Theor. Appl. Genet. 58, 39-42 (1980)

THEORETICAL AND APPLIED GENETICS

Isolation of a Nitrate Reductase Deficient Mutant of *Pisum sativum* by Means of Selection for Chlorate Resistance

W.J. Feenstra and E. Jacobsen
Department of Genetics, Centre of Biological Sciences, University of Groningen, Haren (the Netherlands)

Summary. After EMS treatment of seeds of the *Pisum* variety 'Rondo' a chlorate resistant mutant was isolated which showed a decrease in the in vitro activity of the enzyme nitrate reductase of roughly 95%. The mutation is monogenic and recessive. The mutant shows a decrease in protein content, and an increase in the amount of nitrate accumulated and in the activity of the enzyme nitrite reductase. On a liquid nutrient medium containing nitrate as the sole nitrogen source and in soil, the mutant grows very poorly due to necrosis of the leaves. On liquid medium containing ammonium, either with or without nitrate, growth is as good as that of the parent variety.

Key words: Pea – Chlorate resistance – Nitrate reductase deficient – Mutant

Introduction

Genetic control of nitrate reduction can be studied in higher plants. Spontaneous genetic variation in nitrate reductase (NaR) activity has been detected in *Zea mays* (Warner et al. 1969) and wheat (Duffield et al. 1972).

A number of artificially induced NaR deficient mutants have been obtained of *Arabidopsis thaliana* (Oostindiër-Braaksma and Feenstra 1973, 1975a) by selection for chlorate resistance. *Arabidopsis* was chosen for the selection of biochemical mutants because of its small plant size and short life cycle. Selection for chlorate resistance was also employed in cell cultures for the isolation of NaR less mutants of *Nicotiana tabacum* (Müller and Grafe 1978).

Kleinhofs et al. (1978) isolated NaR deficient mutants of pea (*Pisum sativum*) by direct selection for the absence of in vivo NaR activity. In the present study we show that a NaR deficient mutant of pea can also be obtained by selection for chlorate resistance.

Material and Methods

a. Growth conditions

For mutagenic treatment the variety 'Rondo' was used. M2 and F2 generations were grown in a temperature controlled glass-house at 25°C unless indicated otherwise, with high humidity and a day-length of at least 16 hours. M1 and F1 generations were germinated in soil in pots in the glass-house for two weeks and then transferred to the field. From each M1 plant a few pods were harvested.

b. Mutagenic Treatment

Series 1: 800 g of dry seeds (± 2800 peas) in portions of 200 g were soaked in tapwater for 8 hours at 12°C, after the seed coat had been damaged with sandpaper. Each portion of soaked seeds was submerged in 800 ml of an ethylmethane sulphonate (EMS) solution (0.3% EMS in 0.01 M phosphate buffer pH 5,7) in a 2 l beaker under continuous aeration and occasional stirring for 4 hours at 20°C. After EMS treatment seeds were rinsed in running tapwater for one hour. M1 plants were individually harvested.

Series 2: Seed coats were not damaged. Portions of 200 g of seeds were soaked and then submerged in 500 ml of a 0.3% unbuffered EMS-solution in a 2 l erlenmeyer flask and shaken for two hours. The solution was then replaced by 500 ml of a fresh EMS-solution of the same concentration and the treatment was continued for another two hours. Further treatment was identical to series 1; only completely swollen seeds were planted.

c. Selection for Chlorate Resistance

Twelve M2 seeds per family were germinated in vermiculite moistened with tapwater. After 7 days cotyledons were removed and 7 families with maximally 7 seedlings per family were transferred to a seeding-pan (22,5 × 22,5 × 5,5 cm) containing vermiculite moistened with 200 ml standard mineral solution (SMS) of the following composition (mg/l): KH_2PO_4 1160; K_2HPO_4 480; $MgSO_4.7H_2O$ 1000; $CaCO_3$ 340; trace elements according to Langridge (1955) but in a twofold concentration. Per litre SMS were added: NH_4NO_3 500 mg and KNO_3 400 mg. Two days later 200 ml NO_3^- solution containing KNO_3 (808 mg/l) and $Mg(NO_3)_2 \cdot 6H_2O$ (512 mg/l) were administered; this was followed 3 days

0040-5752/80/0058/0039/$ 1.00

later by 200 ml sodium chlorate solution (2.13 g/1). Seeding-pans were covered by PVC foil to minimize evaporation. Five days after the addition of chlorate 'Rondo' control plants showed severe chlorate damage and M2 seedlings were screened for chlorate resistance. Presumptive mutants were transplanted in soil, irrigated with SMS.

d. Determination of Nitrate Reductase Activity

After germination in vermiculite, seedlings were transferred to soil. Sixteen days after sowing, SMS + NH_4NO_3 (500 mg/1) and KNO_3 (400 mg/1) was administered to the seedlings in order to induce NaR activity. Four days later NaR activity in vitro was determined according to Sanderson and Cocking (1964) using young grown leaves, and in vivo according to Jaworski (1971).

e. Determination of Protein and Nitrate Content

Protein concentration in the young fully grown leaves was determined by the procedure of Lowry et al. (1951). Casein was used as a standard. Nitrate was determined according to the brucine method of Ranney and Barlett (1972).

Results

Table 1 gives data on germination and fertility in M1 and the percentage of M2-families showing one or more chlorophyll mutants among 12 seedlings. Damaging the seed coat had a positive effect on the swelling of seeds. Germination frequency of seeds after EMS-treatment, however, was lower in series 1 than in series 2. M1 fertility and M2 mutant frequency did not differ much. Almost all M2-progenies segregating for chlorophyll mutants showed only one mutant among 12 seedlings tested.

Series 1 contained one M2-family segregating for chlorate resistance (Table 1). Of 14 seedlings in this family which could be tested, only one seedling was resistant. This seedling was tested for in vitro NaR activity and turned out to be NaR deficient. Unfortunately its growth (in soil) was very poor. When the plants were young the oldest leaves, when fully expanded, became necrotic, starting at the margin. Consequently the mutant flowered very poorly and produced only a few seeds. It could be crossed, however, with 'Rondo' and yielded a few selfed and some F1 seeds. Growth and seed production of the F1 were not different from those of 'Rondo'. In the F2, seedlings were tested for in vivo NaR activity. Two groups of plants could be distinguished: 1) a group with low activity (0.1-0.4; average 0.2 μ mol NO_2^-/g fresh weight h), and 2) a group with high activity (0.7-1.2; average 0.9 μ mol NO_2^-/g fresh weight/h). An in vitro NaR activity test carried out simultaneously showed that plants of the low in vivo NaR activity group had activities comparable to the original mutant. Segregation was tested both for the in vivo NaR activity and for chlorate resistance (Table 2). In both ways the mutant proved to be monogenic and recessive. At the same time the F2 showed segregation for a chlorophyll deficiency (light green leaf color) and 2 different growth type mutations. The inheritance of the chlorophyll mutation was also monogenic and recessive. The chlorophyll mutation showed recombination with the chlorate resistance. These characters are therefore not closely linked.

Over 150 F2-seedlings have been tested for recombination between NaR deficiency and necrosis visible after transfer into soil. Recombination could not be detected, indicating that necrosis and lack of NaR activity are probably pleiotrophic effects of the mutant gene. Some biochemical data were obtained with F3 mutant seedlings and were compared to those of F1 and 'Rondo' (Table 3).

Table 2. Analysis of the F2 of mutant X 'Rondo' segregating for chlorate resistance, in vivo NaR-activity and chlorophyll deficiency

Character	–	+	χ_1^2(3:1)	P
Chlorate resistant	36	5	3.58	0.10 – 0.05
'NaR-less' in vivo	62	21	0.04	0.90 – 0.80
Chlorophyll deficient	28	12	0.53	0.50 – 0.30

Table 1. Germination and fertility of M1 and chlorophyll mutation frequency and number of chlorate resistant mutants in M2

Series	Number of seeds treated	Number of seeds swollen	Number of M1-seeds germinated[a]	Number of fertile M1-plants	Percentage of M2-families with chlorophyll mutants	Number of M2-families[b] with chlorate resistant plants
1	2500	2500	708 (28.3%)[c]	633 (89.4%)[d]	3.1	1
2	2500	900	528 (58.7%)	457 (86.6%)	4.4	0

[a] All germinated seeds were planted out
[b] All M2-families available were tested
[c] Of swollen seeds
[d] Of germinated seeds

Table 3. Biochemical characterization of the chlorate resistant mutant determined with 2 F3-progeny seedlings compared with F1- and 'Rondo' seedlings

	NaR-activity[a]	NiR-activity[b]	protein content[c]	Nitrate content[d]
'Rondo'	2.2 ± 0.3	22.2 ± 9.6	3.6 ± 0.7	7.2 ± 2.4
F1	1.7 ± 0.05	19.3 ± 8.2	4.2 ± 0.5	2.7 ± 2.0
Mutant	0.084 ± 0.004	50.1 ± 0.3	2.9 ± 0.02	75.2 ± 27.2

[a] Assayed in vitro expressed as μ mol NO_2^- per g of fresh weight per hour
[b] Assayed in vitro expressed as μ mol NO_2^- per g of fresh weight per hour
[c] Expressed as 10 μg protein per g of fresh weight
[d] Expressed as μ mol NO_3^- per g of fresh weight

The in vitro NaR activity of the mutant is very low, whereas the nitrite reductase (NiR) activity and nitrate content are high. Protein content is somewhat lower than that of 'Rondo'. Differences between the F1 and 'Rondo' are small.

When mutant plants were grown at 25°C on a aerated liquid medium containing NH_4^+ as the nitrogen source necrosis was not observed and growth was almost indistinguishable from that of 'Rondo'. Both 'Rondo' and the mutant showed a more vigorous growth on NH_4NO_3 (SMS + 250 mg/1 NH_4NO_3) than on $(NH_4)_2SO_4$ (SMS + 195 mg/1 $(NH_4)_2SO_4$). When grown on the former solution mutant plants yielded a fair amount of seeds. On a solution lacking nitrogen completely (no special precautions were taken to avoid N-fixation by *Rhizobium*; no root nodules were observed) both 'Rondo' and the mutant showed poor growth, but without the characteristic necrosis symptoms. A solution containing NO_3^- as the sole nitrogen source (SMS + 400 mg/1 KNO_3 + 250 mg/1 $Mg(NO_3)_2.6H_2O$) induced severe necrosis of the mutant plants within three days, but had no harmful effect on 'Rondo'.

Discussion

Several important factors affecting the induction of mutations in pea by ethylmethane sulphonate have been studied by Blixt (1966a; b; c; 1967). However, no readily adoptable procedure for the mutagenic treatment of larger amounts of seeds has been described. The methods applied in the present study were based on the technical facilities available and chosen with the intention to keep the fertility of the M1 at a reasonable level. The variation in the treatments applied was introduced in order to spread risks and does not allow any conclusions as to the conditions to be preferred.

Chlorate resistance in higher plants can be brought about either by a decrease in the uptake of chlorate (Oostindiër-Braaksma and Feenstra 1973; Doddema et al. 1978) or by a reduction of the activity of the enzyme nitrate reductase (NaR) (Oostindiër-Braaksma and Feenstra 1973; Müller and Grafe 1978). The biochemical characterization shows that our pea mutant clearly belongs to the latter class, exhibiting a reduction of the in vitro NaR activity of more than 95%. The high nitrate content and low protein content of our mutant are according to expectation and in agreement with the properties of NaR deficient mutants of *Arabidopsis thaliana* (Oostindiër-Braaksma and Feenstra 1973; Braaksma and Feenstra 1975a) and barley (Warner et al. 1977; Kleinhofs et al. 1980). A high in vitro NiR activity is another common property, indicating that also in pea the induction of this enzyme is by nitrate rather than by nitrite.

The mutant, obtained in our experiments, although not completely inviable, showed a very poor growth after transplantation into soil, together with symptoms indicating the presence of some toxic substance. The typical necrosis symptoms do not develop when the mutant is grown on a liquid nutrient medium lacking nitrogen completely, but are readily evoked by growing the plants on a nutrient medium containing NO_3^- as the sole nitrogen source. This suggests that the necrosis is the result of the accumulation of NO_3^- in the plant.

NaR deficient mutants of *Arabidopsis*, when grown on a defined medium with NO_3^- as the nitrogen source show starvation symptoms but no necrosis (Oostindiër-Braaksma and Feenstra 1973; Braaksma and Feenstra 1975b). It can be assumed that also *Pisum* plants lacking NaR activity will be unable to grow with NO_3^- as the sole nitrogen source. The occurrence of necrosis, however, renders it impossible to observe this effect properly.

As might be expected, NH_4^+ sustains growth of the mutant and the parent cultivar, 'Rondo', equally well. Moreover, it turns out that the presence of NH_4^+ in the nutrient solution prevents the development of necrosis. This may suggest that the toxicity of nitrate becomes evident only when the plant cannot synthesize a sufficient amount of protein. On the other hand the presence of NH_4^+ may partly inhibit the uptake of NO_3^- (Ferguson

and Bollard 1969; Minotti et al. 1969; Doddema and Telkamp 1979).

The necrosis shown by mutant plants grown in soil is readily explained if it is assumed that the concentration of NO_3^- in soil exceeds that of NH_4^+ considerably. Analysis of the soil, used in our experiments revealed the presence of 9 mg N per kg soil as NH_4^+ and 178 mg N per kg soil as NO_3^- (unpublished results). Inability to grow in soil is not a generally occurring character of NaR deficient mutants, since such mutants of *Arabidopsis* (Oostindiër-Braaksma and Feenstra 1973) and barley (Warner et al. 1977; Kleinhofs et al. 1980) do grow well in soil, whereas their residual NaR activity is not appreciably higher, and in some cases, even lower than that of the *Pisum* mutant. The barley mutants, however, were obtained by screening for a low in vivo NaR activity, whereas chlorate resistant barley mutants proved lethal when transplanted into soil (Kleinhofs et al. 1978). Thus, lethality of NaR deficient mutants when transplanted into soil may be restricted to mutants of a certain type. Kleinhofs et al (1978) also isolated NaR deficient mutants of *Pisum* on the basis of low NaR activity, but do not report upon the viability of these mutants when grown in soil, nor on their residual NaR activity. Therefore, the question whether susceptibility to nitrate is a specific character of chlorate resistant NaR deficient pea mutants can only be answered after the isolation of further mutants. In the meantime we have adapted our screening procedure and now grow all suspected mutants on liquid culture.

Acknowledgement

We thank Misses B. Brus and T.H. Rausch and Mrs. A. Hamminga and H. Nijdam for excellent technical assistance. Seeds of variety 'Rondo' were kindly supplied by CeBeCo, Dronten.

Literature

Blixt, S. (1966a): Studies of induced mutations in peas. XV. Effect of environment of the X_1-generation on ethyl-methanesulphonate-treated and Gamma-irradiated Weitor-pea. Agri Hort. Genetica **24**, 62-74

Blixt, S., Ehrenberg, L., Gelin, O., (1966b): Studies of induced mutations in peas. XVI. Effect of duration of treatment with ethyleneimine and ethyl-methanesulfonate. Agri Hort. Genetica **24**, 111-127

Blixt, S., Ehrenberg, L., Gelin, O., (1966c): Studies of induced mutations in peas. XVIII. EMS-treatment of different duration of the varieties Weitor and Witham Wonder. Agri Hort. Genetica **24**, 138-146

Blixt, S., (1967): Studies of induced mutations in peas. XXI. Effect of hydrogen ion concentration on seed-treatments with EMS. Agri Hort. Genetica **25**, 112-120

Braaksma, F.J.; Feenstra, W.J. (1975a): Nitrate reduction in *Arabidopsis thaliana*. Arabidopsis Inf. Serv. **11**, 16-17

Braaksma, F.J.; Feenstra, W.J. (1975b): Revertants of the nitrate reductaseless mutant. B25. Arabidopsis Inf. Serv. **11**, 17

Doddema, H.; Hofstra, J.J.; Feenstra, W.J. (1978): Uptake of nitrate by mutants of *Arabidopsis thaliana*, disturbed in uptake or reduction of nitrate. I. Effect of nitrogen source during growth on uptake of nitrate and chlorate. Physiol. Plant. **43**, 343-350

Doddema, H.; Telkamp., G.P. (1979): Uptake of nitrate by mutants of *Arabidopsis thaliana* disturbed in uptake or reduction of nitrate. II. Kinetics. Physiol. Plant **45**, 332-338

Duffield, R.D.; Croy, L.J.; Smith, E.L. (1972): Inheritance of nitrate reductase activity, grain protein and straw protein in a hard red winter wheat cross. Agron. J. **64**, 249-251

Ferguson, A.R.; Bollard, E.G. (1969): Nitrogen metabolism of *Spirodela oligorrhiza* I. Utilization of ammonium, nitrate and nitrite. Planta *88*, 334-352

Jaworski, E.G. (1971): Nitrate reductase essay in intact plant tissues. Biochem. Biophys. Res. Comm. **43**, 1274-1279

Kleinhofs, A.; Warner, R.L.; Muehlbauer, F.J.; Nilan, R.A. (1978): Induction and selection of specific gene mutations in *Hordeum* and *Pisum*. Mutation Res. **51**, 29-35

Kleinhofs, A.; Kuo, T.; Warner, R.L. (1980): Characterization of nitrate reductase-deficient barley mutants. Molec. gen. Genet. **177**, 421-425

Langridge, J. (1955): Biochemical mutations in the Crucifer *Arabidopsis thaliana* (L) Heynh. Nature **176**, 260-261

Lowry, O.H.; Rosenbrough, N.J.; Farr, A.L.; Randall, R.J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265-275

Minotti, P.L.; Williams, D.C.; Jackson, W.A. (1969): The influence of ammonium on nitrate reduction in wheat seedlings. Planta **86**, 267-271

Müller, A.J.; Grafe, R. (1978): Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Molec. gen. Genet. **161**, 67-76

Oostindiër-Braaksma, F.J.; Feenstra, W.J. (1973): Isolation and characterization of chlorate-resistant mutants of *Arabidopsis thaliana*. Mutation Res. **19**, 175-185

Ranney, T.A.; Bartlett, R.J. (1972): Rapid field determination of nitrate in natural waters. Comm. Soil Sci. Plant Analysis **3**, 183-186

Sanderson, G.W.; Cocking, E.C. (1964): Enzymic assimilation of nitrate in tomato plants. I. Reduction of nitrate to nitrite. Plant Physiol. **39**, 416-422

Warner, R.L.; Hageman, R.H.; Dudley, J.W.; Lambert, R.J. (1969): Inheritance of nitrate reductase activity in *Zea mays* L., Proc. Natl. Acad. Sci. (Wash.) **62**, 785-792

Warner, R.L.; Lin, C.J.; Kleinhofs, A. (1977): Nitrate reductase-deficient mutants in barley. Nature **269**, 406-407

Received April 22, 1980
Communicated by H.F. Linskens

Prof. Dr. W.J. Feenstra
Dr. E. Jacobsen
Department of Genetics
Centre of Biological Sciences
University of Groningen
Kerklaan 30, 9751 NN Haren (the Netherlands)