 0.1 mM either to the original solution or to the petri dishes when the seedlings were 6 days old. At this concentration wildtype seedlings became bleached. Mutant plants remained green but showed severe symptoms of wilting and died when transplanted into soil. In some cases plants could be grown to maturity on a floating support on liquid medium in closed glass jars (Steingröver and Feenstra 1981). Just before flowering the lid of a jar was removed and liquid medium was added in order to raise the upper part of the inflorescence above the rim of the jar. In this way the rosette was protected from water stress and pollination was effective in producing seeds. Chlorate resistant cell lines from seeds were selected by incubating calcium hypochlorite sterilized M2-seeds on medium PG11 supplemented with 10 mM sodiumchlorate.

Biochemical assays

The NR activity in vivo and in vitro, the cytochrome-c-reductase (CcR) activity and the nitrate content of callus was determined as described by Braaksma and Feenstra (1982a) for intact plants. One unit of NR activity is defined as the amount of enzyme catalyzing the formation of 1 nmol NO_2^- in 1 min at 28 °C. One unit of CcR activity is defined as the amount of enzyme catalyzing the reduction of 1 µmol cytochrome c per minute. Fractionation of extracts by sucrose gradient centrifugation was described by Braaksma and Feenstra (1982a). Xanthine dehydrogenase (XDH) activity was assayed as described by Jacobsen et al. (1984). One unit of XDH is defined as the increase in absorbance brought about by 0.25 ml of a 4×10^3-fold diluted suspension of xanthine oxidase from buttermilk.

For induction of NR activity callus was grown on medium PG11 with 10 mM KNO_3. XDH activity was measured in extracts of callus grown on medium PG11.

Results

In plating experiments only a few colonies developed when sieved suspension cultures of wildtype were plated in soft agar (0.3% in PG2) on solid PG2, or directly on solid PG2. However, the plating efficiency showed a remarkable increase when the cells were concentrated by filtration over a double layer of filter paper followed by washing with excess liquid PG2 and culturing the retained cells on the filters on solid PG2. Despite the beneficial effect of plating on filters, colonies developed neither on PG2 nor on PG11 when wildtype cells had been grown previously in medium PG11 with amino acids as the nitrogen source. The best results were obtained when about 5 ml of sieved suspension in PG2 (2.5×10^4 aggregates) was filtered and cultured.

Cell suspensions of wildtype in PG2 (5×10^3 aggregates/ml, together 1.1×10^6 aggregates) were given a mutagenic treatment and collected on filters. Following the same procedure, 3.8×10^5 aggregates were plated without the addition of the mutagen. After one month on selective medium 30 vital colonies could be selected from the mutagenized cells and 9 colonies from the untreated cells. The selected colonies were transferred to medium PG11. After subculturing one year on PG11, 8

out of these 39 lines were still chlorate-resistant (Table 1). After another year, 4 of them had lost resistance. The resistant lines could proliferate on ammonium nitrate (PG5) and the upper limit of tolerance to chlorate was about 8 mM.

Probably due to the long period of growth required by the selection procedure with cell suspensions, the NR^- cell lines could not be regenerated for genetical analysis at the plant level.

Selection of resistant cell lines from M2-seeds

Seeds, when incubated on medium PG11, had a germination rate of close to 100% within 2 weeks. Further development of the seedlings was retarded and callus formed at the hypocotyl. After one month, sufficient callus was formed for biochemical assays. No callus was formed and the seedlings subsequently became whitish when wildtype seeds were placed on PG11 with 10 mM chlorate. At the lower concentrations of chlorate which can be tolerated by NR^- lines with residual enzyme activity, some wildtype seeds also developed callus (Scholten and Feenstra, submitted).

M2-groups from two mutagenic treatments (EMS 39 and EMS 42) were screened on perlite for seedlings resistant to 0.1 mM chlorate (Table 2). Each M2-group contained seeds that were harvested from a group of six M1-plants. From the 59 groups, which segregated one or more resistant plants in this pre-selection, 42 groups were tested for callus production, ultimately yielding 17 resistant cell lines, 9 of which grew well enough to allow a reliable biochemical analysis.

From three M2-groups that gave rise to resistant cell lines, some resistant plants could be grown to maturity and crossed to wildtype to yield a number of F1-seeds. Since it cannot be excluded that the M2-groups in question yield more than one independently arisen mutant, cell lines were established from F2-seeds, which are referred to as G1, G2 and G3, respectively (Table 3). Contrary to the mutant plants found in the other groups, G3 plants could be grown to maturity upon transplantation into soil provided that measures were taken to maintain a high humidity. However, when sown on perlite with nitrate as the sole nitrogen source, G3 plants turned yellow and died.

Callus of G1 and the other lines from seeds died when ammonium nitrate (PG5) was the sole nitrogen source. However, when added to PG11, nitrate in concentrations up to 50 mM had no detrimental effect on callus and even increased the efficiency of callus-formation on seeds. The nitrate content of G1 callus, grown on PG11 with 10 mM KNO_3, was found to be almost twice that of the wildtype (8.4 and 4.5 μmol NO_3^-/g. fresh weight, respectively). Apparently nitrate can be accumulated in G1, but G1 is protected from the toxic effects by the amino acids in the medium. When aspartic acid was omitted from PG11 with 10 mM nitrate, G1 could no longer proliferate (Scholten 1985). The results thus suggest that the protection can be ascribed mainly to aspartic acid.

In order to gain insight into the stability of chlorate-resistance in the variants derived from M2-seeds, the cell lines were regularly examined for sensitivity to chlorate and ability to proliferate on ammonium nitrate. After one year of cell culture on PG11 or PG11

Table 1. In vivo NR activity (percentage of wildtype) in callus after one year on PG5, and chlorate-resistance of callus on PG11 + 8 mM chlorate after one and two years of cultivation of selected cell lines

Cell line	EMS-treatment	NR activity	Chlorate-resistance	
			After 1 year	After 2 years
Wildtype		100	–	–
VW124A2	–	25	+	–
VW124C	–	56	+	+
VW158	–	46	+	–
VW164A	–	39	+	–
VW165A5	+	125	+	+
VW165F2	+	41	+	–
VW169B	+	44	+	+
VW169F2	+	44	+	+

Table 2. Selection of chlorate-resistant cell lines in M2-groups of mutagenic treatment EMS 39 and EMS 42

	EMS 39	EMS 42
No. of M2-groups screened (100 seeds/group)	308	175
Groups with resistant plants	37	22
Groups screened at the cellular level	20	22
No. of seeds tested per group	500	500
Groups with resistant calli	13	7
Resistant calli lost on cultivation	2	1
Well-growing lines, suited for biochemical analysis	3	6

Table 3. Chlorate-resistant cell lines isolated from F2-seeds. In vivo NR activity on PG11 + 10 mM KNO_3 and XDH activity on PG11 are expressed as percentages of wildtype

Cell line	Mutagenic treatment	NR activity	XDH activity
Wildtype		100	100
G1	EMS 39	0	0
G2	EMS 39	0	0
G3	EMS 42	198	70

with 10 mM KNO_3 all lines except G1 and G3 were resistant. G3 had completely lost resistance and was able to grow on PG5. As for G1, several lines were tested, each originating from a different F2-seed. Some of these lines behaved like G3, whereas others kept their resistance. The chlorate-sensitive lines possessed wildtype NR activities. Although no decisive conclusions could be drawn, maintenance of callus of G1 on PG11 with nitrate seemed to favour and accelerate the loss of resistance.

Genetical analysis of lines G1 and G3

On perlite with ammonium sulphate as the nitrogen source and 0.1 mM chlorate, 19 mutants were recovered from 92 F2-plants of G1 × wildtype, showing that the mutant character is monogenic and recessive (P: 0.3–0.5). Moreover, callus could be induced on about 25% of the seedlings grown on PG11 with chlorate. Crossings were made between G1, into which a recessive signal character (*gl*, glabra) had been crossed (no indication of linkage was found), and two cofactor mutants of *A. thaliana* (B25 and B73). Thirty-eight F1-plants of G1 × B25 and 34 F1-plants of G1 × B73 could be tested and proved to be chlorate-sensitive on ammonium sulphate with 0.1 mM chlorate. Therefore, G1 is supposed not to be allelic with B25 or B73.

An F2 of G2 × wildtype, when tested for resistance on callus producing medium, segregated 23% resistant calli. However, when tested for growth on perlite with chlorate, no segregation into mutant and clearly wildtype plants was found.

The F1 of G3 × wildtype showed the wildtype phenotype when grown on nitrate; an F2 segregated 1017 wildtype and 347 mutant plants, indicating that the mutant character is monogenic and recessive (P: 0.7–0.8). In addition, about 25% of the calli induced on F2-seeds were chlorate-resistant. G3 was crossed with B25 and B73 which contained the marker *gl*. In both cases the F1 showed wildtype phenotype, indicating that G3 is neither allelic with B25 nor with B73. Crossings between G1, G2 and G3 have not yet been successful.

Biochemical classification of the isolated lines

Seven out of the eight cell lines which had been isolated from suspension cultures had a decreased level of in vivo NR activity (Table 1). Line VW165A5 exhibited a NR activity even higher than that of the wildtype. After another year the activity of the then still resistant lines had not changed.

All but one (G3; Table 3) well-growing lines isolated from M2-seeds lacked in vivo NR activity when grown on inductive medium (PG11 + 10 mM KNO_3). Since neither XDH activity could be detected, probably no functional cofactor, which is common to NR and XDH, is present in these variants. Line G3 showed a high NR activity and was, contrary to the other lines, not able to grow on 20 mM chlorate. However, its XDH activity was distinctly lower than that of the wildtype. The relatively high NR activity in G3 may possibly be explained by its growth rate, which is considerably higher than that of the wildtype. Therefore, G3 could be a cnx-type mutant with residual NR activity like mutant B25 of *A. thaliana* (Scholten and Feenstra, submitted). As stated previously, eight out of the 17 cell lines which were originally isolated showed poor growth characteristics. Therefore, no conclusions were drawn from the absence of detectable levels of NR and XDH activities in five of these lines. In the remaining three lines the absence of NR activity was combined with low but clearly detectable levels of XDH activity (6–32% of wildtype). Together with the observation that the cell lines were resistant to 20 mM chlorate, these data point to the possibility that these lines are fully NR^- and have an intact cofactor but no functional apoenzyme (nia-type).

Detailed biochemical analysis of line G1

Line G1, as representative of the fully NR^- lines, was further analysed. Mutant plants in an F2 could only be selected on a medium with chlorate, which rendered the plants unsuitable for a reliable biochemical analysis. Therefore, callus of G1 induced on F2-seeds was used as the source of mutant cells. In callus of G1 neither in vivo nor in vitro NR activity and no XDH activity could be measured (Table 4). The CcR activity in G1 was inducible by nitrate and relatively higher than in the wildtype. The results presented in Table 4 indicate that G1 is a cofactor mutant. To determine whether the cofactor is disturbed in its role in dimerization of the subunits, extracts of wildtype and G1 were fractionated by sucrose gradient centrifugation (Fig. 1). All CcR activity in G1 banded in the fractions in which also wildtype NR and CcR was found. Apparently in G1 a complete NR molecule can be assembled and the cofactor has lost its

Table 4. NR activity in vivo and in vitro, CcR activity and XDH activity (units/g fresh weight) in callus of wildtype and G1 grown on PG11 and on the inductive medium PG11 + 10 mM KNO_3

Line	Medium	NR		CcR	XDH
		in vivo	in vitro		
Wildtype	PG11	4.8	30	16.4	497
	PG11 + nitrate	9.2	136	60.7	173
G1	PG11	0	0	55.7	0
	PG11 + nitrate	0	0	111.4	0

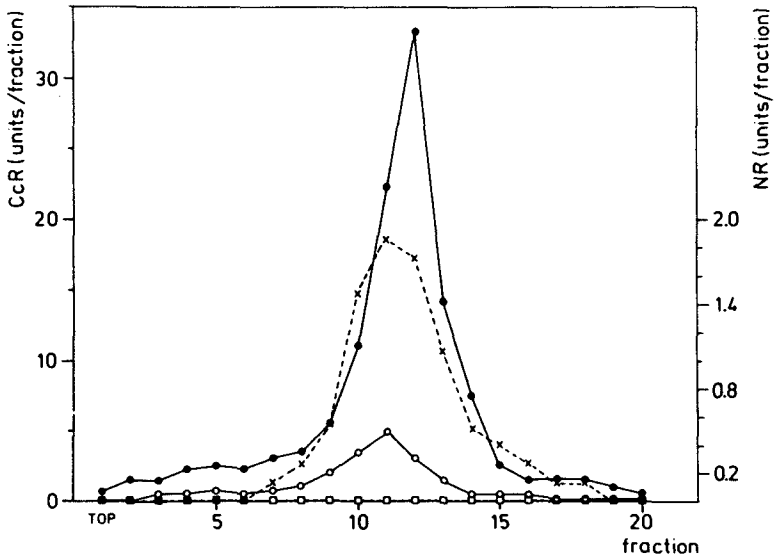

Fig. 1. Fractionation of extracts of wildtype and G1 grown on PG11 + 10 mM KNO_3 by sucrose gradient centrifugation. The crude extract of wildtype contained 5.7 units NR and 21.0 units CcR. The G1 extract contained 48.0 units CcR. In the fractions 8.5 units in vitro NR (×---×) and 23.5 units CcR (○——○) of wildtype and 0 units in vitro NR (□——□) and 121.1 units CcR (●——●) of G1 were recovered

Table 5. Effect of cultivation on medium with 1 mM molybdenum on enzyme activities (units/g fresh weight) in callus of wildtype and G1

Enzyme	Medium	Wildtype	G1
NR	PG11 + KNO_3	55	0
	PG11 + KNO_3 + Mo	24	14
XDH	PG5 + Mo	254	109

catalytic properties. As in plants of *Arabidopsis* (Braaksma and Feenstra 1982a, c) the sum of the activities found in the fractions was higher than in the crude extract applied to the gradients, which suggests that the NR and CcR activities had been separated from some inactivating factor(s).

The NR activity and XDH activity in some mutants of the cnx-type can be restored by addition of excess molybdenum. The results presented in Table 5 showed that G1 belongs to this group of mutants.

Stomatal behaviour in plants of G1

To study the physiological basis of the wilting process in plants of G1 under water stress, the stomatal response to changes in environmental conditions was examined by preparing silicone impressions (Sampson 1961) of leaves of nine-day-old seedlings grown on perlite in petri dishes with 1.5 mM ammonium nitrate as the nitrogen source and 0.5 mM chlorate. Water stress was induced by removing the lid of the dishes at the beginning of a 14 h photoperiod (15 W m^{-2}). At an ambient relative humidity of 40% the stomata of wildtype closed completely within 2 h whereas even after 6 h most of the stomata of G1 remained open despite severe symptoms of wilting.

Discussion

The plating technique on filters proved to be useful for overcoming the barrier of the first divisions in plated cells from suspension cultures. From 1.1×10^6 mutagenized cells, thirty chlorate-resistant colonies could be isolated. Among 3.8×10^5 untreated cells nine resistant colonies were found. Although the suspensions consisted predominantly of aggregates, which renders calculation of the variant frequency less accurate, the number of isolated stably resistant lines suggests that the mutagenic treatment had not increased the frequency with which variants were isolated (respectively 2.7×10^{-6} and 2.6×10^{-6}). In non-mutagenized allodiploid *N. tabacum* cultures, the frequency of NR^- lines was 1.5×10^{-9} (Müller and Grafe 1978). The frequency of resistant lines of *A. thaliana* was considerably higher, but, as can be expected, lower than the frequency of resistants in non- mutagenized haploid cultures of *N. plumbaginifolia* (3.5×10^{-5}; Márton et al. 1982). Induced frequencies were about 10^{-3}. Nevertheless, the results show that in diploid cell cultures of *A. thaliana* NR^- cell lines with residual NR activity can be isolated. Only 8 out of the 39 lines which had been isolated from suspension cultures had kept their resistance after one year of cultivation on non-selective medium, compared to 7 out of 9 lines isolated from M2-seeds. This might indicate that in the former the variation is epigenetic. But the loss of resistance in isolated lines needs not necessarily lead to this conclusion, in view of the instability in cell cultures of G1 and G3, which both are monogenic and recessive mutants. Regaining of wildtype properties can be explained in three different ways. (1) Back mutation and (2) suppressor mutation. Braaksma and Feenstra (1982b) found revertants of mutant B25 with high frequency. Reversion was caused by mu-

tations in a suppressor gene, which were either recessive or dominant. From mutant B73 revertants could also be isolated with high frequency (Braaksma, personal communication). (3) Contaminations with wildtype cells: in the case of suspension-culture-derived variants, the presence of wildtype cells in a cluster next to mutant cells from which a resistant line developed could be an important factor. Similarly, contamination of the primary callus at the seedlings with maternal, heterozygous cells of the perisperm of the M2- or F2-seeds may have been instrumental in bringing about the loss of chlorate-resistance in cell lines G1 and G3. Wildtype contaminations may have had a selective advantage because of the ability to use the nitrate as additional nitrogen source when callus was grown on PG11 with nitrate.

A total of 483 M2-family groups were screened at the plant stage and 17 chlorate-resistant cell lines were selected from promising groups. Since each M2-family group was derived from six M1-plants, the frequency of M1-plants bearing a mutation is about 5.9×10^{-3}. The available data suggest that the variants isolated from M2-seeds are predominantly of the cnx-type. On media without nitrate both variants without and with residual enzyme activity have been isolated in most species (e.g. Müller and Grafe 1978). Apparently *Arabidopsis* is more sensitive to higher concentrations of chlorate (10 mM) and most mutants with residual NR activity have not been able to survive selective pressure (c.f. results with mutant B25; Scholten and Feenstra, submitted). At 10 mM chlorate only one cell line not belonging to the zero-activity type was found (G3; Table 3).

Line VW165A5 from suspension cells and G3 showed characteristics of uptake mutants: high NR activity together with moderate resistance to chlorate (Oostindiër-Braaksma and Feenstra 1973; Scholten and Feenstra, submitted). However, in contrast to callus of such an uptake mutant (B1), callus of G3 is resistant to 10 mM chlorate and is not able to proliferate on PG5. Moreover, G3 seedlings turned yellow and died when sown on perlite with nitrate as the sole nitrogen source whereas B1 seedlings can grow on such a medium. Therefore, in the case of G3, the indications are that we are dealing with a cnx-type mutant.

From biochemical assays on callus it appeared that G1 is disturbed in the catalytic properties of the cofactor of the NR molecule (Table 4). Sucrose gradient fractionation revealed that in G1 a complete molecule can be assembled (Fig. 1). This is also the case in mutant B73 of *Arabidopsis* (Braaksma and Feenstra 1982c). The partial restoration of NR and XDH activities by excess molybdenum is also a common property of mutants G1 and B73. In this respect these mutants are similar to the *cnx E*-mutants of *Aspergillus* (Cove 1979) and to several cnx-mutants in higher plants, like the cnx-68 line (*cnx A*) of *N. tabacum* (Mendel et al. 1981; Mendel and Müller 1985). Our complementation studies indicated that G1 is neither allelic with B73, nor with B25. This would mean that in *Arabidopsis* three genes have been identified which are involved in the functioning of the cofactor, two of them being related to the interaction with molybdenum. In *N. tabacum* complementation between molybdenum-repairable cnx-mutants has not been found (Grafe and Müller 1983). However, with the data available, interallelic complementation between G1 and B73 cannot be excluded. Nevertheless, the most likely explanation for the complementation between G1 and B73 is that in *Arabidopsis* alterations in a least two steps in the assembly of the cofactor can lead to a non-functional molecule which is repairable by excess molybdenum.

Plants of G1 have an absolute need for high humidity to be grown to maturity. Bright et al. (1983) reported the isolation of a conditional lethal, cnx-type mutant (R9401) from M2-seeds of barley. The similarity between G1 and R9401 suggests that wilting at the plant level is a more common feature of this type of mutant which may hamper both isolation as an intact plant and regeneration from cell cultures. The relation between the inability to close the stomata under water stress and the altered nitrogen metabolism in G1 is not clear. However, for analysis of the stomatal behaviour of G1, mutant seedlings were extracted from F2-seeds on ammonium sulphate with 0.5 mM chlorate. In the presence of chlorate the G1 seedlings may have accumulated considerable amounts of the latter compound resulting in a disturbed ionic balance in the stomata. In favour of this explanation is the observation that under mild water stress seedlings of G1 showed symptoms which correspond with those of wilting nitrate-accumulating mutants of pea (Feenstra and Jacobsen 1980).

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References

Braaksma FJ, Feenstra WJ (1982a) Nitrate reduction in the wildtype and a nitrate reductase deficient mutant of *Arabidopsis thaliana*. Physiol Plant 54:351–360

Braaksma FJ, Feenstra WJ (1982b) Reverse mutants of the nitrate reductase deficient mutant B25 of *Arabidopsis thaliana*. Theor Appl Genet 61:263–271

Braaksma FJ, Feenstra WJ (1982c) Isolation and characterization of nitrate reductase deficient mutants of *Arabidopsis thaliana*. Theor Appl Genet 64:83–90

Braaksma FJ, Feenstra WJ, Hermans E (1979) Nitrate reduction in *Arabidopsis thaliana*. IV. Arabidopsis Inf Serv 16:68–70

Bright SWJ, Norbury PB, Franklin J, Kirk DW, Wray JL (1983) A conditional lethal cnx-type nitrate reductase-deficient barley mutant. Mol Gen Genet 189:240–244

Cove DJ (1979) Molecular genetics of nitrate and nitrite reductases. In: Hewitt EJ, Cutting CV (eds) Nitrogen assimilation of plants. Academic Press, London New York San Francisco, pp 289–297

Feenstra WJ, Jacobsen E (1980) Isolation of a nitrate reductase deficient mutant of *Pisum sativum* by means of selection for chlorate resistance. Theor Appl Genet 58:39–42

Grafe R, Müller AJ (1983) Complementation analysis of nitrate reductase deficient mutants of *Nicotiana tabacum* by somatic hybridization. Theor Appl Genet 66:127–130

Jacobsen E, Braaksma FJ, Feenstra WJ (1984) Determination of xanthine dehydrogenase activity in nitrate reductase deficient mutants of *Pisum sativum* and *Arabidopsis thaliana*. Z Pflanzenphysiol 113:183–188

Kleinhofs A, Warner RL, Muehlbauer FJ, Nilan RA (1978) Induction and selection of specific gene mutations in *Hordeum* and *Pisum*. Mutat Res 51:29–35

Márton L, Dung TM, Mendel RR, Maliga P (1982) Nitrate reductase deficient cell lines from haploid protoplast cultures of *Nicotiana plumbaginifolia*. Mol Gen Genet 182:301–304

Mendel RR, Müller AJ (1978) A common genetic determinant of xanthine dehydrogenase and nitrate reductase in *Nicotiana tabacum*. Biochem Physiol Pflanz 170:538–541

Mendel RR, Müller AJ (1985) Repair in vitro of nitrate reductase-deficient tobacco mutants (*cnx A*) by molybdate and by molybdenum cofactor. Planta 163:370–375

Mendel RR, Alikulov ZA, Lvov NP, Müller AJ (1981) Presence of the molybdenum-cofactor in nitrate reductase-deficient mutant cell lines of *Nicotiana tabacum*. Mol Gen Genet 181:395–399

Müller AJ, Grafe R (1978) Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol Gen Genet 161:67–76

Murphy TM, Imbrie CW (1981) Induction and characterization of chlorate-resistant strains of *Rosa damascena* cultured cells. Plant Physiol 67:910–916

Negrutiu I, Beeftink F, Jacobs M (1975) *Arabidopsis thaliana* as a model system in somatic cell genetics. 1. Cell and tissue culture. Plant Sci Lett 5:293–304

Nelson RS, Ryan SA, Harper JE (1983) Soybean mutants lacking constitutive nitrate reductase activity. 1. Selection and initial plant characterization. Plant Physiol 72:503–509

Oostindiër-Braaksma FJ, Feenstra WJ (1973) Isolation and characterization of chlorate-resistant mutants of *Arabidopsis thaliana*. Mutat Res 19:175–185

Raveh D, Huberman E, Galun E (1973) In vitro culture of tobacco protoplasts: use of feeder techniques to support division of cells planted at low densities. In Vitro 9:216–222

Sampson J (1961) A method of replicating dry or moist surfaces for examination by light microscopy. Nature 191:932–933

Scholten HJ (1985) Isolation and characterization of chlorate-resistant cell lines and plants of *Arabidopsis thaliana*. Thesis, Groningen

Scholten HJ, Feenstra WJ (1983) Characteristics of mutants of *Arabidopsis thaliana* selected by resistance to low concentrations of chlorate. Arabidopsis Inf Serv 20:100–103

Scholten HJ, Feenstra WJ, Nijdam H, Datema G (1982) The use of cell culture of *Arabidopsis thaliana* for the study of nitrate reductase-less mutants which are lethal as intact plants. Arabidopsis Inf Serv 19:108–110

Steffen A, Schieder O (1984) Biochemical and genetical characterization of nitrate reductase deficient mutants of *Petunia*. Plant Cell Rep 3:134–137

Steingröver E, Feenstra WJ (1981) Resistance to low concentrations of chlorate on nitrate free medium. Arabidopsis Inf Serv 18:143–145

Strauss A, Bucher F, King PJ (1981) Isolation of biochemical mutants using haploid mesophyll protoplasts of *Hyoscyamus muticus*. I. A NO_3^- non-utilizing clone. Planta 153:75–80

Wakasa K, Kobayashi M, Kamada H (1984) Colony formation from protoplasts of nitrate reductase-deficient rice cell lines. J Plant Physiol 117:223–231

Warner RL, Lin CJ, Kleinhofs A (1977) Nitrate reductase-deficient mutants in barley. Nature 269:406–407