ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 53, nr 4: 507-513
1984

Sources of reducing equivalents for nitrite reductase in *Pisum arvense* roots

GRAŻYNA KŁOBUS

Department of Plant Physiology, Institute of Botany, Wrocław University, Kanonia 6/8, 50-328 Wrocław, Poland

(Received: November 30, 1983. Accepted: May 10, 1984)

Abstract

Glucose-6-phosphate and $NADP^+$ as well as malic acid and $NADP^+$ present in the incubation mixture enhanced nitrite reductase (EC 1.6.6.4) activity in *Pisum arvense* roots. This was manifested by a depression of the nitrite level in the tissues and an increased reduction of nitrites by plastids isolated from *P. arvense* roots. A marked stimulation of plastid malate dehydrogenase was also observed under the influence of nitrates present in the medium. These results suggest that pyridin nucleotides utilised by NiR during nitrite reduction may be formed not only in processes of glucose-6-phosphate oxidation in the pentosephosphate cycle, but also of malic acid by $NADP^+$-dependent malate dehydrogenase.

Key words: nitrite reductase, reducing equivalents, Pisum arvense, roots

INTRODUCTION

The results of Canvin and Atkins (1974) indicate a strict dependence of nitrite reduction on light. Miflin (1974) and Wallsgrowe et al. (1979) demonstrated that the process of nitrite reduction to ammonia is localised in the chloroplasts, and that reduced ferredoxin formed during the light phase of photosynthesis is the physiological source of the reducing equivalent utilised in this process. It is known that assimilation of nitrites occurs in darkness as well (Jones and Sheard 1978, Mann et al. 1979), both in green and not green tissues. Sarkissian and Fowler (1974) noted a stimulating action of nitrates on a number of enzymes of the pentosephosphate pathway in cells of pea

---

Abbreviations used: NiR — nitrite reductase, MDH — malate dehydrogenase

roots. On this basis the authors advanced the supposition that carbohydrate oxidation may be a potential source of reducing power for nitrite reduction in roots. Investigations on the intracellular localisation of enzymes of the pentosephosphate pathway (Emes and Fowler 1979b) and nitrate reductase (Emes and Fowler 1979a, Kłobus et al. 1983) confirm this supposition. The present study was undertaken on the problem of reducing equivalents for nitrite reduction in not green tissues.

MATERIAL AND METHODS

The experiments were carried out on *Pisum arvense* L. roots. The plants were cultured under conditions described in the preceding paper (Kłobus 1984).

Measurement of nitrite reduction in tissues. Roots of 9-day-old *P. arvense* seedlings were used in the experiments. The cut off roots were placed, after thorough washing in tightly closed flasks (50 cm^3) filled with argon and stored in darkness for 1 h at 30° C for nitrite accumulation in the tissues (Srinivasan and Naik 1981). For increasing the permeability of the tissues the roots were frozen for 2 min in liquid nitrogen (Mann et al. 1978). After defrosting (15 min at 27° C) 250-mg portions were placed in 2 cm^3 of 50 mM phosphate buffer, pH 6.8, containing glucose-6-phosphate and $NADP^+$ or malic acid, $NADP^+$ and ATP in concentrations given in Table 1. After 1-h incubation at 30° C the samples were brought to boil (2 min) and NO_2^- was determined in the solution by the method of Harper and Hageman (1972).

Isolation of plastids in a discontinous gradient of sucrose density. For plastid isolation the method was applied described by Suzuki et al. (1981) in a suitable modification. An extract was prepared from the *P. arvense* roots in 50 mM HEPES-NaOH buffer (pH 6.8) containing 0.4 M sucrose, 2 mM EDTA and 5 mM cystein. The homogenate obtained was filtered through two layers of Micra cloth and placed on the earlier prepared gradient. The discontinuous gradient of sucrose density consisted of 4 cm^3 60%, 8 cm^3 50% and 8 cm^3 of 30% (w/w) sucrose. All sucrose solutions were prepared in 50 mM HEPES-NaOH buffer (pH 6.8) containing 2 mM EDTA and 5 mM cystein. The samples were centrifuged for 5 min at 2900 × g, then for 30 min at 18 000 × g. Subsequently 0.87 cm^3 fractions were collected and glucose-6-phosphate dehydrogenase (marker enzyme for plastids) activity was determined in them. Fractions exhibiting the highest activity of the marker enzyme were twofold diluted with HEPES-NaOH buffer, pH 6.8, containing EDTA and cystein in concentration given above and were combined. The suspension obtained was centrifuged for 15 min at 14 000 × g yielding the

plastid fraction. The sediment was then resuspended in 50 mM HEPES--NaOH buffer (pH 6.8) with 0.2 M sucrose, 0.3 M mannitol, 2 mM EDTA and 5 mM cystein added. The whole procedure was run at 0°-4° C.

Nitrite reduction by plastids isolated from P. arvense roots. The plastid suspension in the amount of 0.5 cm^3 was preincubated for 1 h at 30° C in 50 mM HEPES-NaOH buffer, pH 6.8, containing 0.2 M sucrose and 0.3 M mannitol with 5 mM glucose-6-phosphate or malate, $NADP^+$ and 1.5 mM ATP added. Then $NaNO_2$ in a final concentration of 1 mM was added to the mixture. After 30-min incubation the samples were cooled, the plastids were centrifuged off (15 min at 14 000 × g) and 0.05 cm^3 of the solution was taken for colorimetric determination of nitrites (Harper and Hageman 1972).

Nitrate reductase activity was determined *in vivo* after Jaworski (1971).

Malate dehydrogenase activity dependent on $NADP^+$. For determination 0.2 cm^3 of plastid suspension treated for 30 min with triton ×-100 in a final concentration of 0.1 per cent was used. Enzyme activity was determined according to Vidal and Gadal (1981). The reaction mixture contained in 3 cm^3 4 μM oxaloacetic acid, 0.8 μM $NADPH_2$, 200 μM Tris HCl buffer, pH 7.5, and 0.2 cm^3 of plastid suspension. Malate dehydrogenase activity was expressed as the rate of $NADPH_2$ oxidation determined spectrophotometricaly at 340 nm.

RESULTS

In the preliminary experiments on nitrite reduction in intact *P. arvense* roots (Table 1) it was found that both glucose-6-phosphate + + $NADP^+$ and malic acid, $NADP^+$ and ATP present in the incubation medium cause a distinct fall of the nitrite level in the tissues. Such an effect was not observed if only $NADP^+$ or ATP was added to the incu-

Table 1

Influence *in vivo* of various substrates present in the incubation mixture on the nitrite content in *Pisum arvense* roots

Substrates	NO_2^- content in roots, µmoles $NO_2 \cdot g^{-1}$ fr.wt.
Control	119.4± 9.8
$NADP^+$ (5 mM)	120.9± 11.2
ATP (1.5 mM)	117.6± 10.6
Glucose-6-phosphate (5 mM)+$NADP^+$ (5 mM)	78.0± 4.1
Malic acid (5 mM)+$NADP^+$ (5 mM)+ +ATP (1.5 mM)	50.0± 3.9

The results are means from 5 replications. The differences between the arithmetic means of two different combinations exceeding twice the SE value were considered significant for a 5 per cent error.

bation mixture. The nitrite level in both these cases was similar to that in the control.

In vivo measurements of nitrate reductase activity (Table 2) in the presence of glucose-6-phosphate and $NADP^+$ or of malic acid $NADP^+$ and ATP demonstrated that the named substrates do not affect significantly the activity of this enzyme. These results indicate that the depressed nitrite level in *P. arvense* roots observed in the presence of glucose-6-phosphate and $NADP^+$ and of malic acid; $NADP^+$ and ATP (Table 1) was rather due to changes in NiR activity, and did not result from the depression of nitrate reductase activity.

Table 2

Influence of various substrates added to the incubation mixture on nitrate reductase activity

Substrates	NR activity, μmoles $NO_2^- \cdot g^{-1}$ fr. wt. $\cdot h^{-1}$
Control	1122.3±56.8
Glucose-6-phosphate (5 mM)+$NADP^+$ (5 mM)	1123.6±51.9
Malic acid (5 mM)+$NADP^+$ (5 mM)+ +ATP (1.5 mM)	1190.0±52.6

The results are means from five replications.

Since the nitrite reduction takes place in the plastids (E m e s and F o w l e r 1979a, K ł o b u s et al. 1983), further investigations on the sources of the NiR reductor were performed on the fraction of these organelles isolated from *P. arvense* roots. Study of nitrite reduction by intact plastids (Table 3) showed that addition into the incubation mixture of both glucose-6-phosphate with $NADP^+$ and of malic acid with $NADP^+$ and ATP stimulated markedly nitrite reduction. Similarly as in the preceding experiment performed with intact *P. arvense* roots (Table 1), addition of $NADP^+$ or ATP only into the incubation solution did not affect the nitrite reduction.

Table 3

Influence of various substrates present in the incubation mixture on nitrite reduction by plastids isolated from *P. arvense* roots

Substrates	Amount of reduced nitrites, μmoles $NO_2^- \cdot g^{-1}$ fr. wt. $\cdot h^{-1}$
Control	223.8±12.1
$NADP^+$ (5 mM)	221.9±10.8
ATP (1.5 mM)	220.9±11.2
Glucose-6-phosphate (5 mM)+$NADP^+$ (5 mM)	470.1±19.4
Malic acid (5 mM)+$NADP^+$ (5 mM)+ +ATP (1.5 mM)	447.7±17.3

The resul's are means from five replications.

Table 4

Influence of nitrate present in the growth medium on the activity of malate dehydrogenase isolated from *P. arvense* root plastids

Medium	MDH activity, μmoles $NADPH_2 \cdot min^{-1} \cdot g^{-1}$ fr.wt.
$-NO_3^-$	97
$+NO_3^-$	193

The results are means of three replications.

Table 4 shows the changes in malate dehydrogenase activity dependent of $NADP^+$ isolated from *P. arvense* root plastids under the influence of nitrates present in the medium. Addition of nitrates into the medium caused a distinct enhancement of the activity of this enzyme.

DISCUSSION

The marked fall of the nitrite level in *P. arvense* roots incubated in the presence of glucose-6-phosphate and $NADP^+$ with simultaneous lack of change in nitrate reductase activity may be explained by the increased activity of nitrite reductase. This supposition is supported by the results of experiments performed on plastid isolated from *P. arvense* roots. In the presence of glucose-6-phosphate and $NADP^+$ in the incubation mixture a higher NiR activity than in the control was observed appearing in enhanced nitrite reduction by plastids (Table 3). Stimulation of nitrite reductase localised in root plastids by glucose-6-phosphate in the presence of $NADP^+$ indicates that $NADPH_2$ formed during glucose-6-phosphate oxidation in the pentosephosphate cycle may be the reducing equivalent utilised in the process of nitrite reduction to ammonia in *P. arvense* roots. This is in agreement with the hypothesis of Sarkissian and Fowler (1974) claiming that the transformations in the pentosephosphate cycle localised in the plastids (Emes and Fowler 1979b) are the source of reducing equivalents for nitrite reductase activity in roots, localised also in the plastids (Dalling et al. 1972, Emes and Fowler 1979a, Suzuki et al. 1981, Kłobus et al. 1983). In the present experiments, however, malic acid present in the incubation mixture also stimulated nitrite reduction in *P. arvense* roots. This seems to suggest the possibility of utilisation in the process of nitrite reduction of $NADPH_2$ as well derived from oxidation of malic acid by the malate enzyme or $NADP^+$-dependent malate dehydrogenase. There are in the literature reports on localisation in chloroplasts of malate enzyme (Santarius and Stocking 1969). Washitani and Sato (1977) woring on tobacco cell cultures detected the presence of this enzyme in the proplastid fraction. In investigations performed on *P. arvense* roots, ho-

wever, activity of the malate enzyme was not noted in the plastid fraction (unpublished data). Since in experiments performed with intact roots the technique of brief freezing of the tissues in liquid nitrogen was applied, this may have caused damage to the plasmic membrane structure, and the possibility could not be ruled out of utilisation by the plastid nitrite reductase of $NADPH_2$ formed in the cytoplasm. The results of further experiments, however, excluded such a possibility. Malic acid, $NADP^+$ and ATP present in the reaction mixture, caused, namely, stimulation of nitrite reduction by plastids isolated from *P. arvense* roots. It therefore seems more probable that $NADP^+$-dependent malate dehydrogenase is involved in the supply of reducing equivalents for nitrite reductase activity. The activity of the latter enzyme was detected in the plastid fraction of *P. arvense* roots (unpublished data). This supposition is, moreover, confirmed by the fact of marked stimulation of MDH isolated from plastids by the nitrate present in the medium.

Acknowledgment

This work was financed form MR II/7 problem.

REFERENCES

Canvin D. T., Atkins C. A., 1974. Nitrate, nitrite and ammonia assimilation by leaves: effect of light, carbon dioxide and oxygen. Planta 116: 207-224.

Dalling M. J., Tolbert N. E., Hageman R. H., 1972. Intracellular location of nitrate reductase and nitrite reductase. II. Wheat roots. Biochim. Biophys. Acta 283: 513-519.

Emes M. J., Fowler M. W., 1979a. The intracellular location of the enzymes of nitrate assimilation in the apices of seedling pea roots. Planta 144: 249--253.

Emes M. J., Fowler M. W., 1979b. Intracellular interaction between the pathways of carbohydrate oxidation and nitrate assimilation in higher plant roots. Planta 145: 287-292.

Harper J. E., Hageman R. H., 1972. Canopy and seasonal profiles of nitrate reductase in soybeans (*Glycine max* L. Merr.). Plant Physiol. 49: 146-154.

Jaworski E. G., 1971. Nitrate reductase assay in intact tissues. Biochem. Biophys. Res. Commun. 43: 1274-1279.

Jones R., Sheard R. W., 1978. Accumulation an stability of nitrate in intact aerial leaves. Plant Sci. Lett. 11: 285-291.

Kłobus G., 1984. Sources of reducing equivalents for nitrate reductase in *Pisum arvense* roots. Acta Soc. Bot. Pol. 53: 499-505.

Kłobus G., Kubik-Dobosz G., Buczek J., 1983. The intracellular location of nitrogen metabolism enzymes in *Pisum arvense* L. roots. Acta Soc. Bot. Pol. 52: 215-222.

Mann A. F., Hucklesby D. P., Hewitt E. J., 1978. Source of reducing power for nitrate reduction in spinach leaves. Planta 140: 261-263.

Mann A. F., Hucklesby D. P., Hewitt E. J., 1979. Effect of aerobic and anaerobic conditions on the *in vivo* nitrate reductase assay in spinach leaves. Planta 146: 83-89.

Miflin B. J., 1974. Nitrate reduction in leaves. Studies on isolated chloroplasts. Planta 116: 187-196.

Santarius K. A., Stocking C. R., 1969. Intracellular localization of enzymes in leaves and chloroplast membrane permeability to compounds involved in amino acid synthesis. Z. Naturforsch. 246: 1170-1179.

Sarkissian G. S., Fowler M. W., 1974. Interrelationship between nitrate assimilation and carbohydrate metabolism in plant roots. Planta 119: 335--349.

Srinivasan R. C. S., Naik M. S., 1981. Origin of reductant for reduction of nitrate and nitrite in rice and wheat leaves *in vivo*. The New Phytologist 87: 517-537.

Suzuki A., Gadal P., Oaks A., 1981. Intracellular distribution of enzymes associated with nitrogen assimilation in roots. Planta 151: 457-461.

Vidal J., Gadal P., 1981. Evidence for *de novo* synthesis of nicotinamide-adenine-dinucleotide phosphate malate dehydrogenase during greening of corn leaves. Physiol. Veget. 19: 483-489.

Wallsgrowe R. M., Lea P. J., Miflin B. J., 1979. Distribution of the enzymes of nitrogen assimilation of the pea leaf cell. Plant Physiol. 63: 232-236

Washitani I., Sato S., 1977. Studies on the function of proplastids in the methabolism of *in vitro*-cultured tabacco cells. III. Source of reducing power for amino acid synthesis from nitrite. Plant Cell Physiol. 18: 1235-1241.

Źródła równoważników redukcyjnych reduktazy azotynowej w korzeniach Pisum arvense

Streszczenie

Glukozo-6-fosforan i $NADP^+$ oraz kwas jabłkowy i $NADP^+$ obecne w mieszaninie inkubacyjnej powodowały wzrost aktywności reduktazy azotynowej (EC 1.6.6.4) w korzeniach *Pisum arvense*. Zaznaczyło się to obniżeniem poziomu azotynów w tkankach a także wzmożoną redukcją azotynów przez plastydy wyizolowane z korzeni peluszki. Obserwowano również wyraźną stymulację plastydowej dehydrogenazy jabłczanowej pod wpływem azotanów obecnych w pożywce. Powyższe wyniki sugerują, że nukletydy pirydynowe wykorzystywane przez NiR podczas redukcji azotynów mogą pochodzić z procesów utleniania nie tylko glukozo-6-fosforanu w cyklu pentozofosforanowym ale także kwasu jabłkowego przez dehydrogenezę jabłczanową zależną od $NADP^+$.