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Isolation and Characterization of Nitrate Reductase-Deficient Mutants of *Arabidopsis thaliana*

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Summary. Chlorate resistant mutants of *Arabidopsis thaliana* were isolated, of which 10 exhibited a lowered nitrate reductase activity and 51 were chlorate-resistant because of an impaired uptake of chlorate. The 51 mutants of this type are all affected in the same gene. The mutants with a lowered nitrate reductase activity fall into 7 different complementation groups. Three of these mutants grow poorly on media with nitrate as the sole nitrogen source, while the others apparently can reduce sufficient nitrate to bring about growth. In all cases a low nitrate reductase activity coincides with an enhanced nitrite reductase activity. After sucrose gradient centrifugation of wildtype extracts nitrate reductase is found at the 8S position, whereas cytochrome-c reductase is found both at 4 and 8S positions. It is suggested that the functional nitrate reductase is a complex consisting of 4S subunits showing cytochrome-c reductase activity and a Mo-bearing cofactor. All mutants except B25 are capable of assembling the 4S subunits into complexes which for most mutants have a lower S value and exhibit a lower nitrate reductase activity than the wildtype complexes. Since the mutants B25 and B73 exhibit a low xanthine dehydrogenase activity, the Mo-bearing cofactor is probably less available in these mutants than in the wildtype. B73 appears to be the only mutant which is partly repaired by excessive Mo. The possible role of several genes is discussed.

Key words: *Arabidopsis thaliana* – Mutant – Chlorate resistance – Nitrate reductase – Cytochrome-c reductase – Xanthine dehydrogenase

Introduction

Mutants deficient in nitrate reductase are important tools for studying the genetic regulation of the nitrate reduction pathway.

Nitrate reductase-deficient mutants have been extensively characterized in fungi (especially in *Aspergillus nidulans*, MacDonald et al. 1974, and *Neurospora crassa*, Coddington 1976) where the structure of nitrate reductase is well understood (Cove 1979). However, the genetic control of nitrate assimilation in higher plants is relatively unclear. Though in higher plants nitrate reductase-deficient mutants were isolated in *Arabidopsis* (Oostindiër-Braaksma and Feenstra 1973a; Braaksma and Feenstra 1975), barley (Kleinhofs et al. 1978), and cell cultures of *Datura* (King and Khanna 1980) and tobacco (Müller and Grafe 1975, 1978; Mendel and Müller 1978, 1979), they are less well characterized than in fungi. However, Mendel and Müller (1976) showed by means of mutants that also in higher plants there is a common Mo-containing cofactor for the enzymes nitrate reductase and xanthine dehydrogenase (XDH) as was earlier shown to be the case in fungi (Pateman et al. 1964). Therefore, they adhere for nitrate reductase in higher plants to the model of MacDonald et al. (1974) for nitrate reductase in fungi. In this model the enzyme is a complex of subunits (exhibiting cytochrome-c reductase activity) kept together by a Mo-containing cofactor which moreover is needed for the functional enzyme. Although MacDonald et al. (1974) proposed that the complex enzyme comprised two subunits, Lewis (1975, cited by Cove 1979) showed that the mopl.wt. of the complex is 200,000 and of the subunit approximately 50,000 and suggested that nitrate reductase of *Aspergillus* is an aggregate of 4 subunits.

Previously we described a simple isolation technique for the selection of nitrate reductase-deficient mutants: selection for resistance to chlorate, which in the wildtype has a strongly toxic effect (Oostindiër-Braaksma and Feenstra 1973a). Several chlorate resistant mutants were isolated; most, however, did not show nitrate reductase-deficiency but were allelic with a mutant gene *chl-1* giving a decreased uptake of chlorate (Doddema et al. 1978). Nevertheless, mutants with a low level of nitrate reductase activity could also be isolated as chlorate resistant mutants. The mutant B2-1 (Oostindiër-Braaksma and Feenstra 1973a) exhibits only 20–30% of the nitrate reductase activity of the wildtype and the mutant B25 (Braaksma and Feenstra 1982a) exhibits hardly any in vitro nitrate reductase activity. The mutated gene of B25 was

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designated *rgn* (Feenstra and Braaksma 1976) since the gene was thought to be involved in the regulation of the synthesis of nitrate reductase. The other genes of which mutation could bring about chlorate resistance were called *chl-1* for the gene giving the impaired uptake of chlorate and *chl-2* for the gene involved in B2-1.

More data are presented here about other nitrate reductase-deficient mutants and about the relationship between the enzymes nitrate reductase, cytochrome-c reductase and xanthine dehydrogenase.

Materials and Methods

Plant Material

Seed stocks of *Arabidopsis thaliana* (L.) Heinh. were of the mutant *erecta* of the ecotype "Landsberg" (Rédei 1962). The marker line carrying *ttg* (transparent testa and hairless) and *fg* (late flowering) was kindly supplied by Mr. M. Koornneff, Wageningen.

Handling of Plant Material

Growth conditions were as described previously (Oostindiër-Braaksma and Feenstra 1973a). In most cases plants for biochemical assays were grown for 10 days in petri dishes on perlite moistened with a mineral solution under continuous illumination of 25 W/m² (TL-33). However, for the determination of xanthine dehydrogenase (XDH) plants were harvested after growth in the dark for the previous two days since Nguyen (1980) found that the XDH activities of leaves from several species increased considerably after such a treatment.

Crosses were made according to Feenstra (1964, 1965). As mutagen, 40 mM ethylmethane sulphonate was used; otherwise mutagenic treatment was carried out as described previously (Oostindiër-Braaksma and Feenstra 1973a) with the exception that possible mutants were grown after transplantation until producing inflorescences at a lowered light intensity (about 15 W/m², TL-33) with a daily light period of 16 h. Some mutants grew better under these conditions than with the usual continuous illumination of about 25 W/m² (TL-33) (Oostindiër-Braaksma and Feenstra 1974).

Genetic Analyses

Linkage studies between two chlorate resistant mutants were carried out as follows: the F1 obtained by crossing the two mutants was crossed with the wildtype, the resulting generation was self-fertilized and the seeds were harvested from each plant. These progenies were tested for the occurrence of chlorate resistant plants. The amount of recombination is calculated as twice the percentage of progenies containing chlorate sensitive plants only. For other determinations of linkage between chlorate-resistance and marker genes, the same strategy was adopted in most cases; i.e. the F1 was crossed with the wildtype and the second generation was tested for the various phenotypes. This was a reliable method, giving the same information as a test cross.

Biochemical Analyses

The assays for nitrate reductase (in vitro), nitrite reductase, determination of protein content and nitrate content, and the cytochrome-c reductase assay by means of sucrose gradient centrifugation, have been described previously (Braaksma and Feenstra 1982a). One unit of nitrate (nitrite) reductase activity is defined as the amount of enzyme catalyzing the formation (consumption) of 1 nmol NO_2^- per 30 min at 28 °C. One unit of cytochrome-c reductase activity is defined as the amount of enzyme catalyzing the reduction of 1 nmol cytochrome-c per 5 min at 0 °C (in melting ice).

Finding a reliable method to assay xanthine dehydrogenase (XDH) appeared to be difficult (data on different attempts will be published elsewhere). A modification of the double-beam test given by Scazzocchiao et al. (1973) proved to be sensitive enough for the low activities which are found in extracts of *Arabidopsis*. One part of fresh plant material was ground with four parts icecold 0.1 M phosphate buffer (pH 7.6) containing 0.01% mercaptoethanol. After centrifugation the XDH in the supernatant was precipitated in 40% ammonium sulphate and redissolved in the same volume of extraction buffer. This solution could be used for XDH-assays. The precipitation step was found to be necessary to separate XDH from an inhibitor which is soluble in up to 70% ammonium sulphate. The assay was run in a double-beam spectrophotometer. The assay cell contained 0.45 ml 0.1 M pyrophosphate buffer pH 9.4, 0.05 ml benzyl viologen (90 mg/ml in distilled water), 0.25 ml cytochrome-c (4.8 mg/ml in pyrophosphate buffer pH 9.4), 0.25 ml enzyme preparation and 0.2 ml hypoxanthine (0.2 mg/ml in the same buffer). The reference cell omitted the hypoxanthine solution for which buffer was substituted. The reaction was followed by recording the increase in absorbance by reduction of cytochrome-c at 550 nm. One unit of XDH activity is defined as the increase in absorbance brought about by 0.25 ml 10^{-6} of the suspension of xanthine oxidase from buttermilk (Sigma Chemical Company, no. X-1875).

Results

Isolation of Chlorate Resistant Mutants

After a mutagenic treatment with ethylmethanesulphonate (EMS) we screened the M2 progenies of the first 17,000 M1 seedlings for chlorate resistance. This yielded one mutant with hardly any nitrate reductase activity (isolation number B25, Oostindiër-Braaksma and Feenstra 1973b, 1974; Braaksma and Feenstra 1975, 1982a), seven mutants with a lowered level of nitrate reductase activity (isolation numbers B29, B31-1, B31-2, B33, B35, B36 and B40) and 37 mutants with an impaired uptake of chlorate. After screening M2 progenies of another 5,000 seedlings treated with EMS, another promising nitrate reductase-deficient mutant, B73, was isolated. Previously one mutant with a lowered nitrate reductase activity, B2-1 (Oostindiër-Braaksma and Feenstra 1973a), was isolated among the M2 progenies of 18,000 M1 seedlings treated with 1 mM N-methyl-N¹-nitro-N-nitrosoguanidine (NG). Besides B2-1, 14 independently arising mutants were isolated which are chlorate resistant because of an impaired uptake system (Braaksma and Feenstra 1975). The total number of chlorate resistant mutants with defective nitrate reduction is 10.

Table 1. Complementation table for chlorate-resistant mutants

	B2-1	B25	B29	B31-1	B31-2	B33	B35	B36	B40	B73
B2-1		+	+	+	+	+	+	+	+	+
B25			+	+	+	+	+	+	+	+
B29				+	−	−	−	+	+	+
B31-1					+	+	+	+	+	+
B31-2						−	−	+	+	+
B33							−	+	+	+
B35								+	+	+
B36									+	+
B40										+

+: the F1 is chlorate-sensitive; −: the F1 is chlorate-resistant

Table 2. Survey of nitrate and nitrite reductase activity of nitrate reductase deficients mutants of 9 days old, growing on media with different nitrogen sources

Line	Nitrogen-source	Nitrate reductase activity units/mg protein	Nitrite reductase activity units/mg protein	Growth on nitrate medium
Wild type	Nitrate	150 – 250	± 100	Strong
	Ammonium nitrate	100 – 200	± 100	
	Ammonium	25 – 50	25 – 50	
B2-1	Nitrate	10 – 20	150 – 250	Poor
	Ammonium nitrate	15 – 30	150 – 250	
	Ammonium	5 – 10	50 – 100	
B25	Nitrate	0 – 3	300 – 500	Poor
	Ammonium nitrate	0 – 3	200 – 400	
	Ammonium	0 – 3	50 – 100	
B29, B31-2, B33, B35	Nitrate	20 – 50	150 – 250	Strong
	Ammonium nitrate	20 – 50	150 – 250	
	Ammonium	5 – 10	50 – 100	
B31-1	Nitrate	± 30	± 250	Strong
	Ammonium nitrate	± 50	± 150	
	Ammonium	± 10	± 50	
B36	Nitrate	± 80	± 200	Strong
	Ammonium nitrate	± 50	± 100	
	Ammonium	± 30	± 30	
B40	Nitrate	± 90	± 160	Strong
	Ammonium nitrate	± 55	± 70	
	Ammonium	± 20	± 25	
B73	Nitrate	5 – 20	200 – 400	Poor
	Ammonium nitrate	15 – 30	200 – 400	
	Ammonium	5 – 10	50 – 100	

Genetic Analysis of Nitrate Reductase Deficient Mutants

All mutants are monogenic and recessive. Complementation tests (Table 1) in which the F1's were tested for chlorate resistance show that B29, B31-2, B33 and B35 did not complement each other. We therefore conclude that these four mutants are mutated in the same gene. Since other combinations show complementation, the mutations of B2-1, B25, B31-1, B36, B40, B73 and the group mentioned above concern different genes, since linkage studies make interallelic complementation unlikely. We therefore conclude that mutations in at least 7 different genes can bring about a lowered nitrate reductase activity. Linkage studies between the nitrate reductase-deficient mutants were carried out (as described in Materials and Methods) using chlorate resistance as criterion. Close linkage was found between B40 and B2-1, while B31-1 and B36 are also linked with

B2-1, though not closely. Since *chl-2* (the mutation of B2-1) is located on chromosome 2 (Oostindiër-Braaksma and Feenstra 1973a) the mutations of B40, B31-1 and B36 are also located on this chromosome. No further linkage between nitrate reductase deficient mutants could be demonstrated. The other mutations were localized with the help of marker genes. The mutation in B25, *rgn*, is tightly linked to the marker gene *an* (Feenstra and Braaksma 1976) and is thus located on chromosome 1. The gene mutated in the mutants forming the complementation group (B29, B31-2, B33, B35) which was designated *chl-3*, is linked with the marker gene *ch* at a distance of about 6 map units, and therefore *chl-3* is also located on chromosome 1 (Feenstra 1978; Koornneef and Van der Veen 1978). The genes *rgn* and *chl-3* segregate independently which is in agreement with the great distance between *an* and *ch*. The mutation of B73, designated *cnx* according to the gene symbols used by Müller and Grafe (1978), is linked with the marker genes *ttg* and *fg*, and therefore *cnx* is located on chromosome 5 (Koornneef and Van der Veen 1978). The distance between *cnx* and *ttg* is about 6.1 Morgan units and

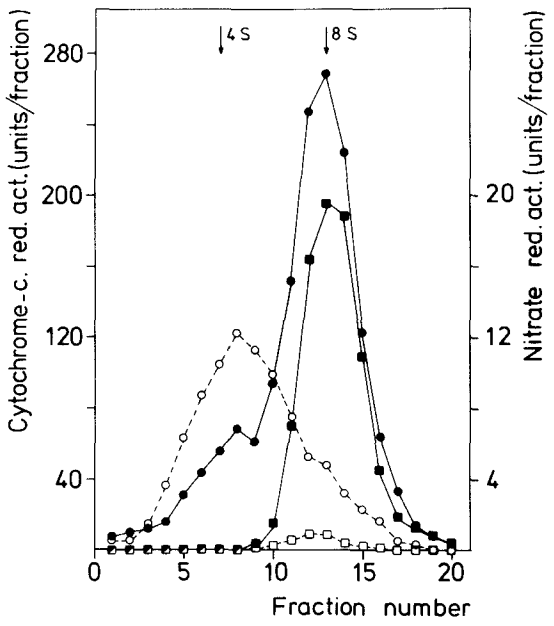

Fig. 1. Sucrose, gradient fractionation of cytochrome-c reductase and nitrate reductase from extracts of wildtype and B25 plants which have grown under 'high level conditions'; cytochrome-c reductase: ●—● wildtype, ○-○ B25; nitrate reductase: ■—■ wildtype, □-□ B25. The amount of wildtype extract applied to the gradient contained 870 units of cytochrome-c activity and 51.45 units of nitrate reductase activity. The sum of the cytochrome-c reductase activities found in the fractions was 2,049 units and that of nitrate reductase activities 112.24 units. The activities in the applied B25 extract were 693 and 3.97 units, respectively, while the sum of the activities found in the fractions was 1,202 and 4.89 units, respectively

data about flowering time indicate that the majority of recombinations between *cnx* and *ttg* also resulted in recombination between *ttg* and *fg*. We therefore conclude that *cnx* is located between *ttg* and *fg* on chromosome 5.

Experiments yielding the data presented below, were carried out with chlorate resistant lines, extracted from the F2's from the crosses mutant X wildtype.

Nitrate and Nitrite Reductase Activity, Growth on the Nitrate Medium

Table 2 shows the range of nitrate and nitrite reductase activities from wildtype and the mutants grown on different nitrogen sources. As was previously mentioned for B2-1 and B25, it appears that a decrease nitrate reductase activity is accompanied by an enhanced nitrite reductase activity. None of the nitrate reductase deficient mutants is constitutive for nitrite reductase. Since their growth on the nitrate medium is poor, the mutants B2-1, B25 and B73 appear to be incapable of using nitrate as the sole nitrogen source. The other mutants grow rather strongly on all media. We can conclude that these mutants can use nitrate as nitrogen source well enough to bring about growth.

The following experiments were carried out with the mutants B2-1, B25 and B73 (the mutants with poor growth on the nitrate medium, carrying *chl-2, rgn* and *cnx*, respectively) and B29 (representative of the complementation group carrying *chl-3*).

Sucrose Gradient Fractionation

As is shown in Fig. 1 (previously reported by Braaksma and Feenstra 1982a and b) the wildtype enzyme complexes, exhibiting both nitrat reductase and cytochrome-c reductase activity, sediment as 8S molecules in fraction 13, whereas the 4S cytochrome-c reductase subunits peak in fraction 7–8. B25 apparently is incapable of assembling the enzyme complexes and exhibits very little or no nitrate reductase activity. Sucrose gradients were made with extracts from B2-1, B29 and B73 plants (Fig. 2) which had grown under "high level conditions" (Braaksma and Feenstra 1982a). In all cases the peak of nitrate reductase is found in fraction 13. For B2-1 the elution pattern is comparable to that of the wildtype, since for both the highest level of cytochrome-c reductase activity is found in fraction 13. However, the ratio nitrate reductase activity/cytochrome-c reductase activity of fraction 13 from B2-1 is only 0.4–0.5 of this ratio for the wildtype, indicating that the 8S complexes exhibit a lower nitrate reductase activity in B2-1 than in the wildtype. In fraction 13 from B73 the ratio nitrate reductase activity/cytochrome-c reductase activity is

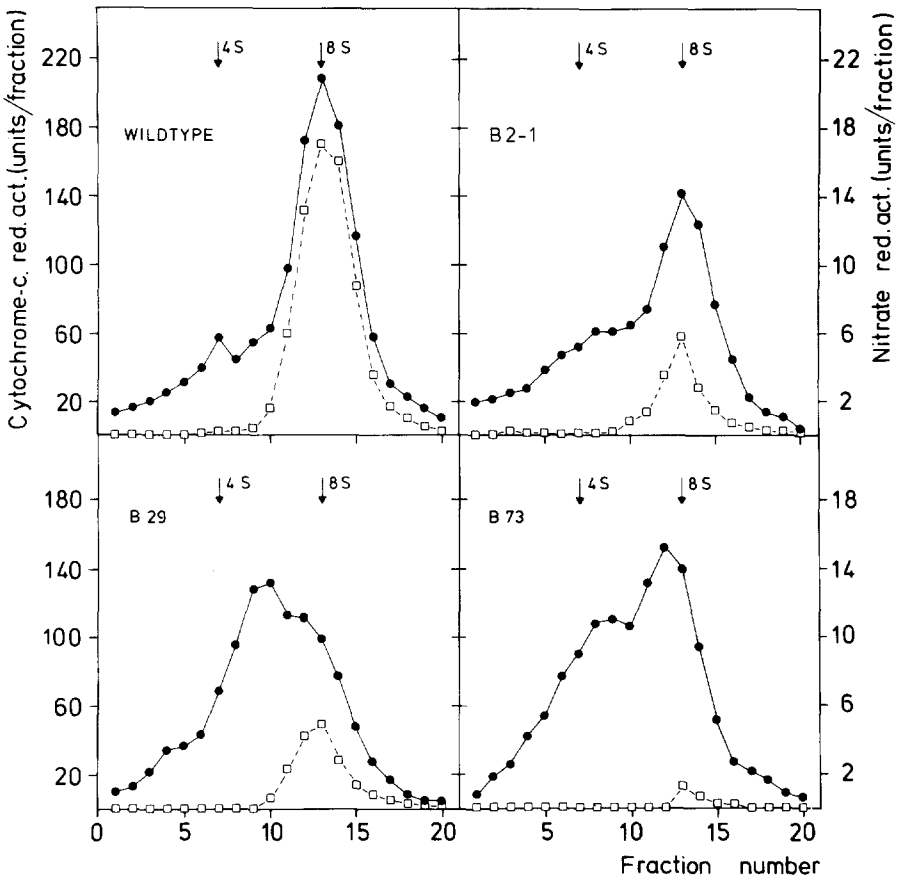

Fig. 2. Sucrose gradient fractionation of cytochrome-c reductase and nitrate reductase from extracts of plants which have grown under 'high level conditions'; cytochrome-c reductase ●—●; nitrate reductase □—□ The added extracts from wildtype, B25, B2-1, B29 and B73 contained cytochrome-c reductase activities of 879, 693, 731, 840 and 750 units resp., though the sums of the activities found in the fraction were 1,703, 1,202, 1,380, 1,458 and 1,721 units resp. For nitrate reductase the activities in the extrcts from these lines were 54.93, 3.97, 20.17, 22.77, 2.28 units resp., while the sums of the activities found in the fractions were 95.79, 4.89, 26.40, 25.47 and 4.45 units, respectively

less than 0.02 of this ratio for the wildtype so that the complexes which apparently are assembled lack almost all nitrate reductase activity. For B73 the peak of the heavy molecules with cytochrome-c reductase activity is in fraction 12 and the peak of light cytochrom-c reductase molecules is in fraction 9. For B29 the highest level of cytochrome-c reductase is found in between the 4S and the 8S position in fraction 10.

The Effect of Excessive Mo

Growth of some nitrate reductase-deficient *cnx* mutants of *Aspergillus nidulans* could be partially restored by a 100 to 1,000-fold increase of the Mo-concentration of the substrate (Arst et al. 1970). We therefore examined the effect of excessive Mo on the mutants (Table 3). The regular Mo amount is 0.05 mg MoO_3 per 1 mineral solution. The 100-fold concentration also contained MoO_3, the 1,000-fold concentration had to be given as K_2MoO_4 since the solubility of MoO_3 is too low. Only the mutant B73 reacted positively to excessive Mo: supplied with a 100- or 1,000-fold Mo-concentration B73 plants grew better on the nitrate medium and exhibited a higher nitrate reductase activity on am-

Table 3. The effect of a 100- and 1,000-fold concentration of Mo in the ammonium nitrate medium on the nitrate reductase activity of plants of 10 days old

Genotype	Mo-concentration compared to normal	Nitrate reductase activity units/mg protein	Relative growth on nitrate medium
Wildtype	1×	153.6	+
	100×	131.7	+
	1,000×	140.4	+/−
B2-1	1×	30.4	−
	100×	29.1	−
	1,000×	32.0	−
B25	1×	1.7	−
	100×	1.1	−
	1,000×	1.6	−
B29	1×	15.3	+
	100×	12.6	+
	1,000×	15.8	+/−
B73	1×	11.1	−
	100×	36.9	+/−
	1,000×	42.3	+/−

Table 4. Xanthine dehydrogenase activity of different genotypes. 3 and 7 days old

Genotype	Age[a] (days)	XDH activity units/mg protein
Wildtype	3	42.4
	7	28.3
B2-1	3	17.6
	7	18.7
B25	3	18.8
	7	3.0
B29	3	21.7
	7	21.7
B73	3	6.1
	7	7.9

[a] For 48 h before harvesting the seedlings were in the dark

monium nitrate. For the wildtype and the other mutants no positive effect of a 100-fold Mo-concentration on growth on nitrate could be noticed and there was no clear effect on the level of nitrate reductase activity. The 1,000-fold Mo-concentration was even harmful, since the plants remained small and showed necrotic spots.

Xanthine Dehydrogenase Activity

It is generally accepted that the Mo-containing cofactor, which is required for assembling and functioning of the complex exhibiting nitrate reductase activity, is also required for xanthine dehydrogenase (XDH) activity (Mendel and Müller 1976). The XDH activity is supposed to be a measure of the availability of this cofactor. Therefore, the XDH activities from the mutants were compared with that of the wildtype. Table 4 shows that the XDH activity of B73 is only about 15–30% of that of the wildtype, and the activity of 7 days old B25 plants drops to 10% of the wildtype activity. The other mutants show smaller divergences from the wildtype activity. These results are in agreement with previously published preliminary data (Braaksma et al. 1979).

Discussion

In our experiments the mutagenic treatments, both with NG and EMS, yielded more chlorate resistant mutants with an enhanced nitrate reductase activity, all mutated in the gene *chl-1* which results in a decreased chlorate uptake, than mutants with a lowered nitrate reductase activity. No mutants of other organisms have been described that are chlorate resistant because of a decreased uptake of chlorate. However, the class of chlorate resistant mutants of *Chlamydomonas* (Nichols and Syrett 1978) that grew well with nitrate as nitrogen source in our opinion could be of this type.

We estimated the mutation frequency of the gene *chl-1* for NG and EMS as 3.6 and 1.3×10^{-4}, respectively, assuming that the embryo contained 3 cells during mutagenic treatment that give sporocyte tissue (Van der Veen and Gerlach 1965; Müller 1965). The concentrations of the mutagens were chosen so that fertility and embryonic aberrations (in the embryo test according to Müller 1963) were equal (unpublished results). Over 50 independently arising chlorate resistant mutants with a high nitrate reductase activity involving one gene, *chl-1*, were found so far, while only 10 mutants, involving 7 different genes, with a decreased level of nitrate reductase activity were isolated. Since we found that B25 can barely endure the usual amount of light during the first week after transplantation into soil (Oostindiër-Braaksma and Feenstra 1974) we may have lost in the beginning some mutants with little or no nitrate reductase activity during the selection procedure when the illumination had not yet been diminished.

The fact that we identified 7 different genes which can result in a lowered nitrate reductase activity contrasts with data on tobacco cell cultures (Mendel and Müller 1976, 1979, 1980; Müller and Grafe 1978) and barley (Kleinhofs et al. 1980) where two genes are supposed to be involved viz. 1. a structural gene coding for the apo-enzyme and 2. a gene *cnx* determining the Mo-containing cofactor. These authors, however, studied only mutants with no or very low nitrate reductase activity.

In all cases (Table 2 and unpublished results) mutants with a low level of nitrate reductase activity exhibit a high nitrite reductase activity and a high nitrate content. This phenomenon is in agreement with other reports on higher plants (Feenstra and Jacobsen 1980; Kleinhofs et al. 1980; Mendel and Müller 1979, 1980; Warner and Kleinhofs 1981). Probably the high nitrite reductase activity can be explained by the high nitrate content since nitrate is the inducer for nitrite reductase (Braaksma and Feenstra 1982a). Since a high level of nitrate reductase activity implies a relatively low nitrate content we think that selection of lines with a high level of nitrate reductase activity might possibly solve the problem of nitrate accumulation in horticultural crops.

Our results with sucrose gradient centrifugation (Figs. 1 and 2) differ from the results of Mendel and Müller (1979) in so far that in extracts of tobacco cell cultures the 4S cytochrome-c reductase was found not to be induced by nitrate. Thus, this band seems to represent enzyme activities not associated with nitrate reductase. However, Mendel and Müller (1980) showed that 7.6S nitrate reductase (also exhibiting cytochrome-c reductase activity) dissociated into subunits sedimenting at 4.1S, which exhibit cytochrome-c reductase activity. For *Arabidopsis* it was shown that the 4S enzyme was induced by nitrate (Braaksma and Feenstra 1982a). Our mutants with an impaired XDH-activity, B25 and B73, show an elution pattern for cytochrome-c reductase which is dif-

ferent from that of the wildtype and of each other. In B25 no 8S peak is found and in B73 the high molecular weight peak shifts from fraction 13 to 12. Mendel and Müller (1979) showed that the tested *cnx* lines of tobacco exhibited the 7.6S band of cytochrome-c reductase activity like the wildtype. In their experiments with the tested *nia* mutants, which are supposed to be mutated in the structural gene for the apo-enzyme, the 7.6S band was absent. This resembles the behaviour of our mutant B25, but it was clearly shown (Braaksma and Feenstra 1982b) that the mutated gene *rgn* is not the structural gene.

Because of the aberrant XDH-activities it is probable that both *rgn* and *cnx* are involved with the presence of functional cofactor. The effect of the mutation to *rgn* is that aggregation of the enzyme complex is no longer achieved. Possibly the cofactor is not available for the assemblage. In the presence of *cnx*, enzyme complexes are formed which are somewhat smaller than in the wildtype (7.6S instead of 8S) and which exhibit no nitrate reductase activity. This effect is partly restored by excessive Mo, so that our *cnx* mutant in this respect resembles the *cnxE* mutants in *Aspergillus nidulans* (Arst et al. 1970). According to their suggestion concerning the *cnxE* mutants, the *cnx* product in Arabidopsis may be an enzyme involved in the cofactor synthesis, perhaps incorporating Mo into the cofactor. Somehow the defective cofactor is capable of assembling the enzyme complex with the omission of part of it (possibly the cofactor itself) resulting in 7.6S molecules.

The mutations in *chl-2* and *chl-3* which also result in a lowered nitrate reductase activity probably do not affect the cofactor as is shown by the XDH-activities in B2-1 and B29. For both *chl-2* and *chl-3* there are arguments that the structural gene of the apo-enzyme is involved. In B2-1 the low ratio nitrate reductase activity/cytochrome-c reductase activity indicates a different exposure of the active sites of the enzymes in B2-1 and the wildtype. This difference might be caused by a mutation in the structural gene which does not affect the cytochrome-c reductase activity. In B29 there are irregularities with the aggregation of the enzyme complexes, which also might be caused by a mutation in the structural gene.

Physiological data for a structural model for nitrate reductase from higher plants are reviewed by Notton and Hewitt (1979). Although no single model fits all available data, all results show the same basic features. The most consistent model shows 4 subunits (probably of two types) and either 1 or 2 molybdenum complexes. Only one type would exhibit cytochrome-c reductase activity though both types are rather close in molecular weight. This model is in accordance with genetic data. It is possible that for the types of subunits there are two different structural genes. In that case *chl-2* and *chl-3* can each be a structural gene for the apo-enzyme.

Our results on the genetic regulation of nitrate reductase are not complete enough to permit the compilation of a satisfactory model.

Apart from genetic mechanisms of regulation, molecular mechanisms also play an important role. The review by Hewitt et al. (1979) shows that several distinct, sensitive and reversible mechanisms of regulation are possible. Mutations affecting these mechanisms can give rise to mutants exhibiting a lower nitrate reductase activity than the wildtype. It is probable that some of our mutants with a low nitrate reductase activity are involved in molecular mechanisms of regulation.

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