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ORIGINAL ARTICLE

**Agnès Massonneau · Nicolas Langlade
Sébastien Léon · Jana Smutny · Esther Vogt
Günter Neumann · Enrico Martinoia**

Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.): relationship between organic acid excretion, sucrose metabolism and energy status

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Abstract Under phosphorous deficiency, plants of white lupin (*Lupinus albus* L.) develop root clusters, which are also called proteoid roots due to their preferential presence in the Proteaceae. In their mature stage, these roots acidify the soil and excrete high amounts of carboxylates [up to 1.5 and 7 μmol $(g\ FW)^{-1}\ h^{-1}$ of malate and citrate, respectively] enabling lupins to utilise sparingly available sources of phosphate. Using the amplified fragment length polymorphism (AFLP) technique, we identified genes predominantly expressed in juvenile and mature cluster roots. Transcripts for two enzymes involved in glycolysis, fructokinase and phosphoglucomutase, were identified in juvenile cluster roots and one, sucrose synthase, in mature cluster roots. In order to verify these observations we performed quantitative reverse transcription-polymerase chain reaction (RT-PCR) and could confirm the increased transcript level. Measurements of enzymatic activities showed that fructokinase and phosphoglucomutase activities increased in juvenile cluster roots, whereas sucrose synthase activity was maximal in mature cluster roots. These results indicate that formation of proteoid roots and citrate excretion increase sink strength locally. Production of citrate and inhibition of respiration are likely to result in an increased $NADH/NAD^+$ ratio, which may be toxic for the plant. The fermentation pathway would allow oxidation of NADH by decarboxylation of pyruvate and subsequent reduction of the resulting acetaldehyde. Determination of alcohol dehydrogenase activity showed that this enzyme is strongly induced in mature proteoid roots. However, ethanol production was not increased, indicating that pyruvate is shunted to citrate synthesis and not to ethanol production.

Keywords Cluster root development · Energy status (root) · *Lupinus* (cluster roots) · Organic acid (excretion, root) · Sucrose metabolism

Abbreviations AFLP: amplified fragment length polymorphism · RT-PCR: reverse transcription-polymerase chain reaction

Introduction

Phosphorus (P), one of the major macronutrients in plants, is a limiting factor for plant productivity due to its low availability in natural and agricultural ecosystems. This problem is overcome in agriculture by the use of phosphate fertilisers, which represents a consumption of about 5 million tons per year each for Europe and North America, and about 3 times more for Asia (FAO data base between 1990 and 1995).

Although P fertilisers are effective in preventing phosphorus deficiency, most of the applied phosphorus (about 75%) is thought to be converted in soils to poorly soluble P forms (Gilbert et al. 1997; Ruiz and Romero 1998). Excessive use of commercial P fertilisers introduces two major ecological problems, the first being the impact on the environment (overfertilisation associated with the risk of pollution of surface waters, waste of energy during fertiliser production), and the second, the use of non-renewable natural P resources (rock phosphate) for the production of fertilisers. Therefore, understanding of plant-induced phosphorus mobilisation

A. Massonneau · N. Langlade · S. Léon
J. Smutny · E. Martinoia (✉)
Laboratoire de Physiologie Végétale,
Institut de Botanique, Université de Neuchâtel,
Rue Emile Argand 13, 2007 Neuchâtel, Switzerland
E-mail: enrico.martinoia@bota.unine.ch
Fax: +41-32-7182271

E. Vogt
Institut für Pflanzenwissenschaften,
Swiss Federal Institute of Technology,
Universitätsstr. 2, 8092 Zürich, Switzerland

G. Neumann
Institut für Planzenernährung (330),
Universität Hohenheim, 70593 Stuttgart, Germany

from different P sources in soils is ecologically and economically of great interest.

Most of the soil phosphorus is present as organic phosphate or insoluble mineral phosphate, neither of which can be absorbed by plants. Organically bound phosphate, which may account for up to 80% of the total phosphorus in some cultivated soils (Li et al. 1997a; Gilbert et al. 1998), has to be mineralised to inorganic phosphate before plants can take it up. Plants and soil microorganisms have developed the capacity to release phosphohydrolases into the rhizosphere, which convert organic phosphate to inorganic phosphate. Among these phosphohydrolases, acid phosphatase is the most documented and widespread enzyme secreted in response to phosphorus deficiency (Lefebvre et al. 1990; Duff et al. 1994), but other enzymes, such as ribonucleases and phytases, have also been found in rhizodepositions and cell culture media (Dodds et al. 1996; Li et al. 1997a, b; Köck et al. 1998).

Rhizosphere acidification, increased reducing capacity of the roots, excretion of carboxylate chelators and phenolic compounds are major root-induced mechanisms used by plants to solubilise sparingly available inorganic phosphate (P_i) forms (Marschner et al. 1987; Hoffland et al. 1989; Dinkelaker et al. 1997; Raghothama 1999). While rhizosphere acidification is a widespread response to phosphorus deficiency, particularly in dicotyledonous plants, the exudation of considerable amounts of carboxylates responsible for P_i solubilisation seems to be restricted to a limited number of plant species (Marschner 1995; Neumann and Römheld 2000). White lupin and members of the Proteaceae are capable of excreting far larger amounts of carboxylates than any other plant species investigated so far (Gardner et al. 1981; Dinkelaker et al. 1997).

White lupin is particularly well adapted to grow on infertile soils containing low amounts of available phosphorus, despite the absence of mycorrhizal associations. In response to phosphorus starvation, this plant develops special root clusters, so called proteoid roots (Purnell 1960; Lamont 1972; Gardner et al. 1981; Watt and Evans 1999). Due to the lack of detailed descriptions of the different parts of cluster roots before 1999, most studies have compared cluster roots with non-cluster roots (Johnson et al. 1994, 1996a, b). However, recent work has shown that cluster roots cannot be taken as a homogeneous material. Different developmental stages of cluster roots have been defined along the secondary roots: juvenile cluster roots are actively growing; mature cluster roots exhibit no more growth activity, but are responsible for the exudation of large amounts of citrate and a concomitant release of protons, which declines again in the senescent cluster roots (Neumann et al. 1999; Watt and Evans 1999). Thus, chemical changes involved in solubilisation of sparingly soluble P sources appear to be most strongly expressed in mature cluster roots. The present study provides a detailed comparison of relations between organic acid metabolism, catabolism of carbohydrates and energy status at different stages of cluster root development.

Materials and methods

Plant material

Seeds of white lupin (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, Rastatt, Germany) were incubated overnight in aerated water and germinated for 4 days on filter paper soaked with 0.2 mM $CaSO_4$. Thereafter, the seedlings were transferred to an aerated nutrient solution [0.1 mM Fe(III)-EDTA, 5 mM $Ca(NO_3)_2$, 1.88 mM K_2SO_4, 1.62 mM $MgSO_4$, 75 μM H_3BO_3, 25 μM $MnSO_4$, 2.5 μM $CuSO_4$, 2.5 μM $ZnSO_4$, 0.75 μM $(NH_4)_6Mo_7O_{24}$, 125 μM KCl] in the presence (+P) or absence (–P) of 0.25 mM KH_2PO_4. The nutrient solution (4 l for 12 plants) was changed every 3–4 days and plants were grown at 22 °C and 65% relative humidity with a light period of 16 h at 200 μmol m^{-2} s^{-1}.

Detection of root-induced changes and harvest of different root parts

Bromocresol purple was used as a pH indicator to visualise pH changes along the roots. The root system was spread between two glass plates covered by a 2-mm layer of 1% agar containing 0.04% (w/v) bromocresol purple. The pH of the gel was adjusted to 6–6.5 in order to get a colour (Fig. 1) which allows acidification (yellow) or alkalinisation (purple) to be determined. Changes were visible after 10 min of contact with the plant.

In order to differentiate the developmental stages of cluster roots for harvesting, the root system was immersed in a solution of bromocresol purple (0.04%, w/v), which indicates acidification in active cluster regions (Neumann et al. 1999).

Acid extraction and determination of P_i and ATP

Roots (250 mg) were frozen in liquid nitrogen, homogenised and resuspended in 1 ml of 10% (w/v) perchloric acid. The homogenate was centrifuged for 10 min at 13,000 *g*. For P_i determination, the supernatant was adjusted to pH 6–7 using 4 M KOH, incubated for 5 min on ice and centrifuged again for 10 min at 13,000 *g*. The amount of P_i was determined according to Chan et al. (1986). For ATP determination, the acid extract was neutralised with a solution containing 3.2 mM EDTA-Na_2, 3.5 mM $MgCl_2$ and 52.6 mM Mops (pH 9.0). One volume of extract was mixed in the cuvette of a Lumac Biocounter M2500 with 0.2 vol. of firefly lantern extract (FLE-250; Sigma) diluted with 60 ml of distilled water. After a delay of 2 s following the addition of FLE, the light signal was integrated for 5 s and compared with ATP standards.

Collection and analysis of exudate

Excised roots were rinsed briefly in distilled water and incubated for 1 h at 22 °C in Eppendorf tubes containing 1 ml of penicillin (250 mg l^{-1}). Citrate and malate contents were determined in the exudates using the citric acid test kit and L-malic acid test kit (Boehringer, Mannheim, Germany). Ethanol was determined in the exudates using the ethanol test kit (Boehringer).

Extraction of total RNAs

Frozen tissues were homogenised in liquid nitrogen using pre-cooled mortars and pestles, extracted with 8 vol. of extraction buffer [20% (v/v) phenol in 10 mM Tris HCl (pH 9.0), 20% (v/v) chloroform, 60 mM NaCl, 6 mM Tris-HCl (pH 7.5), 0.6 mM EDTA, 0.6% SDS, 1.2 mM β-mercaptoethanol, 9 mM Na-ascorbate]

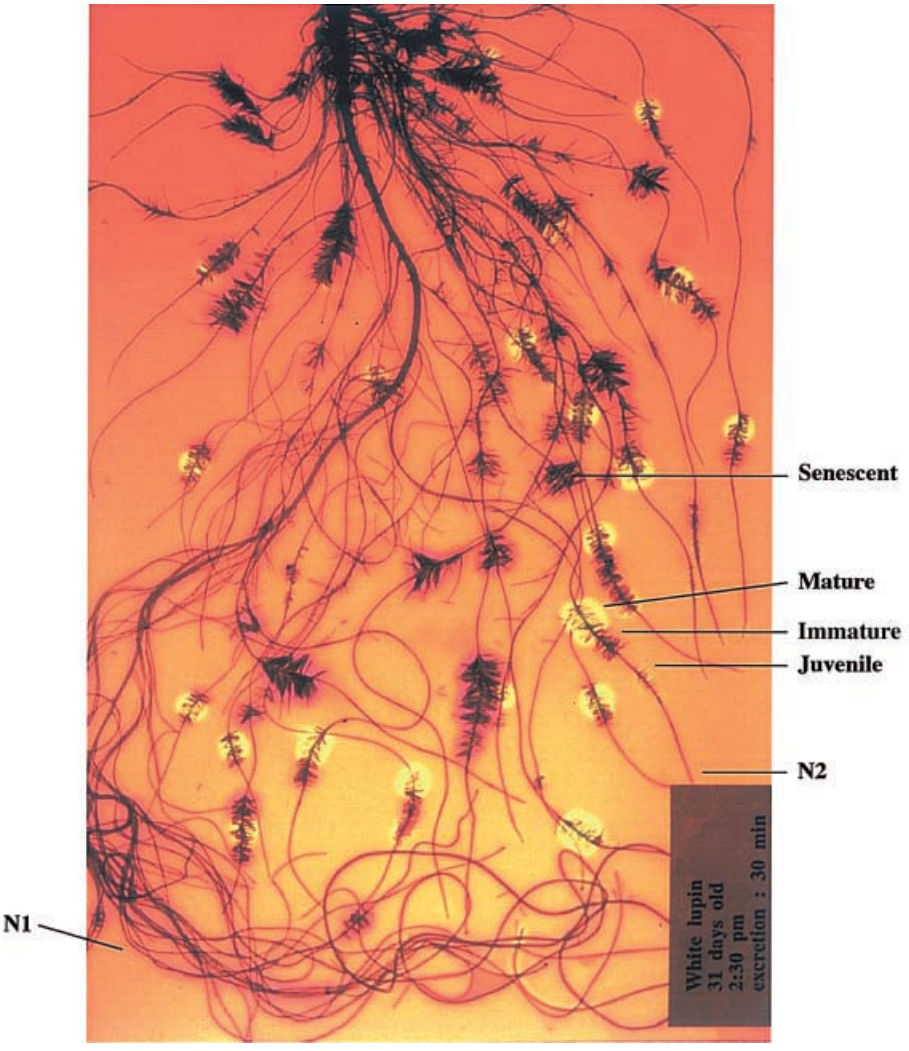

Fig. 1 Root system of a 31-day-old P-deficient white lupin (*Lupinus albus*). The roots were placed between two plates of agar containing 0.04% (w/v) bromocresol purple as a pH indicator. The dye turns to yellow (pH < 5) when the roots are acidifying their surrounding and become purple if alkalinisation (pH > 7) occurs. The different developmental stages of the cluster roots are indicated. *N1* Apical region (1 cm) of secondary roots that do not contain cluster roots, *N2* apical region (1 cm) of secondary roots containing cluster roots

and incubated for 30 min at 4 °C under constant stirring (all subsequent steps were carried out at 4 °C). After centrifugation for 20 min at 9,000 *g*, nucleic acids were recovered from the upper phase and precipitated overnight at 4 °C with 1/10 vol. of 3 M Na-acetate and 2 vol. of ethanol. Nucleic acids were subsequently pelleted for 20 min at 9,000 *g*, washed with 70% (v/v) ethanol and resuspended in water. The RNAs were specifically precipitated with 2 M Li-acetate for 3 h at 4 °C, pelleted and washed as mentioned above. The RNA pellet was resuspended in water and precipitated by adding 1/10 vol. of 3 M Na-acetate and 2 vol. of ethanol. After a 20-min centrifugation at 9,000 *g*, the RNAs were washed with ethanol and resuspended in water.

Amplified fragment length polymorphism (AFLP) of cDNA and identification of clones

The RNAs from juvenile, mature and senescent cluster roots were compared by the cDNA-AFLP technique described by Bachem et al. (1996). First- and second-strand cDNA synthesis was carried out according to standard protocols (Sambrook et al. 1989). Restriction sites (*Taq*I and *Ase*I) and adaptors used corresponded to those described by Bachem et al. (1996) giving rise to a total of 16^2 primer combinations. Thermocycling was done for 35 cycles, including an 11-cycle touch-down (0.7 °C per step) at the beginning of the reaction (65°C to 56 °C). Products from the polymerase chain reaction (PCR) were separated on a 5% polyacrylamide sequencing-type gel. Labelled cDNA fragments were visualised by autoradiography. Amplified fragments were then cloned in pGEM-T easy vector (Promega) and sequenced. Identification of the different genes was carried out using the Blast programme from EMBNET (www.ch.embnet.org).

Reverse transcription(RT)-PCR

Total RNA was treated with RQ1 DNase (Promega) following the manufacturer's instructions and then loaded on a gel to check concentrations. About 2.5 µg of total RNA of each root type, supplemented with 10^6 copies of pAW109 RNA (provided by Perkin-Elmer Biosystems; Roche Molecular Systems, Branchburg, N.J., USA), were reverse-transcribed with oligo-dT using the RT system provided by Promega and following manufacturer's instructions. An aliquot of 1/100 of the RT product was used for each PCR reaction.

The PCRs were performed with Promega's Taq DNA Polymerase in the buffer supplied containing 2.5 mM $MgCl_2$. Final concentrations of nucleotides were 0.1 mM for dATP and 0.2 mM for the other dNTPs. The PCR reaction mixture was supplemented with 0.5 MBq of ^{33}P-labelled dATP. The sucrose synthase, phosphoglucomutase and fructokinase primers were designed based on the clones obtained with the cDNA-AFLP. A pAW109-specific set of primers was introduced into the same tube to verify the RT-PCR processing. Primers for sucrose synthase were 5′-AGTGATGGTCCCTTTGGTGA-3′ and 5′-ACACGCTCAACCTTGTCTCC-3′, amplifying a 554-bp fragment; control RNA was amplified using AW112 5′-CAGAGGGAAGAGTTCCCCAG3-′ and AW113

5′-CCTTGGTCTGGTAGGAGACG-3′ giving a 301-bp fragment. Primers for phosphoglucomutase were 5′-GAGCTCTGAAGG-AGAATCATTG-3′ and 5′-AGGATCATGGAGTGACAGTC-3′, amplifying a 357-bp fragment, and AW125 5′-CAATGTCTC-ACCAAGCTCTG-3′ and AW159 5′-GAGGAGGTGTTGAC-TTCATTC-3′, amplifying an approximately 450-bp fragment. For fructokinase, 5′-TCCTGAAGAAGCTCGTCAAC-3′ and 5′-GAGTACCTCTCTTAACCTTGG-3′, amplifying a 336-bp fragment, and AW125 and AW159 were used. The PCR reactions were 45 s at 95 °C, 1 min at 60°C (sucrose synthase) or 56 °C (phosphoglucomutase and fructokinase) and 1 min at 72 °C for 24 cycles. The number of cycles was checked to be in the linear range of the PCR reactions under these conditions.

Products from RT-PCR were separated by 2.5% (w/v) agarose gel electrophoresis. The gels were treated for 10 min with 0.25 M HCl and then transferred using a vacuoblot system with 2 × SSC (1 × SSC: 15 mM Na_3-citrate, 150 mM NaCl, pH 7) on a polarised nylon membrane (Porablot NY plus, Düren, Germany) for 2 h. Blots were rinsed and exposed to Biomax MR film (Eastman Kodak, Rochester, N.Y., USA) for a few hours.

Extraction of soluble proteins and assay of enzymes

Roots were ground in liquid nitrogen and homogenised with 3 vol. of 0.1 M Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 2.5 mM DTT, 3 mM Na-DEDTC (diethyldithiocarbamate), 1 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 3% polyvinylpolypyrrolidone (PVPP) K30. After 25 min centrifugation at 12,000 *g* (4 °C), the supernatant was used to determine the extractable enzymatic activities and protein concentrations (DC Protein Assay kit; Bio-Rad). All enzymes were assayed spectrophotometrically by monitoring the appearance or the disappearance of NADH at 340 nm. All reactions were initiated by addition of the root extract.

Alcohol dehydrogenase (EC 1.1.1.1) was assayed in 10 mM Na_4-pyrophosphate (pH 8.8), 1.3 mM NAD, 6.3% (v/v) ethanol (omitted in blanks). Fructokinase (EC 2.7.1.4) was assayed in 25 mM Tris HCl (pH 8.0), 50 mM KCl, 0.3 mM NAD, 1 mM ATP, 3 mM $MgCl_2$, 100 μg ml^{-1} BSA, 0.5 mM D-fructose (omitted in blanks), 1.2 U ml^{-1} glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.8 U ml^{-1} phosphoglucose isomerase (EC 5.3.1.9). Pyruvate decarboxylase (EC 4.1.1.1) was assayed in 180 mM Nacitrate (pH 6.0), 0.9 mM KCN, 0.2 mM NADH, 1 U ml^{-1} alcohol dehydrogenase (EC 1.1.1.1), 33 mM Na-pyruvate (omitted in blanks), and calculations of activities were based on the regression curve obtained with baker's yeast pyruvate decarboxylase (Sigma). Phosphoglucomutase (EC 5.4.2.2) was assayed in 25 mM Hepes-NaOH (pH 7.5), 3 mM $MgCl_2$, 0.3 mM NAD, 100 μg ml^{-1} BSA, 20 μM glucose-1,6-bisphosphate, 2 mM glucose-1-phosphate (omitted in blanks), 1.2 U ml^{-1} glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Sucrose synthase (EC 2.4.1.13) was assayed in 20 mM Hepes-NaOH (pH 7.5), 3 mM $MgCl_2$, 100 μg ml^{-1} BSA, 50 mM sucrose, 0.3 mM NAD, 1 mM ATP, 4 mM UDP (omitted in blanks), 1.2 U ml^{-1} glucose-6-phosphate isomerase (EC 1.1.1.49), 0.8 U ml^{-1} phosphoglucose isomerase (EC 5.31.9), 1 U ml^{-1} hexokinase (EC 2.7.1.1).

Respiration measurements

Respiration was measured at 25 °C using a Clark-type O_2 electrode. A saturated Na-dithionite solution and air-equilibrated water were used to calibrate the O_2 concentrations. Five to ten (for non-cluster roots) or three (for cluster roots) root pieces were incubated in air-saturated water under agitation. The rate of O_2 consumption was recorded for about 3 min.

Results

Description of root morphology

Large numbers of cluster roots developed in white lupin under phosphorus deficiency (–P) compared to P-sufficient plants (+P). In order to compare the different root types we defined different zones (Fig. 1): N1 corresponds to the apical root zones (1 cm) of secondary roots not containing cluster roots; N2 corresponds to the apical root zones (1 cm) of secondary roots which contain cluster roots; juvenile roots are young developing cluster roots which have yet not reached their final size. Immature and mature cluster roots are the same size and shape, showing that no growth occurs between the two stages of development. The most striking difference between these roots is that the immature cluster roots do not acidify or only very slightly acidify their environment, while mature cluster roots strongly acidify the medium. In immature cluster roots, citrate excretion is increased by a factor of three compared with juvenile cluster roots (Table 1). A similar increase by a factor of about three can be observed between the immature and the mature stages. Immature cluster roots can be found either on the same root cluster as mature cluster roots or as a separate root cluster. Malate excretion remains fairly constant in these three stages of cluster root development. Senescent cluster roots weakly alkalinise the medium and citrate excretion is largely reduced.

Table 1 Characteristics of different root zones of P-sufficient and P-deficient white lupin (*Lupinus albus*). The different types of roots were identified using bromocresol purple (see Fig. 1). Organic acids were collected for 1 h. Data are means ± SD (organic acids, $n = 5$; proteins $n = 3$; total RNA content $n = 2$–4; root phosphate content and respiration $n = 6$; ATP content $n = 3$). All data are expressed on a gram fresh weight basis. *nd* Not determined

Root zone		Excretion		Root content				Respiration (μmol O_2 min^{-1})
		Citrate (nmol h^{-1})	Malate (nmol h^{-1})	Proteins (mg)	RNA (mg)	Phosphate (μmol)	ATP (nmol)	
+P	N1	330 ± 92	646 ± 126	0.66 ± 0.05	0.247 ± 0.068	nd	nd	nd
	N1	471 ± 142	369 ± 126	0.52 ± 0.06	0.068 ± 0.049	0.15 ± 0.03	24.3 ± 4.5	2.0 ± 0.2
	N2	432 ± 42	712 ± 140	0.91 ± 0.09	0.655 ± 0.003	0.18 ± 0.09	23.0 ± 10.1	2.9 ± 0.4
	Juvenile	756 ± 150	1,654 ± 249	1.18 ± 0.02	0.573 ± 0.073	0.38 ± 0.10	90.9 ± 8.7	3.3 ± 0.4
–P	Immature	2,464 ± 330	1,696 ± 489	0.82 ± 0.01	0.247 ± 0.088	0.21 ± 0.02	47.4 ± 6.8	2.5 ± 0.3
	Mature	7,051 ± 2,066	1,539 ± 327	0.90 ± 0.09	0.062 ± 0.029	0.18 ± 0.05	42.6 ± 8.9	1.9 ± 0.4
	Senescent	2,297 ± 788	90 ± 126	0.47 ± 0.01	0.016 ± 0.002	0.08 ± 0.04	12.1 ± 3.9	0.6 ± 0.1

Organic acid excretion, respiration, protein, RNA, P_i and ATP content in the different root parts

In order to make a general comparison between the different root types we measured some further parameters relating to the phosphate status (Table 1). Comparison of protein contents shows that with the exception of N1 and the senescent cluster roots the protein contents were quite similar (Table 1). In contrast, the total RNA contents decreased strongly from the immature to the mature and senescent cluster roots. This fact is important, since the phosphate liberated and reallocated by the degradation of RNA is likely to play an important role in the growth of young root tissues (see *Discussion*). Phosphate and ATP contents (Table 1) were highest in juvenile roots and lowest in senescent cluster roots. The other root types had very similar phosphate contents but immature and mature cluster roots contained about twice as much ATP as the root apices. Respiration was about 1.5-fold higher in juvenile cluster roots than in most of the other root types. However, there was a decrease in the respiratory activity of mature cluster roots and senescent cluster roots, which exhibited only about 20% of that observed in juvenile cluster roots.

Expression patterns and activities of sucrose synthase, fructokinase and phosphoglucomutase

In order to identify stage-specific expression of mRNA we performed a cDNA-AFLP analysis (Bachem et al. 1996) with juvenile, mature and senescent cluster roots. Cloning and sequencing of a range of differentially expressed bands showed that several enzymes involved in glycolysis, namely fructokinase, sucrose synthase, and phosphoglucomutase were up-regulated in juvenile and mature cluster roots (Table 2). The high similarity observed over stretches of 120 and 227 bases for fructokinase and sucrose synthase makes it very likely that the clones indeed correspond to the genes coding for the enzymes identified in the data base. In the case of phosphoglucomutase a very high similarity was found for a phosphoglucomutase-like gene of *Arabidopsis thaliana*. The similarity to a described phosphoglucomutase from *Spinacia oleracea* was significantly lower. However, closer analysis of the sequences shows that the stretch of the *Arabidopsis* phosphoglucomutase-like gene corresponding to the part of the lupin gene identified by AFLP exhibits a homology to the spinach phosphoglucomutase similar to that of lupin. In contrast, comparison of the whole coding sequence of *Arabidopsis* with spinach resulted in an E-value of –105. This discrepancy is due to the fact that the domains typically conserved in phosphoglucomutases are not located on the stretch identified in our AFLP approach. It is therefore very likely that the clone 1B corresponds to phosphoglucomutase.

Since excretion of citrate depends on available carbohydrates (Johnson et al. 1996a; Neumann et al. 1999), we decided to investigate in more detail the regulation of carbohydrate catabolism. Due to the limited amount of RNA available for some stages of cluster root development (mainly senescent cluster roots) we were forced to perform quantitative RT-PCR analysis instead of Northern blots (Fig. 2). The RT-PCRs with RNA extracted from different plants cultures were performed for each gene and always showed the same pattern. These experiments confirmed the results obtained by AFLP. Fructokinase was induced in juvenile cluster roots and expression was still high in immature cluster roots. The transcript levels started to decrease in the mature clusters and were very low in the senescent cluster roots. Transcript levels for phosphoglucomutase were high in juvenile cluster roots and decreased during development of the root cluster. In the case of sucrose synthase, a slight increase in the transcript levels could be detected in juvenile cluster roots and they were highest in immature and mature cluster roots.

Since mRNA levels may not reflect the enzyme activity in a tissue, and transition of one cluster root stage to another is a rapid process, we measured the in vitro activities of the three enzymes (Fig. 3). Based on fresh weight, the activities of fructokinase and phosphoglucomutase were increased in response to phosphorus starvation. In –P plants, the juvenile and mature cluster roots had the highest activities, whereas senescent clusters and normal N1 exhibited a lower activity. Due to

Table 2 Inventory of white lupin cDNA-AFLP clones with significant homologies, as determined by blastx search (EMBNET). Clone ID and size refer to the identification number of the cDNA-AFLP clones and their size. The clones were identified in the cDNA-AFLP assay comparing normal roots with juvenile proteoid roots

Clone ID	Size (bp)	Similarity to:	Location of the similarity (aa)	% Similarity (significance)
1C	489	Fructokinase (EC 2.7.1.4) *Lycopersicon esculentum*	182–302	88% (4e-50)
28F	882	Sucrose synthase (EC 2.4.1.14) *Pisum sativum*	31–258	86% (e-104)
1B	370	Phosphoglucomutase-like (*Arabidopsis thaliana*)	406–528	88% (4e-51)
		Phosphoglucomutase precursor (EC 5.4.2.2) *Spinacia oleracea*	395–536	60% (1e-10)

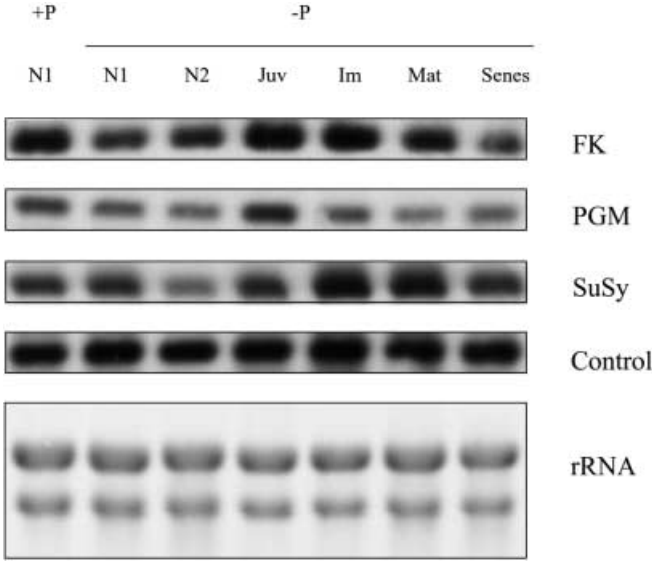

Fig. 2 Transcript levels of fructokinase (*FK*), phosphoglucomutase (*PGM*) and sucrose synthase (*SuSy*) in different parts of lupin +P and –P roots. Quantitative RT-PCR was performed (see *Materials and methods*), including a synthetic RNA as a control (control shown for PGM). As a further control an RNA agarose gel was stained with ethidium bromide. *N1* Apical region (1 cm) of secondary roots that do not contain cluster roots, *N2* apical region (1 cm) of secondary roots containing cluster roots, *Juv* juvenile, *Im* immature, *Mat* mature, *Senes* senescent

the variation in the protein content between the different –P root types, the specific activity of fructokinase showed similar but less pronounced fluctuations. However, in –P plants the specific activities of fructokinase and phosphoglucomutase were much higher in every root part than in +P plants.

The activity of sucrose synthase, unlike that of fructokinase and of phosphoglucomutase, was not enhanced by P starvation in normal roots, but increased in the cluster roots. The highest activity (3- to 5-fold the activity determined in N1 or N2) was observed in mature cluster roots. Similar to fructokinase and phosphoglucomutase activities, sucrose synthase activity decreased strongly in senescent cluster roots.

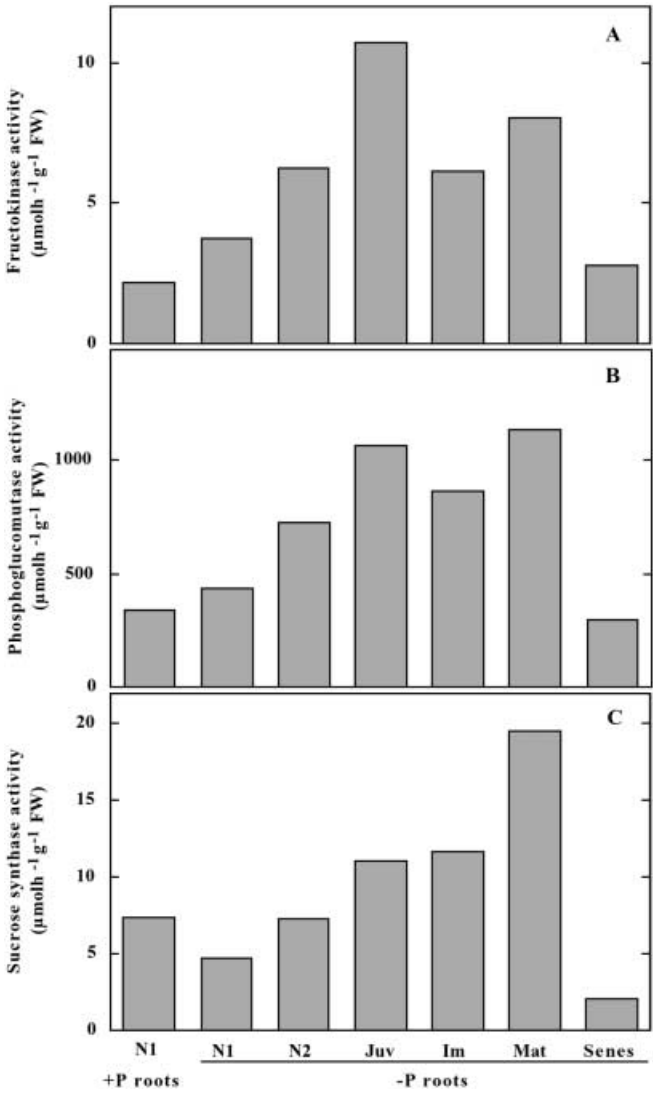

Fig. 3 In vitro activities of fructokinase (**A**), phosphoglucomutase (**B**) and sucrose synthase (**C**), measured in –P and +P roots of white lupin. The experiments were repeated at least four times, and the distribution pattern of enzymatic activities obtained in different root zones was reproducible. Due to the variations in the absolute enzyme activities, one single, typical experiment is shown. Measurements were performed in triplicate; the SD is not shown since it is too small to be visualised. See Fig. 2 for abbreviations

Alcohol dehydrogenase and pyruvate decarboxylase activities, and ethanol excretion

Low respiration rates and production of large amounts of organic acids suggest that lupin plants accumulate reducing equivalents. A pathway to cope with overproduction of NADH consists of the reduction of acetaldehyde originating from pyruvate decarboxylation and formation of ethanol. Indeed, alcohol dehydrogenase activity strongly increased in cluster roots excreting citric acid (Fig. 4). While an increase could already be observed in immature cluster roots compared with N2 roots, a more than 3-fold increase was observed in mature cluster roots. Based on fresh weight, there was even a slight increase in juvenile cluster roots compared with N2. In contrast, induction of pyruvate decarboxylase activity was far lower and no differences could be observed between N2 and juvenile, immature and mature cluster roots. In order to investigate whether the increase in alcohol dehydrogenase is linked to ethanol production and excretion; we determined ethanol excreted by the different root types. Only a very low tissue concentration of ethanol was detected within the different root parts and no differences could be observed (not shown). In contrast, roots grown under P stress excreted substantial amounts of ethanol. Surprisingly, N2 roots and juvenile cluster roots excreted more ethanol than immature or mature cluster roots. Values for ethanol

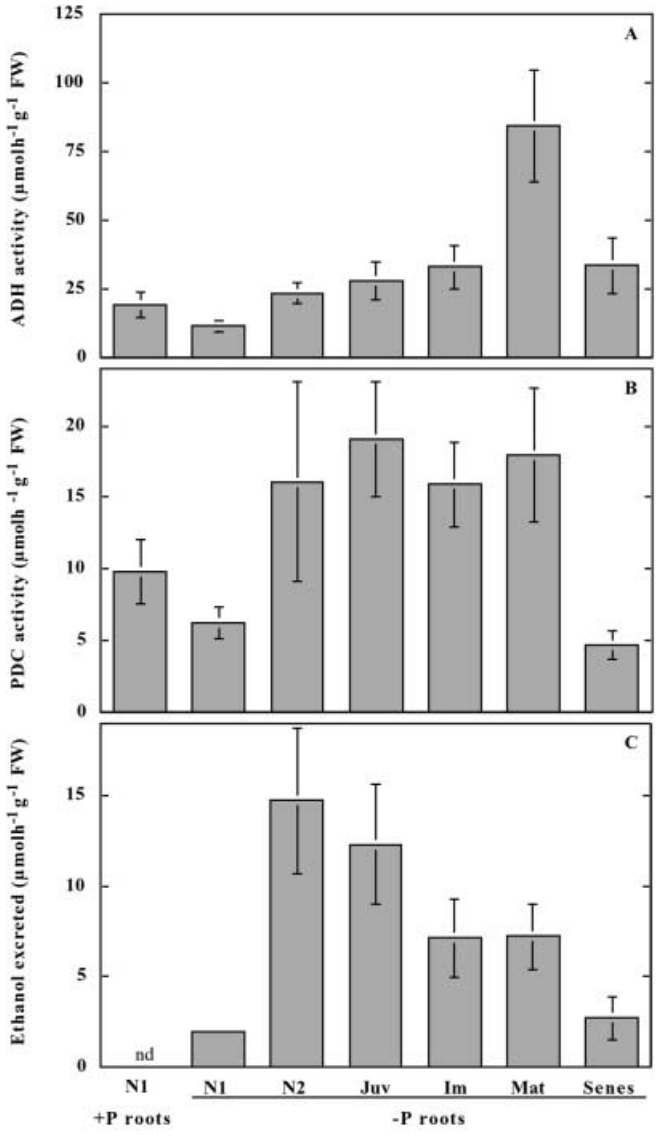

Fig. 4 In vitro activities of alcohol dehydrogenase (*ADH*, **A**), pyruvate decarboxylase (*PDC*, **B**), and excretion of ethanol (**C**) in different zones of +P and –P roots of white lupin. Data represent the mean ± SD of four independent experiments, each with three replicates. See Fig. 2 for abbreviations

excretion similar to those observed for N2 and juvenile roots have been reported for 11-day-old peas subjected to anaerobic conditions. Interestingly, in this case ethanol excretion was higher under light conditions [10.8 μmol (g FW)$^{-1}$ h^{-1}] than in low light (1.1 μmol (g FW)$^{-1}$ h^{-1}].

Discussion

The advantages of localised acidification and root excretion of carboxylates, together with the limited longevity of mature cluster roots, have been already discussed with respect to phosphorus mobilisation in soils (Hoffland et al. 1989; Dinkelaker et al. 1997). Localised activity of mature cluster roots is also of economic importance for cluster root growth. The change from juvenile to mature and finally to senescent cluster roots is accompanied by a drastic decrease in total RNA (Table 1). In soils limited in P_i, the high P requirement of the growing parts of the root, such as the apex and juvenile cluster roots, can only be sustained at the expense of P_i re-translocation from other parts of the plant. Under such conditions, RNA represents an important pool of phosphorus in plant cells. Using our data, an estimation of the size of this pool can be made. Based on weight, RNA contains 8–9% phosphorus. Since juvenile cluster roots are likely to be less vacuolated than cluster roots with fully expanded cells and therefore the data on fresh weight cannot be directly compared, we will calculate only the P_i made available during the transition from immature to senescent cluster roots. The difference in RNA content was 230 μg (g FW)$^{-1}$, which contains about 20 μg P_i (g FW)$^{-1}$. This corresponds to a P_i content of 0.7 μmol (g FW)$^{-1}$. Kakie (1969) showed that in plants grown under limited phosphate supply, about 40% of the phosphate is present in the nucleic acid fraction. Complete degradation of RNA and subsequent re-translocation into growing organs is therefore a prerequisite for growing under phosphate limitations. Therefore, it must be postulated that in P-deficient white lupin the highly co-ordinated senescence of cluster roots plays a crucial role in the development of new cluster roots. It is obvious that in a hydroponic culture, as used in these experiments, re-translocated phosphate will not be sufficient to sustain growth. In nature, cluster roots solubilise and take up sparingly available phosphate. However, it is likely that re-translocation of phosphate is also required for growth in this case. Furthermore, endogenous phosphate is less expensive in terms of energy costs, since the plant does not have to synthesise and excrete citrate. The observation that higher phosphate and ATP levels are present in the juvenile cluster roots is a further hint that phosphate is efficiently translocated to young, growing parts of a plant.

Using cDNA-AFLP we identified three clones that were differentially expressed in different root types and exhibited a high homology to genes coding for enzymes involved in the transformation of sucrose to fructose-6-P: sucrose synthase, fructokinase and phosphoglucomutase. Since these enzymes are the starting points of glycolysis we were interested to see whether the transcript levels for these genes are indeed induced. The RT-PCR confirmed the results obtained with cDNA-AFLP. The observation that sucrose synthase was originally found in juvenile cluster roots may be due to the fact that at the beginning of our studies we were not able to differentiate between immature and mature cluster roots and that immature cluster roots were, at least partially, sampled together with juvenile cluster roots since they exhibit a negligible acidification of the medium. Sucrose unloaded from the phloem sap serves as a carbon source for the roots, either for growth or for organic acid

excretion and is either processed by invertases or sucrose synthase (Ciereszko et al. 1998). Experiments in which P-deficient white lupin shoots were labelled with $^{14}CO_2$ demonstrated carbon translocation from shoots to roots with subsequent conversion of sugars to organic acids in the root tissue (Johnson et al. 1996a). Organic acid excretion (malate and citrate) increased from about 1,000 nmol (g FW)$^{-1}$ h^{-1} in –P or +P normal roots to more than 7,000 nmol (g FW)$^{-1}$ h^{-1} in the mature cluster roots (Table 1). Such $^{14}CO_2$ labelling studies showed that organic acids exuded by the roots of P-deficient white lupin are also linked to phosphoenolpyruvate carboxylase-mediated non-photosynthetic CO_2 fixation in the root tissue (Johnson et al. 1996a, b), which can account for one-third of the exuded carbon. In the case of the mature cluster roots, this implies that root CO_2 fixation is responsible for an exudation of about 2,860 nmol (g FW)$^{-1}$ h^{-1}. Compared with the total organic acids exuded from mature cluster roots [7,051 nmol (g FW)$^{-1}$ h^{-1}], 5,730 nmol (g FW)$^{-1}$ h^{-1} has to be sustained by carbon supplied from the shoots as sugars. Our enzymatic studies showed that fructokinase, phosphoglucomutase and sucrose synthase (Fig. 3) exhibited increased activities in juvenile, immature and mature cluster roots compared with normal roots. We also measured hexokinase activity in –P roots (results not shown), which was found to be very low compared with sucrose synthase and fructokinase. For fructokinase, as well as for phosphoglucomutase, the highest transcript levels and enzymatic activities could be detected in the juvenile cluster roots. However, our RT-PCR data indicate that the decrease in transcript level in immature and mature cluster roots is faster than the decrease in enzymatic activity. It is therefore likely that protein turnover is less rapid than mRNA turnover. The transition of juvenile to immature cluster roots takes approximately 2 days, and about 1 day from the immature to the mature stage. Therefore, enzymes that are synthesised in an earlier stage, and do not exhibit a rapid turnover, may still remain active in a later stage of development. In contrast to phosphoglucomutase and fructokinase, maximal transcript levels of sucrose synthase can be detected in the immature stage. Maximal enzymatic activity of sucrose synthase is found in the mature state, and it must again be postulated that turnover of the enzyme is slower than that of the corresponding mRNA. A decreased protein turnover under phosphate starvation could save ATP and hence help the plant to survive phosphate stress.

In bean root tips, sucrose synthase activity, rather than invertase, has been reported to be responsible for the increased sucrose hydrolysis under P starvation (Ciereszko et al. 1998). The sink strength of growing potato tubers was also found to be closely related to the activity of sucrose synthase (Zrenner et al. 1995). In the present study, sucrose synthase activity, which is likely to indicate the sink strength, displays a pattern very similar to that of organic acid exudation in the different root types. Thus, organic acid excretion is apparently the cause of a strong local sink, since in a small part of a plant up to 20% of the CO_2 fixed during photosynthesis is excreted in the form of citrate.

The use of sucrose synthase by cluster roots is also of energetic importance for the cells. During phosphorus deficiency, pools of P_i and nucleotide triphosphates are significantly reduced, whereas the pyrophosphate (PPi) pool is maintained. A metabolic "bypass" using PPi-dependent enzymes instead of ATP-dependent enzymes has been proposed in P-deficient plants (for reviews, see Theodorou and Plaxton 1993; Plaxton 1996, 1998). The sucrose synthase pathway allows sucrose conversion to hexose phosphates via a PPi-dependent pathway, and compared with the invertase pathway requires half as much ATP.

Carbohydrate catabolism under P-deficient conditions can increase the NADH/NAD^+ ratio (Juszczuk and Rychter 1997) due to inhibition of important pathways for NADH oxidation, such as nitrate assimilation (Rufty et al. 1990; Gniazdowska et al. 1999) and respiration (Theodorou and Plaxton 1993; Neumann et al. 1999). Increased activity of the ADP- and P_i-independent, cyanide-resistant respiration (Juszczuk and Rychter 1997), or decreased citrate turnover (Neumann et al. 1999) by down-regulation of dehydrogenases in the tricarboxylic acid cycle (Lance and Rustin 1984) have been suggested as putative adaptations to counteract P-deficiency-induced overproduction of NADH. The same function may be ascribed to the increased activity of alcohol dehydrogenase observed in the present study, which was most pronounced in mature cluster roots and associated with the highest tissue concentrations of citric acid (Neumann et al. 1999). As observed in other cases, e.g. anaerobiosis (Tadege et al. 1998), the activity of alcohol dehydrogenase is strongly increased compared with the activity of pyruvate decarboxylase. However, our measurements do not show an increased ethanol excretion. Since, in previous experiments, we observed that under the given experimental conditions citrate is not readily used by microorganisms, it is unlikely that ethanol is rapidly degraded. Therefore, our results indicate that, despite the increased activity of alcohol dehydrogenase, ethanol production is not induced and that pyruvate is probably shunted to citrate production. However, the question still remains of how the plant can deal with the increased ratio of NADH to NAD^+.

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