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Reverse Mutants of the Nitrate Reductase-deficient Mutant B 25 of *Arabidopsis thaliana*

F. J. Braaksma and W. J. Feenstra
Department of Genetics, Biology Centre, Haren (the Netherlands)

Summary. Revertants of B25, a nitrate reductase-deficient mutant of *Arabidopsis thaliana* (L.) Heinh, were isolated with a high frequency. All 7 independently arisen revertants were mutations in the same suppressor gene *su*, which is unlinked to the originally mutated gene *rgn*. The mutant character shows up both in growth on nitrate as the sole nitrogen source and in susceptibility to chlorate. When judged for these properties the mutant alleles are either dominant for both, recessive for both or dominant for growth on nitrate and recessive for the effect of chlorate, when compared to the wildtype allele. Whereas the original mutant B25 exhibits no or very little nitrate reductase activity, the activities of the revertants were in the range of 0.4 to 1.5 of the wildtype activity. Physiological characteristics of nitrate reductase from the revertants are the same as those from the wildtype. Probably *rgn* is not the structural gene for nitrate reductase. The ability to assemble the nitrate reductase complex from its subunits, which was absent in mutant B25, appears to have been restored in the revertants.

Key words: *Arabidopsis thaliana* – Suppressor – Nitrate reductase deficient mutant

Introduction

The isolation of revertants is in some cases a suitable means to study the character of a forward mutation. A reversion may imply a second mutation, which restores exactly the base sequence of the parental line. Often, however, reversion is the result of suppression, i.e. a second mutation at a site different from the first mutation site is able to mask the phenotypic expression of the first mutation. Many mechanisms for suppression are known. If the structural gene is involved in the suppression, the characteristic features of the enzyme can be different in the revertant when compared with the wildtype.

Reverse mutants of B25, a nitrate reductase-deficient mutant of *Arabidopsis thaliana*, were isolated and genetically and physiologically characterized. The mutant B25 (Braaksma and Feenstra 1975a; Braaksma et al. 1979; Feenstra and Braaksma 1976), which was isolated as a chlorate resistant mutant, grew poorly on a medium with nitrate as the sole nitrogen source and appeared to be nitrate reductase-deficient. Preliminary reports on the revertants of B25 have been published previously (Braaksma and Feenstra 1975b, 1979, 1980; Feenstra and Braaksma 1976).

Materials and Methods

Plant Material

Seed stocks of *Arabidopsis thaliana* (L.) Heinh used were of the mutant *erecta* of the ecotype "Landsberg" (Rédei 1962). The mutant B25 was isolated as a chlorate resistant mutant and described previously (Oostindiër-Braaksma and Feenstra 1973b, 1974; Braaksma and Feenstra 1975a, b). The marker line carying *ttg* (transparant testa and hairless) and *fg* (late flowering) and the line segregating male sterility were kindly suplied by Mr. M. Koornneef, Wageningen.

Handling of Plant Material

Growth conditions were as described previously (Oostindiër-Braaksma and Feenstra 1973a).

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). Phenotypes of plants were assessed in the rosette-stage of ca. 10 day old seedlings.

Biochemical Analyses

Extraction and determination of NADH-nitrate reductase activity of extracts were as described previously (Oostindiër-Braaksma and Feenstra 1973a) except that the reaction was stopped by addition of activated charcoal (see Stulen 1970) and

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thorough mixing; the amount of nitrite in the filtrate was determined. With intact plants the in vivo nitrate reductase activity was assayed according to Jaworski (1971), using 2×10^{-2} M KNO_3 and 5% propanol. One unit of nitrate reductase activity is defined as the amount of enzyme catalyzing the formation of 1 nmole NO_2^- in 30 min at 28 °C. For the determination of nitrite reductase activity we used the dithionite assay of Joy and Hageman (1966) with chemically reduced benzylviologen as electron donor. One unit of nitrite reductase activity is defined as the amount of enzyme catalyzing the consumption of 1 nmole NO_2^- in 30 min at 28 °C. Sucrose gradient centrifugation and assay for cytochrome-*c* reductase were carried out as described by Wallace and Johnson (1978). Plants with a highly induced cytochrome-*c* reductase activity ("high level conditions") were obtained as follows: after 10 days growth on perlite with ammonium nitrate medium the rosettes were cut off and floated on a nutrient solution without nitrogen for 24 h followed by 20 h on 3.2×10^{-2} M NH_4NO_3; the rosettes were then harvested and extracted. The extraction buffer consisted of 0.1 M K-phosphate buffer (pH 7), 1 mM EDTA and 10 µM FAD. One unit of cytochrome-*c* reductase activity is defined as the amount of enzyme catalyzing the reduction of 1 nmole cytochrome-*c* in 5 min at 0 °C (in melting ice). Protein was determined according to Lowry et al. (1951) and the amount of nitrate according to Ranney and Barlett (1972).

Polyacrylamide gel electrophoresis was carried out according to Ingle (1968); the gels were incubated in a solution containing 3 ml 0.1 M potassium phosphate buffer of pH 6.5, 3 ml 0.1 M KNO3, 1.5 ml 0.5% (w/v) sulphanilamide, 1.5 ml 0.02% (w/v) N-(1-naphthyl)ethylene diamine dihydrochloride and 1 mg NADH; after incubation for 30 min at 28 °C the gels were transferred into 1N HCl, after which the position of nitrate reductase became visible by the red azo dye formed under these conditions.

Results

The Isolation of Revertants of B25

The chlorate-resistant mutant B25 grows very poorly on a medium with nitrate as the sole source of nitrogen. On this medium the plantlets are yellowish, the cotyledons and the first rosette leaves remain very small, and the development of the plants stops at this stage, whereas the wildtype plants are green and grow well. Therefore, a simple criterion can be used for the selection of reverse mutants: healthy green plants on a nitrate medium.

A mutagenic treatment with EMS was given to seeds of B25. Since dominant revertants could be expected, M1 plants were screened; however, among ca 30,000 seedlings no green plants or chimera's could be found. In order to find recessive mutants M1 plants growing on an ammonium nitrate medium (on which both B25 and wildtype grow well) were transplanted into soil and grown to seedset. About 1,000 M2 progenies were screened on the nitrate medium; 7 independently arisen revertants were isolated with isolation number B25R1, B25R2, . . ., B25R7.

Considering that probably 3 meristem cells in the mutagenized seeds will produce flowers and seeds (Müller 1965; Van der Veen and Gerlach 1965; Balkema 1971) and that every cell contains 2 sets of chromosomes that can give rise to a reversion, the reversion frequency is 1.2×10^{-3}.

General Characterization of the Revertants

Eventually, homogenous revertant lines consisting of strongly growing green plants were obtained from all 7 revertants. With some lines, especially B25R7, it took several generations to accomplish purity, suggesting that at least some revertant lines carried a dominant reverse mutation of which the homozygous type might have a lowered viability.

All revertant lines appeared to be as chlorate sensitive as the wildtype. Thus two different criteria can be used to distinguish the revertant (wildtype) from the B25 phenotype: 1. growth on the nitrate medium 2. effect of chlorate on the ammonium nitrate medium. Though the former holds true for all 7 revertants, there are morphological differences between them. While most revertants give rise to plants that are hardly distinguishable from wildtype plants, plantlets of B25R2 and B25R4 are smaller, the latter having narrow leaves.

Crosses of revertants × wildtype and reciprocal yielded in all cases an F1 with a wildtype phenotype, as could be expected with parents of this type. In the F2, however, in all cases some plants with the B25 phenotype appeared, indicating that the revertants arose by a mutation in a gene different from *rgn*, the mutated gene of B25. The second mutations thus happen to be suppressor mutations. Testing the F1's of crosses revertant × B25 (Table 1) revealed the remarkable fact that the F1 can have the B25 phenotype for both criteria (for B25R3 and B25R4), or the revertant phenotype for both criteria (for B25R2 and B25R7), or the revertant phenotype for criterion 1 and the B25 phenotype for criterion 2 (as for B25R1, B25R5 and B25R6). Therefore, it can be concluded that the suppressors in B25R3 and B25R4 are recessive, while those in B25R2 and B25R7 are dominant but that the suppressors in B25R1, B25R5 and B25R6 are either dominant or recessive depending on whether criterion 1 (growth on nitrate) or criterion 2 (effect of chlorate) is applied.

Complementation Tests Between Revertants

All possible crosses between revertants were made. All F1's appeared to be strongly growing on the nitrate medium and chlorate sensitive, so that for both criteria the F1's exhibit the revertant phenotype. Since the suppressors from B25R1, B25R3, B25R4, B25R5 and

Table 1. Growth of the F1's B25 × revertant for 2 criteria and thus dominance or recessiveness of the corresponding suppressors

Revertant line	Properties of the F1 from the cross revertant × B25 and reciprocal		Dominance/recessiveness of the suppressors with respect to the wildtype allele	
	Growth on nitrate (criterion 1)	Effect of chlorate (criterion 2)	According to criterion 1	According to criterion 2
B25R1	good [a]	resistant	dominant	recessive
B25R2	good	sensitive	dominant	dominant
B25R3	poor [b]	resistant	recessive	recessive
B25R4	poor	resistant	recessive	recessive
B25R5	good	resistant	dominant	recessive
B25R6	good	resistant	dominant	recessive
B25R7	good	sensitive	dominant	dominant

[a] Like wildtype; [b] Like B25

B25R6 appeared to be recessive for at least criterion 2 it can be concluded that they all belong to the same complementation-group. Thus the suppressor mutations of these revertants are mutations in the same suppressor-gene, designated *su*. The suppressors are thus su^1, su^3, su^4, su^5, and su^6. Concerning B25R2 and B25R7 no conclusion can be drawn since their suppressors are dominant for both criteria.

Possible Linkage Relationship Between su and the Suppressors from B25R2 and B25R7

Since it could not be determined by means of complementation tests whether the suppressor from B25R2 and B25R7 (tentatively designated su^2 and su^7) also belonged to the gene *su* or not, we tested whether these suppressor mutations were tightly linked to each other and to *su*.

The crosses B25R2 × B25R7, B25R2 × B25R5 and B25R5 × B25R7 were made. These F1's were of the wildtype phenotype as was expected since at least one of the parents was carrying a dominant suppressor. When these experiments were being carried out it was known that *su* and *rgn* are not linked. The F1 from the cross B25R2 × B25R7 will therefore be *rgn/rgn*, su^2/su^7 when su^2 and su^7 belong to the same gene or *rgn/rgn*, $su^2+/+su^7$ when they do not belong to the same gene. If, in the latter case, recombination between suppressors occurs, gametes of the type *rgn*, + + will arise, giving rise, eventually, to F3 families in which either all or one quarter of the plants are of the B25 phenotype.

From each of the above mentioned crosses about 400 F3 families were tested, but in none of the families B25 phenotypes could be discovered. This indicates that su^2 and su^7 are tightly linked to each other and to the gene *su* with distances smaller than 0.25 map units. In view of these results and considering the existence of suppressor alleles which are either recessive or dominant according to the criterion applied, the most obvious conclusion is that all suppressor mutations belong to the same gene. Therefore, the designations su^2 and su^7 for the suppressors from B25R2 and B25R7 are maintained.

Table 2. Segregation in the F2 of the crosses B25R1 and B25R4 × wildtype for 2 different criteria

Revertant line	Test	Total number of plants	Number of plants with the B25-phenotype	Number of plants with the wildtype phenotype	P (1/16 B25)	P (3/16 B25)
B25R1	criterion 1 (growth on nitrate medium)	305	20	285	$0.75 < P < 0.9$	$P \ll 0.001$
	criterion 2 (reaction to chlorate)	254	53	201	$P \ll 0.001$	$0.25 < P < 0.5$
B25R4	criterion 1 (growth on nitrate medium)	331	56	275	$P \ll 0.001$	$0.25 < P < 0.5$
	criterion 2 (reaction to chlorate)	329	60	269	$P \ll 0.001$	$0.75 < P < 0.9$

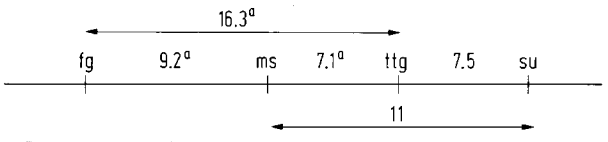
Linkage Studies Between su and rgn

Since in the F2's of the crosses revertants × wildtype, plants with the B25 phenotype appeared (see above) it could be concluded that *su* and *rgn* were not tightly linked, but further studies had to be conducted in order to reveal whether both genes showed linkage or independent segregation. In the case of independent segregation for *su* and *rgn* we expected 1/16 B25 phenotypes for a dominant suppressor and 3/16 for a recessive one in the F2 of the crosses revertant × wildtype. All data concerning crosses where linkage between *rgn* and *su* could have been established are in accordance with the hypothesis that *su* and *rgn* segregate independently with a probability >0.25. As an example, in Table 2 data are given from the crosses B25R1 and B25R4 × wildtype. These data also demonstrate the above mentioned remarkable fact that the suppressor gene from B25R1 behaves as a dominant gene when tested for growth on the nitrate medium (criterion 1) and as a recessive gene when tested for chlorate resistance (criterion 2).

Localization of su

Results from a cross between a revertant line and a line segregating for male sterility (*ms*-1, Koornneef and Van der Veen 1978) suggested a rather close linkage between *su* and *ms* (ca 11% recombination).

In order to localize *su* more accurately, a line of Koornneef was used, carrying the marker *ttg* (transparent testa and hairless) and *fg* (late flowering), both of which are on chromosome 5 and show linkage with *ms*-1 (Koornneef and Van der Veen 1978; Koornneef pers. comm.). From the F2 of the cross revertant × marker line (*rgn/rgn, su³/su³, fg⁺ttg⁺/fg⁺ttg⁺ × rgn⁺/rgn⁺, su⁺/su⁺, fg ttg/fg ttg*) only plants with a B25 phenotype were transplanted and grown to seedset. This was done by growing the F2 on nitrate medium, removing the green plants and then adding 5 ml of a solution containing 2 g/l $(NH_4)_2SO_4$ in order to rescue the plants with poor growth on nitrate. The genotype of the harvested F2 plants was therefore *rgn/rgn; su⁺/-*. The genotype for *su* and *ttg* could be determined from the phenotypes occurring in the F3. Close linkage was estimated between *ttg* and *su*, since recombinants occurred in only 35 of the 251 tested F3 families. Therefore the distance between *ttg* and *su* is estimated as 7.5±1.2 M.U. (Koornneef et al. 1980a). The majority (14 from a total of 16) of recombinations between *ttg* and *su* did not result in recombination between *ttg* and *fg* so that it can be concluded that *su* is located on the other side of *ttg* than *fg* (linkage between *ttg* and *fg* is about 16.3 M.U., Koornneef, pers. comm.). All data are in accordance with the map of Fig. 1.



[a] Data, corrected according to Kosambi, by Koornneef, (pers. comm.)

Fig. 1. Map of part of chromosome 5

The Construction of an su/su, rgn⁺/rgn⁺ Line

This could only be performed in an indirect way, since it was expected that the effect of *su* in a wildtype background would not be visible. The cross (revertant × wildtype) × wildtype yields the following genotypes: *rgn/rgn⁺, su/su⁺*(A); *rgn/rgn⁺, su⁺/su⁺*(B), *rgn⁺/rgn⁺, su/su⁺*(C), *rgn⁺/rgn⁺, su⁺/su⁺*(D). All genotypes have the wildtype phenotype, but in the selfed progenies of A and B some B25 phenotypes can be found that are absent in the progenies of C and D. In the progenies of type C, plants of the wanted genotype (*rgn⁺/rgn⁺, su/su*) are present, but they cannot be distinguished from the other genotypes found in the progenies of C and D. Therefore, the genotype of several plants from progenies in which no B25 phenotype turned up was further investigated by crossing the plants with the original revertant. The tested plant was of the required genotype when no B25 genotypes appeared in the F2. Selfed seeds of these plants yielded suppressor lines, cleared from the B25 background. Such lines were constructed for su^1 and su^3. The presence of the suppressor genes in these lines was tested as follows.

The crosses B25 × suppressor lines were made. From the F2 the chlorate resistant plants were transplanted and grown to seedset. Since it is known that *su* and *rgn* are not linked, it can be expected that the genotypes of the chlorate resistant F2 plants are *rgn/rgn, su/su⁺* and *rgn/rgn, su⁺/su⁺* with a ratio of 2:1. Thus it is expected that 2/3 of the progenies of these chlorate resistant F2 plants will segregate revertant phenotypes. Some progenies did segregate revertant phenotypes for chlorate resistance, both in the case of su_1 and of su_3, although the number of segregating progenies was lower than expected, whereas the ratio of revertant type to B25 type plants in the segregating families approached 3:1 rather than 1:3. This suggests that the suppressor gene behaves no longer as a clearly recessive gene but in some cases is dominant. However, in the chlorate resistant plants in the F2 (genotypically *rgn/rgn; su/su⁺*) the allele *su* must still have acted as a recessive gene, otherwise plants with this genotype would not have been chlorate resistant. Notwithstanding these rather unexpected change of dominance relationships, this experiment proves that the isolated suppressor lines do contain the suppressor, though the

Table 3. Relative in vitro nitrate and nitrite reductase activities, nitrate content per mg of protein, and in vivo nitrate reductase activity, per g fresh weight of plants of wildtype, B25 and revertants, grown on different media.

Line	Ammonium nitrate medium				Ammonium medium		Nitrate medium		
	in vitro nitrate reductase	Nitrite reductase	Nitrate content	in vivo nitrate reductase	in vitro nitrate reductase	Nitrite reductase	in vitro nitrate reductase	Nitrite reductase	Nitrate content
Wildtpye	1 [a]	1 [b]	1 [c]	1 [d]	0.10	0.07	1.5	1.5	0.61
B25	0.03	3.6	1.6	0.26	0.02	0.28	0.04	3.5	3.0
B25R1	0.38	1.3	0.89	0.84	0.07	0.14	0.57	1.8	0.49
B25R2	2.0	1.0	0.36	2.1	0.37	0.12	1.6	1.2	0.15
B25R3	1.3	0.93	0.75	1.3	0.37	0.19	2.1	1.5	0.62
B25R4	2.1	0.95	0.59	2.3	0.17	0.17	2.3	1.3	0.34
B25R5	0.45	1.1	0.88	0.88	0.14	0.15	1.5	1.7	1.2
B25R6	1.3	1.0	0.69	1.1	0.16	0.12	1.6	1.3	0.36
B25R7	1.5	1.1	0.63	1.1	0.12	0.13	1.7	1.3	0.89

[a] 108.4 units/mg protein; [b] 157.9 units/mg protein; [c] 3.12 μ-equiv/mg protein; [d] 174.9 units/g fr.w

lines are phenotypically indistinguishable from the wildtype.

Nitrate Reduction in the Revertants

Since all revertants behaved as the wildtype for both criteria (growth on the nitrate medium and reaction to chlorate) it was interesting to test whether the extremely low activity of nitrate reductase from B25 had also changed into the wildtype level of activity. Table 3 shows the results of a typical experiment where the nitrate and nitrite reductase activities and nitrate content of rosettes from wildtype, B25 and revertants grown on different media are given relative to those of wildtype grown on the ammonium nitrate medium. It is shown that the extremely low in vitro nitrate reductase activity, the high nitrite reductase activity and the high nitrate content, which B25 exhibits, have changed in the revertants towards the values of the wildtype or even beyond. For instance, while B25 on the ammonium nitrate medium exhibits 0.03 of the wildtype in vitro nitrate reductase activity, the revertants exhibit 0.38 to 2.1 of this activity. Concerning the in vivo nitrate reductase activity it is marked that the activity (per g of fresh weight) of B25R2 and B25R4 is very high, 2.1 and 2.3 of the wildtype activity. However, plants of these revertants are much smaller than wildtype plants. If the in vivo nitrate reductase activity is calculated per plant, all revertants exhibit 0.8 to 1.1 of the activity of the wildtype. The differences between revertants are rather pronounced. However, the results do not permit the classification of revertants into three groups, which correspond to the classification according to dominance relationships: 1. dominant for both criteria; 2. recessive for both criteria; 3. dominant concerning growth on nitrate and recessive concerning reaction to chlorate. In order to investigate whether the level of nitrate reductase in the F1 was responsible for dominance or recessiveness, from every group a revertant and the F1 of the cross revertant × B25 were tested for nitrate reductase activity with the usual in vitro method as well as with the in vivo assay.

Table 4. Relative in vitro and in vivo nitrate reductase activity of some parental lines and the F1's

Genotype	Relative nitrate reductase activity per gram fresh weight	
	in vitro [a]	in vivo [b]
Wildtype	1 [c]	1 [d]
B25	0.05	0.31
B25R1	0.85	0.87
B25R3	1.5	1.6
B25R7	1.3	1.6
F1 B25R1 × B25	0.13	0.42
F1 B25R3 × B25	0.20	0.33
F1 B25R7 × B25	0.64	0.70

[a] Means of 2 experiments; [b] Means of 4 experiments; [c] 282.1 units/g fr.w; [d] 158.3 units/g fr.w

The results in Table 4 are in agreement with the dominance of su^7 since the F1 of the cross B25R7 × B25 exhibits sufficient nitrate reductase activity for good growth on a nitrate medium and for chlorate sensitivity. The difference between su^1 and su^3 (groups 3 and 2, respectively) is not quite clear and will be discussed below.

Physiological Characteristics of Nitrate Reductase

The Km of nitrate reductase depends on growth conditions of the *Arabidopsis* plant material and on purifica-

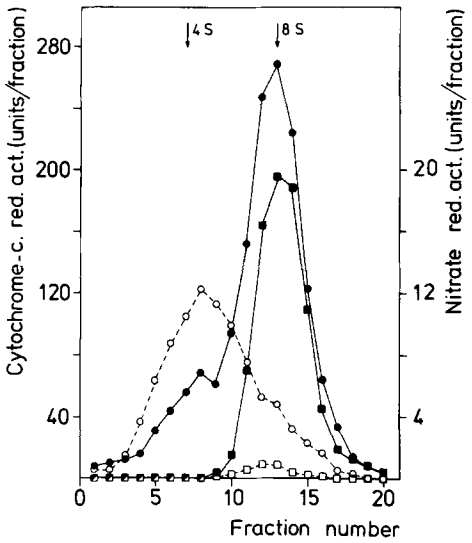


Fig. 2. Sucrose gradient fractionation of cytochrome-*c* and nitrate reductase from extracts of wildtype and B 25 plants which were grown under "high level conditions" (see Materials and Methods). Cytochrome-*c* reductase, ●—● wildtype, ○---○ B 25; nitrate reductase: ■—■ wildtype, □---□ B 25. The amount 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 2049 units and that of nitrate reductase activity 112.24 units. The activities in the applied B 25 extract were 693 and 3.97 units, respectively, while the sum of the activities found in the fractions was 1202 and 4.89 units, respectively

tion of the crude extracts. However, under comparable circumstances the Km is constant. Since we were interested to know whether the characteristic features of the enzyme nitrate reductase of the revertants had altered compared to those of the wildtype, the Km's of nitrate reductase from the wildtype, B25R1, B25R2, B25R3, B25R4 and B25R5 in crude extracts of plants grown on solid ammonium nitrate medium were determined. In all cases the apparent Km (NO_3^-) was 0.67 mM and the Km (NADH) was 24 mM. Therefore, these results are an indication that the features of nitrate reductase from the revertants are not altered compared to the wildtype. More support for this statement is found in experiments concerning thermostability and electrophoretic mobility. Thermostability of nitrate reductase from the revertants is comparable to that from the wildtype, in all cases only half of the starting activity remains after 3–4 min at 37 °C. Polyacrylamide gel electrophoresis showed that nitrate reductase from the wildtype and from the revertants had the same electrophoretic mobility. In all experiments one band of nitrate reductase activity could be discovered at the same distance from the origin, whether one used extracts of the wildtype or the revertants or a mixture of wildtype extract with one of the revertant extracts.

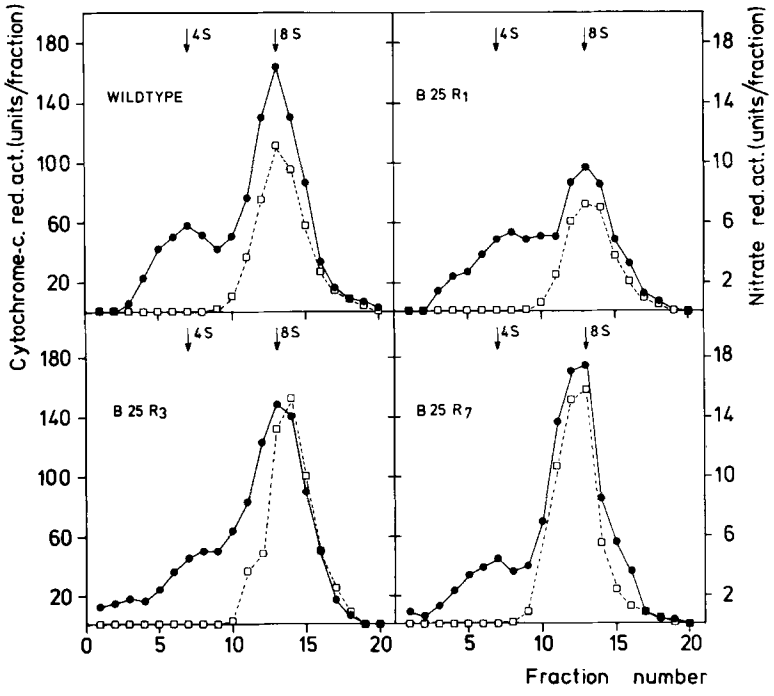


Fig. 3. Sucrose gradient fractionation of cytochrome-*c* and nitrate reductase from extracts of plants which were grown under "high level conditions"(see Materials and Methods). Cytochrome-*c* reductase, ●—●; nitrate reductase, □---□. Extracts from wildtype, B25R1, B25R3 and B25R7, applied to the gradient, contained cytochrome-*c* reductase activities of 615, 465, 720 and 786 units, respectively. The sums of the cytochrome-*c* reductase activities found in the fractions were 1307, 956, 1381 and 1318 units, respectively. The nitrate reductase activities in the applied extracts from the lines were 36.62, 24.64, 48.03 and 48.81 units, respectively. The sums of the nitrate reductase activities found in the fractions were 60.19, 42.16, 76.91 and 77.83 units, respectively

From these results it can be concluded that these characteristic features of nitrate reductase are alike for the revertants and the wildtype.

Cytochrome-c Reductase Activity

Wildtype and B25 showed a remarkable difference in the cytochrome-*c* reductase elution pattern on a sucrose gradient (Braaksma and Feenstra in press), which is also shown in Fig. 2. Under induced conditions, a high 8s peak of nitrate reductase activity is found in wildtype extracts. Most of the cytochrome-*c* reductase also sediments at the 8s position, but a minor fraction sediments as 4s type. All cytochrome-*c* reductase from B25 extracts, however, sediments at the 4s position, while no nitrate reductase is present. In Figure 3 it is shown that the revertants exhibit an elution pattern comparable to that of the wildtype. In this experiment the extracts of B25R1, B25R3 and B25R7 exhibited 0.76, 1.2 and 1.3 respectively of the cytochrome-*c* reductase activity and 0.67, 1.3 and 1.3 respectively of the nitrate reductase activity of the wildtype.

The Effect of the Suppressor on Other Chlorate Resistant Mutants

Other mutants could be tested for susceptibility to the suppressing effect of su_1 and su_3 by a procedure similar to the method by which the suppressor lines were tested for the presence of these genes. These other mutants which were tested for the effect of the suppressor are B1 and B2-1. The mutant B1 carries the gene *chl*-1, which is responsible for a lowered uptake of chlorate (Oostindiër-Braaksma and Feenstra 1973a; Doddema et al. 1978) and which is located on chromosome 1 and thus unlinked with *su* on chromosome 5. The mutant B2-1 carries the gene *chl*-2, resulting in a lowered nitrate reductase activity (Oostindiër-Braaksma and Feenstra 1973a). Since *chl*-2 is located on chromosome 2, it is unlinked with *su*. On these mutants, however, the suppressors appeared to have no suppressing effect, since all progenies of chlorate resistant F2 plants were homogenously chlorate resistant. It can therefore be concluded that neither su^1 nor su^3 has effect on the genes *chl*-1 and *chl*-2.

Discussion

It was easy to isolate reverse mutants from B25 in the M2, and these comprised both recessive and dominant mutants for the selection criterion used (green growing plants on the nitrate medium). However, in the M1 no revertants could be discovered, though many dominant reverse mutations must have been present. The M1 plantlets have a chimeric structure (Balkema 1971) which may be the reason why the reversion is not expressed in the M1 but is easily found in the M2. Revertants from several pyrimidineless mutants of *Arabidopsis* (which are dominant) could be found in the M1 and the chimerical structure could be established without doubt (Heyting and Feenstra 1966; Van den Berg et al. 1967). However, Koornneef et al. (1980b) had to isolate revertants of non-germinating gibberellin sensitive lines in the M2, since they could not be found in the M1. These revertants were recessive. Somerville and Ogren (1980) also isolated in the M2 recessive revertants of chlorophyll mutants. In the M1 potential dominant mutations are present in only a minor part of the plantlet in either roots or shoot. For the pyrimidineless mutants it is apparently enough that part of the shoot is able to synthesize thiamine to let the plant grow. With B25 it appears that a dominant suppressor in only part of the M1 seedling does not result in growth on nitrate medium. All investigated revertants of B25 appeared to be mutated in a suppressor gene *su*, which is unlinked with the original mutation (*rgn*) of B25. The mutation frequency for *su*, 1.2×10^{-3}, is relatively high compared to other genes. For instance the mutation to *rgn* was found only once when screening 35,000 M2 families (Braaksma and Feenstra 1975a).

The suppressor mutation of a revertant can be recessive (su^3 and su^4), dominant (su^2 and su^1) or dominant for criterion 1 (growth on nitrate) and recessive for criterion 2 (effect of chlorate) as is found for su^1, su^5 and su^6. There seems to be no connection between dominance or recessiveness of the suppressor and the level of in vitro or in vivo nitrate reductase activity of the corresponding revertant. For example B25R4 (recessive suppressor) exhibits higher activities than the revertants with a dominant suppressor, while B25R1 and B25R5 (suppressor dominant or recessive depending on the criterion used) exhibit low activities (Table 3). This is again shown in Table 4. Though in the revertant lines this connection cannot be found, in the F1's revertant × B25 the activities (Table 4) are fairly in agreement with the dominance or recessiveness of the corresponding suppressors. The F1 of the cross B25R7 × B25 has the wildtype phenotype, apparently because the level of nitrate reductase is approximately 70% of the wildtype. The F1's of the crosses B25R1 and B25R3 × B25 exhibit so little nitrate reductase activity that they are chlorate resistant. Though this holds true for both crosses, growth on the nitrate medium is different for both crosses since growth of the F1 from B25R1 × B25 is good and and from B25R3 × B25 is poor (Table 1). The in vitro nitrate reductase assay gives the best indication of the actual amount of enzyme activity, and therefore 0.13 and 0.20 of the

wildtype activity is apparently low enough to allow chlorate resistance. Probably (see also Braaksma and Feenstra in press) the in vivo activity is partly due to peroxidases, which may also play a substantial role in growth on a nitrate medium and which may not reduce chlorate. This may account for the F1 from B25R1 × B25 exhibiting a somewhat higher in vivo activity though a lower in vitro activity compared with the F1 from B25R3 × B25. It is remarkable, however, that such a small difference in nitrate reduction activity can affect a clear phenotypic difference. The fact that no revertants have been found, which were recessive for growth on nitrate and dominant for the effect of chlorate, indicates that a lower level of nitrate reductase activity is sufficient for good growth on nitrate than for sensitivity to chlorate. The observation that the suppressor can become dominant after being recessive for several generations is remarkable. Possibly relatively slight differences in enzyme activity can effect such a change, though it is not clear how this effect is obtained.

In addition to the change of *su*^{1} and *su*^{3} from recessiveness to dominance, which also was found in some other experiments, a few more remarkable observations were made. Firstly, the fact that for several generations B25R7 could not be found as homozygous line, which indicates that during the first generations after selection B25R7 had a low viability as homozygous plant. After the isolation of the homozygous line, however, no growth problems were noticed. Secondly, when B25R3, a strongly growing revertant, was tested for nitrate reductase activity for the first time, the level of activity was a factor of 3 higher than in the wildtype. When progenies from sister plants of this line were tested, such a high level was not found anymore. All these observations show that the extreme features in the revertants tend to become more like those of the wildtype after some generations. This is the case for the change from recessiveness to dominance of some suppressors, for the viability of homozygous B25R7 plants and for the nitrate reductase activity of B25R3. The shift from recessiveness towards dominance has consequences for the reliability of the method by which the effect of the suppressors on other mutant genes was tested. However, the crosses with the suppressor lines made for the latter purpose were made simultaneously with the crosses made for testing the presence of *su*^{1} and *su*^{3} in the suppressor lines free from the B 25 background, for which sister plants were used. Since in the latter case sufficient plants were found in which the genes still behaved as recessives, the conclusion that the suppressors do not act on genes *chl*-1 and *chl*-2 seems justified.

For nitrate reductase of the wildtype and the revertants, Km, thermostability and electrophoretic mobility were similar. Though Stulen (pers. comm.) and Ingle (1968) report that after electrophoresis of extracts from radish cotyledons two clearly separated bands with nitrate reductase activity could be detected, in our experiments with *Arabidopsis* only one band could be discovered. Since the same Km values and the same thermostability were obtained and the same electrophoretic mobility was observed for nitrate reductase from the wildtype and the revertants, it can be concluded that the same enzyme is synthesized in these genotypes. Moreover, the wildtype elution pattern of cytochrome-*c* reductase on a sucrose gradient has appeared in the revertants, while the elution pattern in B25 is different. If the structural gene was mutated in the original mutant, it is improbable that a suppressor gene, of which different mutations can result in recessiveness or dominance and in different levels of enzyme activities, in all revertants brings about the same enzyme features as were present in the wildtype. Therefore, it is concluded that the structural gene for nitrate reductase is not involved in the mutation of *rgn*. Based on the same arguments it seems highly improbable that the gene *su* is the structural gene for nitrate reductase.

Mendel and Müller (1979) adhere also for higher plants to the model of MacDonald et al. (1974) that nitrate reductase is an assembly of two molecules of cytochrome-*c* reductase kept together by the Mo-containing co-factor which also is necessary for the activity of nitrate reductase. This assembly is formed under "high level conditions" in the wildtype and not in the mutant B25. The simpliest explanation for the lesion in B25 would be a defective co-factor as in the *cnx* mutants in fungi (Pateman et al. 1964; see Coddington 1976) and tobacco cell cultures (Mendel and Müller 1976, 1979). The *cnx* mutants lack xanthine dehydrogenase as well as nitrate reductase and are supposed to lack a functional Mo-containing cofactor, which is shared by both enzymes. B25 has lost almost all activity of xanthine dehydrogenase after 7 days from germination whereas the activity of the wildtype is still present at this age (preliminary results). It is therefore tempting to conclude that the mutation of B25 is of the *cnx*-type.

If this is the case the effect of the mutations of *su* must be a restored co-factor. To our knowledge no suppressor genes which restore enzyme activities in higher plants have been reported by other authors. The mutations of *su* appear not to affect the chlorate resistance genes *chl*-1 and *chl*-2. Further investigations will be made in order to learn more about the nature of this suppressor.

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Dr. F. J. Braaksma
Dr. W. J. Feenstra
Department of Genetics
Biology Center
Kerklaan 30,
9751 NN Haren (the Netherlands)