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# Adaptation of Plasma Membrane $H^+$ ATPase of Proteoid Roots of White Lupin (*Lupinus albus* L.) under Phosphorus Deficiency

**A thesis submitted for the requirement of the doctoral degree in agriculture**
**Faculty of Agricultural and Nutritional Sciences,**
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Content

# 1. Introduction

## 1.1 Soil Phosphate

Phosphorus (P) is one of the key elements in energy metabolism and biosynthesis of nucleic acids and membranes in higher plants. It plays an important role in photosynthesis, respiration, and regulation of a number of enzymes and, therefore, is one of the essential nutrient elements for plants. Crop production can be severely compromised through lack of available P, which is of particular concern in the highly weathered and volcanic soils of the humid tropics and subtropics, in many sandy soils of the semiarid tropics, and in calcareous soils of the temperate regions (Raghothama, 1999). In addition, the recovery of applied P by crop plants in a growing season is very low. In soil more than 80% of the applied P becomes immobile and unavailable for plant uptake due to precipitation with Ca, adsorption on Al or Fe oxides/hydroxides, or conversion to organic forms (Holford, 1997).

In neutral to calcareous soils, the $Ca^{2+}$ concentration is high enough that any $Ca(H_2PO_4)_2$ added to the soil will be transformed quickly into $CaHPO_4$ that shows lower solubility as compared to $Ca(H_2PO_4)_2$. This transformation of applied $Ca(H_2PO_4)_2$ is especially characterized in calcareous soils with high pH and $Ca^{2+}$ concentration in soil solution. $CaHPO_4$ can undergo further transformation to less soluble hydroxyapatite. In neutral and acid soils phosphate adsorption is the dominant process affecting phosphate availability for plants. Specific phosphate adsorption is brought about by ligand exchange in which the $OH^-$ on the adsorbing surface is replaced by phosphate. Phosphate adsorbing surfaces include Fe oxides/hydroxides, Al hydroxides, allophanes, clay minerals, organic Fe complexes and calcite (Parfitt 1978). In this way, the phosphate pool is rendered immobile. This process of phosphate aging is especially rapid in acid soils with a high adsorption capacity.

Additionally, soil microbes immobilize phosphate by effectively converting it into organic forms. Organic phosphate is an important fraction, which makes up 20 – 70% of total P in soil (Ron Vaz et al . 1993). Most of them are present in the form of inositol phosphate ester, which are not readily utilized by plant roots. Because of different transformation processes of phosphate fertilizers, the phosphate concentration

of the soil solution is very low and even in fertile arable soils it ranges 1-10 µM (Hossner et al. 1973)

## 1.2 Adaptation of proteoid roots to P deficiency in soils

Higher plants have developed a fascinating array of strategies to obtain otherwise unavailable P of soil reserves. Roots exhibit an impressive plasticity in response to low availability of P and can modify their morphological structure and physiological function including (1) mycorrhizal associations between roots and soil fungi, (2) alterations in root architecture and branching, (3) increases in root hair density and length, (4) root exudation of various compounds, and (5) development of proteoid roots.

Regarding P efficiency, the development of proteoid roots is a particularly interesting adaptation strategy of higher plants. Proteoid roots are bottlebrush-like root clusters with densely located rootlets of limited growth with an average length of 0.5 to 1 cm. The rootlets are usually covered with long and dense root hairs (Purnell, 1960; Dinkelaker et al., 1995; Watt and Evans, 1999b). Purnell (1960) observed this root structure in plant species of the family *Proteaceae* for the first time and named it ‘proteoid root’. Most species of this family are dominant in the natural ecosystems of the Mediterranean, south Africa and Australia (mainly Western Australia), a few genera of *Proteaceae* are also distributed in central and south America, India and southeast Asia (Beadle, 1981). These are slow-growing sclerophyllous shrubs and trees and are abundant on nutrient-poor habitats, such as highly-leached sands, sandstones and laterites (Bradle, 1981; Lamont, 1982; Grose, 1989). They are regarded as highly efficient at extracting soil phosphorus and are also characterized by a very efficient utilization of P within the plant (Grundon, 1972; Grose, 1989). Interestingly, *Proteaceae* lack mycorrhizal associations (Lamont, 1982; Brundrett and Abbott, 1991). Proteoid roots are found in various families including *Leguminosae, Proteaceae, Casuarinaceae, Myricaceae, Eleagnaceae,* and *Betulaceae* (Skene, 1998). Of the species that form proteoid roots, white lupin is the only crop currently used in agriculture and the one that has been most intensively studied (Watt and Evans, 1999b). In the investigations on adaptation mechanisms of higher plants to P

deficiency, white lupin has become a model plant (Johnson et al., 1996b; Neumann et al., 1999; Watt and Evans, 1999b).

Besides the morphological adaptation, one of the most remarkable characteristics of proteoid roots is the release of a large amount of organic acids, predominantly citric and malic acid, into rhizosphere under P deficiency conditions (Gardner et al., 1983; Dinkelaker et al., 1989; Li et al., 1997; Keerthisinghe et al., 1998; Neumann et al., 1999). About 0.1 mmol citric acid per g soil has been reported in the rhizosphere soil of proteoid roots of white lupin (Dinkelaker et al., 1989; Gerke et al., 1994; Li et al., 1997), which is high enough to release P from sparingly soluble Fe and Al phosphate (Gerke et al., 1994) by mechanisms of ligand exchange or chelation of metal ions (Hinsinger, 1998). Besides organic acids, proteoid roots also release large amounts of acid phosphatases into rhizosphere (Dinkelaker et al., 1995; Gilbert et al., 1999; Neumann et al., 1999; Miller et al., 2001), which can efficiently mobilize P from organic compounds (Li et al., 1997; Neumann et al., 2000). In addition, proteoid roots can exudate phenolic compounds, mainly isoflavonoids (Wojtaszek et al., 1993), which can prevent the microbial degradation of other root exudates in the rhizosphere (Dinkelaker et al., 1995).

## 1.3 Exudation of organic acids by proteoid roots

It has been repeatedly demonstrated that citric acid is the predominant organic acid released by proteoid roots of white lupin under P-deficient conditions (Dinkelaker et al., 1989; Johnson et al., 1996a, 1996b; Li et al., 1997; Neumann et al., 1999). The amount of released citric acid can represent as much as 11% (Gardner et al., 1983) to 23% (Dinkelaker et al., 1989) of the total plant dry weight, depending on physiological development stages and severity of the P deficiency. The highest exudation activity of citric acid is related to the mature root clusters, whereas young and old clusters release only a limited amount of acids (Keerthisinghe et al., 1998; Neumann et al., 1999; Watt and Evans, 1999a).

Biochemical studies reveal that increased release of citric acid by proteoid roots is related to the enhanced synthesis of organic acid in proteoid root cells (Johnson et al., 1994; Neumann et al., 1999). Although the exuded organic acids resulted from increased PEPC activity in the shoot of P-deficient rape plants was reported (Hoffland

1990), it may not be true for the proteoid roots of white lupins. Root and shoot $^{14}C$-labeling studies showed that about 30% of released carbon in the form of organic acids, mainly citric acid, originated from dark $CO_2$ fixation by PEPC in roots of P-deficient white lupin (Johnson et al., 1996a). Accordingly, in proteoid roots enhanced gene expression and *in vitro* activities of enzymes involved in catabolism of carbohydrates (sucrose synthase, phosphoglucomutase, fructokinase) (Neumann et al., 2000; Massonneau et al., 2001), biosynthesis of organic acids (PEPC, malate dehydrogenase, citrate synthase) (Johnson et al., 1996a; Uhde-Stone et al., 2003) have been reported (Fig.2).

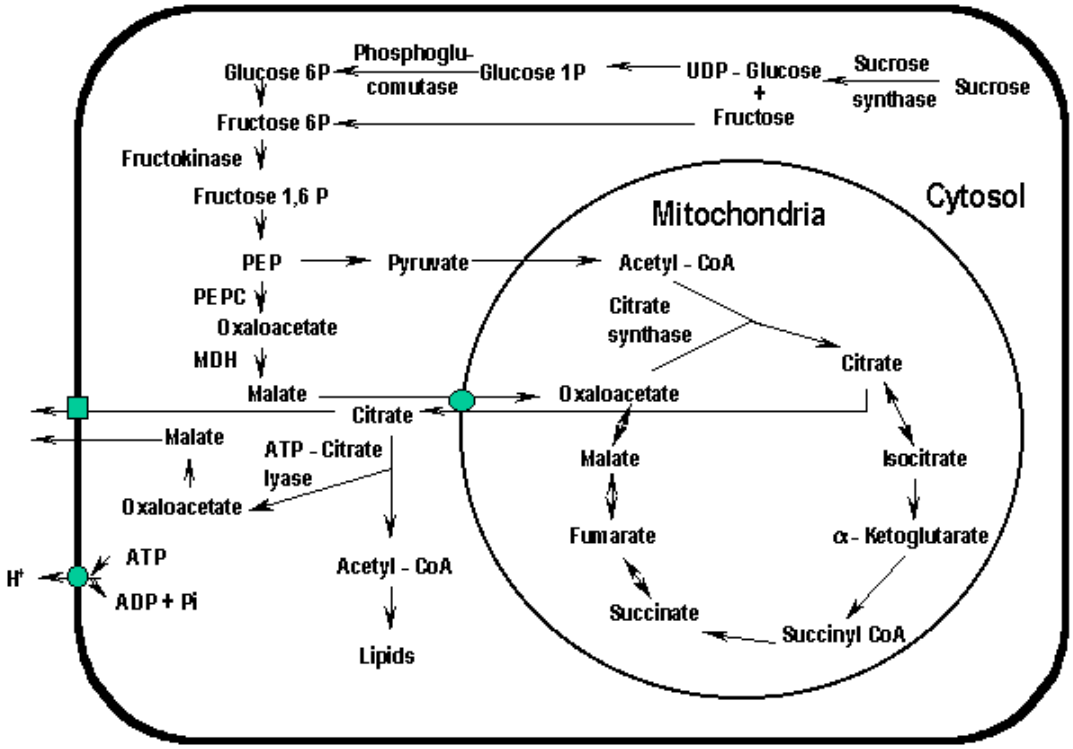


Figure 1. Model for phosphorus deficiency-induced metabolic changes related to intracellular accumulation and exudation of citrate in cluster roots of *Lupinus albus* (Neumann und Martinoia, 2001).

So far, the transport processes involved in the exudation of organic acids from proteoid roots have not been elucidated. In earlier studies, rhizosphere acidification was related to imbalance in nutrient uptake between cations and anions (cations > anions) (Marschner 1995) such as in the cases of $NH_4^+$ nutrition (Jungk & Claassen, 1986) or symbiotic $N_2$ fixation (Mengel & Steffens, 1982; Marschner & Römheld, 1983).

Accordingly, it was suggested that rhizosphere acidification by proteoid roots of white lupin was due to the excess uptake of cations under P deficiency (Dinkelaker et al., 1989; Sas et al., 2002). However, it was found that about half of total $H^+$ exudation was not accounted for by uptake of excess cations, and was suggested to be attributed to organic anion extrusion (Sas et al., 2002). It must be pointed out that in these studies only the correlations (but not the mechanisms) of $H^+$ release and uptake of various nutrient ions by plant roots were calculated. Investigations druing the last decade have yielded much evidence that the mechanism of $H^+$ release is a primary process caused by $H^+$ ATPase activity in the plasmalemma of root cells (Mengel and Schubert, 1985; Schubert and Matzke, 1985; Schubert 1995).

Because of high cytosolic pH (above pH 7) organic acids, like citric acid, dissociate into $H^+$ and citrate. These two ions are then transported across plasma membrane via different transporters. The exudation of citrate from proteoid roots of white lupin was reduced by anthracene, an anion channel blocker, which implied an involvement of anion channel in the citrate exudation (Neumann et al., 1999).

There is general agreement that plasma membrane $H^+$ ATPase (EC 3.6.1.35) is responsible for the export of protons out of plant cells (Serrano, 1989, Palmgren 1998; Michelet & Boutry 1995; Sze et al., 1999). This enzyme acts as a primary transporter by pumping protons out of the cell, thereby creating pH and electric potential differences across the plasma membrane. The basic functions of this enzyme are, therefore, to keep $H^+$ homeostasis of the plant cells and supply driving force for membrane transport of various substances. This enzyme may be especially important for proteoid roots which release large amounts of citric acid. On the one hand, during synthesis of citric acid a large amount of $H^+$ should be removed to keep cells from acidosis. On the other hand, $H^+$ release by $H^+$ ATPase can also supply counterions for citrate release to maintain overall charge balance. Therefore, it can be hypothesized that in proteoid root cells of white lupin, both $H^+$ ATPase and anion channel-like transporters may be up-regulated during the adaptation to P deficiency to match the requirement of citric acid exudation (Fig. 2).

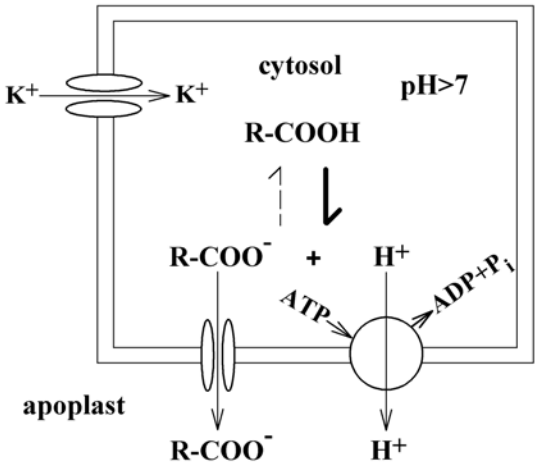


Figure 2. Hypothetical mechanism for the release of organic acids by proteoid root cells of white lupin.

In fact, it has been demonstrated that plasma membrane $H^+$ ATPase responds to a number of environmental factors, such as salt stress (Niu et al., 1993), low solution pH (Yan et al., 1998), nutrient supply (Schubert and Yan, 1997), and Fe deficiency (Dellórto et al., 2000).

The aim of the present study is to investigate the adaptation of plasma membrane $H^+$ ATPase in proteoid roots of white lupin in response to P deficiency. The following experimental approaches were chosen to elucidate the adaptation mechanisms:

1. Quantification all ions released by active proteoid roots to study quantitative relationships between citrate release and the release of other ions, especially $H^+$.
2. Isolation of plasma membrane from proteoid roots to study the hydrolytic activity and the $H^+$-pumping activity of plasma membrane $H^+$ ATPase activity *in vitro*.
3. Study of $H^+$ ATPase enzyme kinetics to characterize differences between the $H^+$ ATPases from active proteoid roots and those from other types of roots.
4. Analysis of $H^+$ ATPase by means of Western-blot technique to quantify enzyme concentration of $H^+$ ATPase in active proteoid roots.
5. Study of the gene expression of $H^+$ ATPase in proteoid roots during adaptation to P deficiency
6. Study of the relationships of gene expression between plasma membrane $H^+$ ATPase and the enzymes, which are important for the synthesis of citric aid during

the development of proteoid root to reveal the importance of $H^+$ ATPase for citric acid exudation by proteoid roots of white lupin

# 2. Material and Methods

## 2.1 Plant cultivation

Seeds of white lupin (*Lupinus albus* L. cv. Amiga) were immersed in 6% $NaClO_3$ for 5 min and thorougly washed with deionized water for several minutes. These seeds were soaked in aerated 1 mM $CaSO_4$ for 1 d and germinated at 22°C in the dark between two layers of filter paper moistened with 1 mM $CaSO_4$. After 4 d, seedlings were transferred to a container with 50 L of one-fourth-strength concentrated nutrient solution. Plants were grown in a growth chamber under controlled conditions. Lamps gave a light intensity of approximately 400 µE $m^2$ $s^{-1}$ at shoot height, with a day/night cycle of 16 h/8 h at 22°C/15°C. Relative humidity was 60%. After 2 and 4 d of cultivation, the concentration of the nutrient solution was increased to one-half and full strength concentration, respectively.

The full-strength nutrient solution had the following composition:

| Macroelements | | Microelements | |
|---|---|---|---|
| 0.5 mM | $Ca(NO_3)_2$ | 25 µM | $H_3BO_3$ |
| 1.75 mM | $K_2SO_4$ | 1.5 µM | $MnSO_4$ |
| 0.25 mM | KCl | 1.5 µM | $ZnSO_4$ |
| 1.25 mM | $MgSO_4$ | 0.5 µM | $CuSO_4$ |
| | | 25 nM | $(NH_4)_6Mo_7O_{24}$ |
| | | 20 µM | Fe(III)-EDTA |

For the control plants, the nutrient solution received an additional 0.25 mM $KH_2PO_4$. The solution pH was kept constant at 6.0 by continuous titration with 0.1 M NaOH or 0.05 M $H_2SO_4$ using a pH stat system (Schott). The complete solution in the containers was changed every 3 d.

At the age of 3 weeks under P deficiency, plants were used to collect exudation or harvested to isolate plasma membrane. At this time, secondary clusters of proteoid

roots along lateral roots were in mature stage and showed strong rhizosphere acidification.

## 2.2 Plant growth, number of proteoid roots and P concentrations of plant shoots

After 3 weeks of cultivation, plant shoots and roots were separated. After determination of fresh weights, plant shoots were dried at 80°C for 2 d and then the dry weight of shoots was determined. For determination of fresh weights, roots of white lupin were thoroughly washed with deionized water three times and blotted dry with tissue paper. The number of proteoid roots per plant was determined. For determination of phosphate concentration of shoot, plant matter was dry-ashed and the resulting ash was dissolved in $HNO_3$. The phosphate concentration was then determined photometrically by using molybdate-vanadate reagent (yellow method for P determination).

## 2.3 Detection of rhizosphere acidification by intact proteoid roots

The exudating activity of proteoid roots was determined according to Römheld et al. (1984). The roots of 3-week-old plants were thoroughly washed with deionized water and spread on the agar sheet. The agar sheet contained: 0.75% (w/v) agar, 0.006% (w/v) bromocresol purple, 2.5 mM $K_2SO_4$, 1 mM $CaSO_4$, The pH was adjusted to 6.

The roots were carefully pressed into agar without being damaged. The incubation for visualization of rhizosphere acidification was conducted in a growth chamber under light for 5 h. For the determination of vanadate sensitivity of rhizosphere acidification of intact proteoid roots, two agar sheets were separately prepared. The agar sheet for the control had the same composition as mentioned above. For vanadate treatment, the agar sheet contained an additional 1 mM $Na_3VO_4$.

## 2.4 Collection of exudate from intact proteoid roots

Active proteoid roots, which can stongly acidify rhizosphere after 3 weeks of cultivation, were used for collection of root exudates. The entire root system was

incubated overnight in a matrix solution containing 0.5 mM $K_2SO_4$, 0.5 mM $Na_2SO_4$ and 0,5 mM $CaSO_4$. The solution pH was kept constant at 6. The next morning, individual plants were removed to a plastic box. The root system was fully spread on the bottom of the box. Two active proteoid roots were enclosed in a plexiglass cuvette (4*1*1cm, L*W*H) through two notches, to prevent damage to the lateral root axis. The notches were sealed with Baysilone-Paste (Mittelviskos, GE Bayer Silicones, Germany) to create a compartment that was separated from the rest of the root system (Fig. 3). After active proteoid roots had been sealed in cuvettes, the box was filled with 200 ml matrix solution to submerge the entire root system, but not to overflow the cuvettes. If the cuvette was proven to be sealed, 3 ml matrix solution were filled in, which was sufficient to submerge encased proteoid roots. Before the incubation started, the solution in the cuvettes was exchanged twice (each after 10 min incubation). The matrix solution in the plexiglass cuvettes was not stirred or aerated during the period of incubation. Experiments were conducted in growth chamber under the same conditions as for the plant cultivation. For all experiments the collection of root exudates began always at 10:00 a.m. after plants had been exposed to light for 4 h to avoid a possible variation of citrate release due to diurnal rhythm (Watt and Evan 1999a).

Figure 3. Collection of proteoid root exudates.

The solution volume change was determined during incubation periods from 15 to 120 min and was found to be less than 1% of original volume (3 ml). For each treatment, one sample solution for exudate analysis was pooled from eight different cuvettes.

In order to study the effect of incubation time on the root exudation, different incubation periods were applied to the proteoid roots of one plant. Eight plants were used for root exudation in each experiments.
To study the effect of pharmacological drugs on root exudation, various drugs were applied to the proteoid roots of various plants to avoid a possible cross-talking effect of drugs through the root system. Each sample solution was then collected from 16 proteoid roots of four plants.

At the end of the collection period, the solution was removed from the plexiglass cuvette with a pipette, filtered through filter paper and transferred to a plastic vial. Proteoid roots in the cuvette were harvested to determine fresh mass, frozen in liquid nitrogen, and stored at -20°C for analysis of citrate and malate.

For the investigation of the effects of various pharmacological drugs on root exudation, all solutions were prepared with the matrix solution immediately before use. In the solution containing 350 µM sodium vanadate, $Na_2SO_4$ was omitted in the matrix solution to keep a comparable $Na^+$ concentration as the matrix solution. Fusicoccin was dissolved in ethanol and diluted with the matrix solution to 5 µM. Anthracene-9-carboxylate (Abb. Anthracene) was dissolved in ethanol with ultra-sonification and diluted with the matrix solution to 150 µM (Neumann et al., 1999). The pH of all solutions was adjusted to 5.6.

## 2.5 Root exudate analysis

The concentration change of individual ion species before and after incubation with proteoid roots was determined. The quantity of root exudates was then calculated by multiplying the concentration change of ions with the solution volume used for the collection of root exudates. The volume change of the solution during incubation periods was found to be less than 1% and therefore neglected.

### 2.5.1 Quantification of $H^+$ released

Immediately after collection and filtration, 5 ml fresh exudation solution was titrated with 1 mM NaOH to pH 7 using a micro pH electrode (InLab 423, Mettler-Toledo GmbH, Switzerland). Parallel to the exudation solution the corresponding blank solution was also titrated in the same way. The $H^+$ exudation by proteoid roots was calculated as the difference in alkalinity between exudation solution and the corresponding blank solution. The exudation solution was not treated with vacuum before titration since in a preliminary experiment vacuum treatment did not show a significant effect on the titration results of the exudation solution.

### 2.5.2 Quantification of $K^+$, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$ released

For the determination of the concentration of various cations, exudation solution and corresponding blank solution were tenfold diluted with bi-distilled water. The concentration of individual cations ( $K^+$, $Na^+$, $Ca^{2+}$ and $Mg^{2+}$ ) was measured by use of atomic absorption spectrometry (Varian 220FS, Varian Australia Pty Ltd, Australia).
According to the concentration difference of the ion between exudation solution and the corresponding blank solution, the ion exudation by proteoid roots was calculated for each ion species.

### 2.5.3 Quantification of $SO_4^{2-}$, $Cl^-$, $NO^{3-}$ and $PO_4^{3-}$ released

For the determination of the concentration of various inorganic anions, exudation solution was fivefold diluted and passed through a Strata $C_{18}$-E column (Sorbent Lot Number S201-13, Strata, California, USA). Aliquots of 20 µl were injected by means of an autosampler (788 IC Filtration Sample Processor, Methrohm, Switzerland) attached to a Metrohm 761 Compact IC system (Metrohm, Switzerland). Ion separation was conducted on an analytical anion column (A-Supp 4, 4 mm ID * 250 mm, Metrohm, Switzerland) with matching guard column. The solution of 4.00 mM $NaHCO_3$ and 1.00 mM $Na_2CO_3$ was used for elution with a flow rate of 1.00 ml/min at ca. 5.3 MPa, 35°C. The baseline was kept between 16.8 to 17.5 µs/cm. The eluent anions were suppressed with a self-regenerating suppressor and sample anions were detected by conductivity. Standards were prepared for $SO_4^{2-}$, $Cl^-$, $NO^{3-}$ and $PO_4^{3-}$ . Calibration curves were constructed for the quantification of the identified ions present

in the samples. Date acquisition and integration of chromatograms were performed using the system 761-788 in the software IC-Net 2.1 (Metrohm AG, Switzerland). According to the concentration difference of the ion between exudation solution and the corresponding blank solution, the ion exudation was calculated for each ion species.

### 2.5.4 Quantification of citrate and malate

For determination of citrate in the exudation solution 1 ml solution was directly used for analysis. Citrate was analyzed using the citric acid test kit (Biopham, Mannheim, Germany). For the determination of malate, 5-8 ml exudation solution were mixed with 20 µl 1M $H_2SO_4$ and then vacuum-evaporated to dryness by use of a concentrator (Automatic Environmental Speedvac Concentrator, Savant AES1010, USA). The residue was re-dissolved in 1 ml 0.05 M NaOH and the pH of the solution was then adjusted to 8. Malate was analyzed using the L-malic acid test kit (Biopham, Mannheim, Germany). For the determination of citrate and malate in proteoid roots, root tissue samples were homogenized with 5% (v/v) $H_3PO_4$. After centrifugation, the supernatant was adjusted to pH 8. Citrate and malate content were analyzed using the test kits for citric acid and L-malic acid (Biopham, Mannheim, Germany), respectively.

## 2.6 Plasma membrane isolation

To investigate the adaptation of plasma membrane $H^+$ ATPase in the proteoid roots under P deficiency, plasma membrane was isolated from four different types of roots of 3-week old white lupin (see also Fig. 4): Lateral roots of P-sufficient plants [4- to 5-cm apical zone of lateral roots marked as lateral (+P)], proteoid roots of P-sufficient plants [marked as proteoid (+P)], lateral roots of P-deficient plants [4- to 5-cm apical zone of lateral roots marked as lateral (P)], and active (the youngest, fully developed) proteoid roots of P-deficient plants [marked as proteoid (P)]. The whole root system was cut and washed quickly with deionized water. Roots were always immersed in the ice-cold water during selecting and collecting proteoid roots and root apicals for the isolation of plasma membrane.

To investigate plasma membrane $H^+$ ATPase during the development of proteoid roots under P-deficiency, plasma membrane was isolated from young, mature and senescent proteoid roots, respectively. The young proteoid roots were 3 d old, the mature proteoid roots were 5-7 d old , and senescent proteoid roots were more than 9 d old. They were sequentially localized from apex to the base of laterals.

Plasma membrane of roots was isolated according to Yan et al. (2002). Roots were cut and washed three times with chilled, deionized water and ground in ice-cold homogenization buffer with a mortar and pestle.

The homogenization buffer contained:

250 mM sucrose

250 mM KI

2 mM EGTA

10% (v/v) glycerol

0.5% (w/v) bovine serum albumin

2 mM dithiothreitol

1 mM phenylmethylsulfonyl fluoride

5 mM 2-mercaptoethanol

50 mM 1,3-bis(tris[hydroxymethyl]methylamino) propane (BTP),
adjusted to pH 7.8 with MES

The homogenate (adjusted to a grinding medium/tissue ratio of 4 mL $g^{-1}$ fresh weight) was filtered through two layers of Miracloth (Calbiochem-Novabiochem, San Diego) and centrifuged in a swinging bucket rotor at 11,500 g (AH 629 rotor, 36 ml, Sorvall Products, Newtown, CT) for 10 min at 0°C. The supernatants were centrifuged at 87,000 g for 35 min.

The microsomal pellets were resuspended in phosphate buffer, which contained:

250 mM Sucrose

3 mM KCl

5 mM $KH_2PO_4$ (pH 7.8)

The microsomal membrane preparation was fractionated by two-phase partitioning in aqueous dextran T-500 (Sigma) and polyethylene glycol (Sigma) according to the

method of Larsson (1985). Phase separations were carried out in a series of 32-g phase systems that contained:

6.1% (w/w) dextran T-500

6.1% (w/w) PEG 3350

250 mM Sucrose

3 mM KCl

5 mM $KH_2PO_4$ (pH 7.8)

Stock solutions of polymers were prepared with concentrations of 20% and 40% (w/w) for dextran and polyethylene glycol, respectively. The concentration of dextran stock solution was determined by optical rotation (Larsson, 1985). The phase stock was weighed and diluted to 6.1% (w/w, each polymer) with phase buffer to a final weight of 32 g. Polymers in "start tubes" were, however, diluted to 26 g. Six grams of microsomal resuspension (in phase buffer) were added to the upper phase of each start tube. The tubes were sealed with Parafilm (American National Can, Greenwich, CT) and mixed by inversion (30 times). Phase separation was achieved at 4°C by centrifugation at 720 g (Sorvall AH-629 rotor, 36 ml) for 23 min followed by two washing steps in identical phases. Centrifugation times for the second through fourth phases were 15, 10, and 5 min, respectively.

The upper phases obtained after four separations were diluted with phosphate buffer (see above) and centrifuged at 151,200 g for 40 min. The pellets were washed with resuspension buffer and pelleted again.

The resuspension buffer contained;

250 mM Sucrose

3 mM KCl

5 mM BTP/MES, pH 7.8

The pellets were re-suspended in re-suspension buffer, divided into aliquots, and immediately stored in liquid nitrogen.

Protein was quantified according to the method of Bradford (1976) and the reagent was set up as following:

0.01% (w/v) Coomassie Brilliant Blue G-250

4.7% (w/v) alcohol

8.5 % (w/v) phosphoric acid

50-100 µl resuspended membrane protein was mixed with 5 ml reagent and incubated for 40 min at room temperature. The absorption was measured at 595 nm using spectrophotometer. Bovine serum albumin was used as standard.

## 2.7 Enzyme assay

### 2.7.1 Hydrolytic activity of plasma membrane $H^+$ ATPase

Hydrolytic ATPase activity was determined by measurement the $P_i$ amount after 30 min hydrolysis reaction.

The 0.5 ml reaction reagents were composed of

30 mM BTP/MES

5 mM $MgSO_4$

50 mM KCl

50 mM $KNO_3$

1 mM $Na_2MoO_4$

1 mM $NaN_3$

0.02% (w/v) Brij 58

5 mM disodium-ATP.

Reaction was initiated by the addition of 30 µl membrane re-suspension with 1 to 2 µg protein, proceeded for 30 min at 30°C, and stopped with 1 ml of stopping reagent [2% (v/v) concentrated $H_2SO_4$, 5% (w/v) SDS, and 0.7% (w/v) $(NH_4)_2MoO_4$)] followed immediately by 50 µL of 10% (w/v) ascorbic acid. After 10 min, 1.45 mL of arsenite-citrate reagent (2% [w/v] sodium citrate, 2% [w/v] sodium *m*-arsenite, and 2% [w/v] glacial acetic acid] was added to prevent the measurement of phosphate liberated because of ATPase activity from ATP hydrolysis under acidic conditions (Baginski et al., 1967). Color development was completed after 30 min and $A_{820}$ was measured by

means of a spectrophotometer. ATPase activity was calculated as phosphate liberated in excess of boiled-membrane control.

The kinetic characteristics of plasma membrane $H^+$ ATPase were studied in the presence of an ATP-generating system that included 5 units of pyruvate kinase (Sigma) and 5 mM PEP (Boehringer Mannheim/Roche, Basel; Sekler and Pick, 1993). $V_{max}$ and $K_m$ were determined by means of a regression analysis. Activation energy of ATPase was calculated, using the Arrhenius equation, from $V_{max}$ values determined at 25°C and 30°C, respectively.

### 2.7.2 pH gradient

The formation of a pH gradient across the plasma membrane of inside-out vesicles was measured as the quenching of $A_{492}$ by AO. The change of the quenching was continuously monitored by mens of a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia).

The assay mixture contained

5 mM BTP/MES (pH 6.5)

12 µM AO

300 mM KCl

250 mM Sucrose

0.5 mM EGTA (adjusted to pH 6.5 with BTP)

9 µM valinomycin

1 mM $NaN_3$

1 mM $Na_2MoO_4$

50 mM $KNO_3$

0.05% (w/v) Brij 58

50 µg membrane protein in a final volume of 1.5 ml

Brij 58 was used to create inside-out vesicles (Johansson et al., 1995). After equilibration of the membrane vesicles with the reaction medium, the reaction was initiated by the addition of Mg-ATP (mixture of $MgSO_4$ and disodium-ATP, adjusted to pH 6.5 with BTP) to give a final concentration of 5 mM. The reaction temperature

was 25°C. Quenching was calculated after the $H^+$ gradient was disrupted by 10 µl 755 µM gramicidine, a $H^+$ ionophore.

### 2.7.3 Gel electrophoresis and immunodetection of plasma membrane $H^+$ ATPase

Plasma membrane proteins were separated by SDS-PAGE using the system of Laemmli (1970). Membrane vesicles (4 µg membrane proteins) were solubilized in SDS-loading buffer containing
0.125 mM Tris-HCl, pH 7.4
10% (w/v) SDS
10% (v/v) glycerol
0.2 M dithiothreitol
0.002% (w/v) bromocresol blue
5 mM phenylmethylsulfonyl fluoride
0.05% (w/v) trasylol

The standard markers for protein molecular mass were purchased form Sigma. After 30 min shaking at room temperature (22°C), samples were loaded on a discontinuous SDS-polyacrylamide gel (6% [w/v] acrylamide stacking gel and 10% [w/v] acrylamide separating gel). The resulting gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) ethanol overnight. To enhance the image, the gels were then incubated for 10 min with a solution containing 8% (v/v) acetic acid and 25% (v/v) ethanol. Then the gels were destained with a 10% (v/v) acetic acid and 30% (v/v) ethanol solution.

For the Western-blot analysis, after SDS-PAGE separation samples were transferred to PVDF membrane filters (0.2 µm, Pall Specialty Materials, Port Washington, NY) using a semi-dry blotting system with a buffer containing 10 mM 3-cyclonexylamino-1-propane sulfonic acid (pH 11, adjusted with NaOH) and 20% (v/v) methanol for 1.5 h at room temperature and at a current intensity of 0.8 mA $cm^{-2}$. For staining of the obtained blot, the lane of the standard markers of molecular mass was separated from the lanes of the membrane proteins. The former was stained with Coomassie Brilliant Blue R-250, as described above. For the identification and quantification of plasma

membrane $H^+$ ATPase, the remaining blot with plasma membrane proteins was incubated with a polyclonal antibody (kindly supplied by Dr. Michael G. Palmgren, Royal Veterinary and Agricultural University, Copenhagen) specific for the central part of plant $H^+$ ATPase (amino acids 340-650 of AHA2). The antiserum was diluted 1:3,000 in TBS-T buffer (1 mM Tris-HCl [pH adjusted to 8.0 with NaOH], 15 mM NaCl, and 0.1% [v/v] Tween 20) and incubation was carried out for 1 h at room temperature followed by an incubation at 4°C overnight. After rinsing in TBS-T, PVDF membrane filters were incubated at room temperature for 2 h with a 1:30,000 (v/v) diluted secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma). After rinsing in TBS-T, the filters were incubated for 5 min in a buffer containing 100 mM Tris-HCl (pH 9.5, adjusted with NaOH), 100 mM NaCl, and 5 mM $MgCl_2$. After separate staining, the part of the blot with the lane of standard molecular mass and the part of the blot with lanes of plasma membrane proteins were combined.

For quantification of plasma membrane $H^+$ ATPase, the blots were scanned using a video camera (Module CCD, LTF-Labortechnik, Wasserburg-Bodensee, Germany), and the $H^+$ ATPase immuno-reactive bands were quantified densitometrically (software TINA, Raytest Isotopenmessgeräte, Straubenhardt, Germany).

### 2.7.4 Statistical treatment

Variation is indicated by ±SE (if bars exceed symbols in figures). Significant differences between treatments were calculated using the Student's t test. For the studies of root exudation, significant differences between the control and the treatments with pharmacological drugs were calculated using the *t*-paired test. All tests were calculated using the software program SigmaStat (version 2.03).

## 2.8 Molecularbiological analysis

### 2.8.1 Isolation of total RNAs from different roots of white lupins

Total RNAs were extrated from three different types of roots of 3-week-old white lupin: Lateral roots of P-sufficient plants, lateral roots of P-deficient plants, and active

proteoid roots of P-deficient plants. Total RNAs were also extracted from 1 to 8 d-old proteoid roots. Excised roots were rinsed briefly in deionized water, blotted dry and frozen in liquid nitrogen. Frozen tissues were ground in liquid nitrogen using pre-cooled mortars and pestles and aliquoted.

The isolation of total RNA with phenol-chloroform was according to the method of Cox and Goldberg (1988) with some modification. A 100 mg sample was mixed with 1 ml lysis buffer by vigorously vortexing. The lysis buffer was composed as following:
100 mM Tris-HCl (pH 8.6)
2% Lausrolyl-sarcosine
25 mM EDTA pH 8
25 mM EGTA pH8
100 mM β- mercaptoethanol
5 mM DTT
1% SDS
β-mercaptoethanol was always freshly added before using.

After 5 min of incubation on ice to disrupt the cell and denaturate proteins, 400 µl phenol (pH 5.2) and 400 µl chloroform were added, mixed and centifugated at 12 000 rpm (4 °C) for 10 min. The cell crash and tissue fragments were pelleted at the botten, but proteins and lipids floated in the lower phase. Nucleic acids recovered from the upper aqueous phase were collected and purified again by mixing with chloroform and centifugating at 4 °C (12 000 rpm) for 10 min. The upper phase was collected, 60% (v/v) isopropanol and 10% (v/v) 3 M Na-acetate (pH 5.2) were added to precipitate RNAs overnight at -20°C. RNAs were subsequently pelleted by 10 min centrifugation (12 000 rpm*)*, washed with chilled 70% (v/v) ethanol and resuspended in diethylpyrocarbonate-treated water. Because of the susceptibility of RNA to degradation by ribonucleases (RNases), the purification procedure must be done quickly and samples must be kept at 4°C during processing to prevent RNA degradation.

### 2.8.2 Determination of integrity of mRNA

When total RNA is extracted from cells, it consists of a complex mixture of polynucleotide chains that vary in lengths. The 23 S to 25 S and 16 S to 17 S fractions contain rRNAs, while the 4 S fraction includes tRNAs and other small RNAs. Most of the RNAs in these three classes are relatively stable. In contrast, mRNAs are far less abundant in the cell and typically make up only 1% to 2% of the total cellular RNA. To synthesize the cDNA, enough template (mRNA) is necessary. The integrity of the mRNA was checked by agarose-gel electrophoresis, using 18 S and 28 S ribosomal RNAs as markers.

To prepare 30 ml 1% agarose-gel, 0.3 g agarose was mixed in 24 ml 0.04 M morpholinopropanesulfonic acid (MOPS pH 7.0). The slurry was heated in a microwave oven until the agarose was dissolved. When the gel had cooled down to approximately 60°C, 6 ml 37% (v/v) formaldehyde were added, mixed and poured into the mold. 5 µg RNA was mixed with loading buffer, which contained ethidium bromide, and loaded into the slots of the submerged gel. After gel electrophoresis at 80 V for 1 h, the gel was photographed using UV light. Since ethidium bromide contains a planar group that intercalated between the stacked bases of RNA or DNA, the fixed position of this group and its close proximity to the bases causes the dye bound to RNA or DNA to display an increased fluorescence compared to dye in free solution. UV irradiation absorbed by the necleotides at 260 nm and transmitted to the dye, or irradiation absorbed at 300 nm and 360 nm by the bound dye itself, is emitted at 590 nm in the red-orange region of the visible spectrum. As visualized by ethidium bromide staining of gels, the mRNA should form a continuous smear (~10 S - ~30 S) with most of the molecules migrating at about 16 S-18 S. There is usually ribosomal RNA present in the mRNA. The sharpness of the ribosomal RNA bands provides a rough indication of whether the mRNA is degraded.

### 2.8.3 Spectrophotometric determination of DNA or RNA

For quantifying the amount of DNA or RNA, readings were taken at a wavelength of 260 nm, which allows calculation of the concentration of nucleic acid in the sample. An $OD_{260}$ of 1 corresponds to 50 µg/ml for double-stranded DNA, 40 µg/ml for RNA. The ratio between the readings at 260 nm and 280 nm ($OD_{260}/OD_{280}$) provides an

estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have $OD_{260}/OD_{280}$ of 1.8 and 2.0, respectively. If there is contamination with protein or phenol, the $OD_{260}/OD_{280}$ will be significantly less than the values given above, and accurate quantification of the amount of nucleic acid will not be possible.

### 2.8.4 First-strand cDNA synthesis

Eukaryotic mRNA contains a 3‘ sequence of polyadenylic acid, which is different from other RNAs (rRNA, tRNA and prokaryotic RNAs). With an oligo-dT primer, mRNA can be used as a template for reverse transcriptase to synthesize a complementary DNA (cDNA) on it. Compared with genomic DNAs, cDNAs have no introns or non-transcriptable regions, which can directly reflect the activity of gene expression.

The cDNA was synthesized following the manufacturer's instructions in SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). 5 µg total–RNA ( 1-11 µl), 1 µl 10 mM dNTP-mix and 1 µl Oligo-dT-Primer (0.5 µg/µl) were filled up to 12 µl with DEPC-Water. After incubation at 65 °C for 5 min to remove any RNA secondary structure, the following reagents were added:

2 µl 10x PCR-buffer (pH 8.3)

4 µl 25 mM $MgCl_2$

2 µl 0.1 M DTT

1 µl RNaseOUT™ Recombinant RNase Inhibitor

The mixture was incubated at 42°C for 2 min and 1 µl of Superscript™ II RT (50 Units) was added to start the reverse transcription. After incubation at 42°C for 50 min, the reverse transcriptase was denatured by 70°C heat for 15 min. 1 µl RNAse H was added and incubated at 37°C for 10 min to cleave the RNA. The synthesized first-strand cDNA was aliquotated and stored at –20°C.

### 2.8.5 Identification of plasma membrane $H^+$ ATPase by PCR

PCR (Polymerase chain reaction) is a powerful tool to find new genes or gene families. Most genes can be grouped into families, which share structural similarities (Telenius et al., 1992 ). By aligning the plasma membrane $H^+$ ATPase mRNA sequences from a number of plants we found that plasma membrane $H^+$ ATPase of *Vicia faba* can be used as a guide sequence.

The degenerated primers were then designed as follows:

| | | |
|---|---|---|
| s 670 Forward | ACT GG(AG) GT(GT) CA(CT) AC(AC) TT(CT) TT | 57°C |
| as 2082 Reverse | GTC AT(AG) AT(AG) GTA CC(AG) TCA TT | 55°C |
| s 1294 Forward | AGC AAA GG(GT) GC(AT) CC(ACT) GAG CAG AT | 72°C |
| as2082 Reverse | GTC AT(AG) AT(AG) GTA CC(AG) TCA TT | 55°C |
| s 1294 Forward | AGC AAA GG(GT) GC(AT) CC(ACT) GAG CAG AT | 72°C |
| as1776 Reverse | AC(AT) CCA TC(AT) CC(AGT) GTC AT(AGT) CCA | 63°C |

A 10 µl pre-mix for every PCR-tube was set up as following:

1 µl 10 x PCR buffer (containing $MgCl_2$ 15 mM)

0.1 µl forward primer (100mM)

0.1 µl reverse primer (100mM)

0.2 µl dNTP (10 mM)

8.5 µl sterile $H_2O$

0.1µl *Taq* Polymerase

The PCR program is designed as follows:

| | | |
|---|---|---|
| Denaturation | 94°C | 60 s |
| Denaturation | 94°C | 30 s |
| Annealing | 55°C | 30 s |
| Elongation | 72°C | 1 min |

After 24 cycles, the PCR fragments were separated on a 1% agarose gel. Labelled DNA fragments were visualized by UV-graphy. Amplified fragments with apropriate length were then cloned and sequenced. Identification of the different genes was carried out using the blast programme from EMBNET (www.ch.embnet.org).

### 2.8.6 Cloning and Sequencing the PCR Product

**1) Cloning of PCR product**

The TA Cloning® Kit with pCR®2.1(Invitrogene) provides a quick, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into the plasmic vector. *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3‘ ends of PCR prducts. The linearized vector (pCR®2.1) is supplied with single 3‘-thymidine (T). This allows PCR inserts to ligate efficiently with the vector.

The set up of the TA® Cloning is described below:

| | |
|---|---|
| PCR product | 2 µl |
| 10X Ligation Buffer | 1 µl |
| pCR® 2.1 vector (25ng/µl) | 1 µl |
| Sterile water | 4 µl |
| T4 DNA Ligase (4.0 Weiss units) | 1 µl |

The ligation reaction was incubated overnight in the water bath at 14°C.

For transformation, not more than 2 µl reaction mixture were added into a tube of one shot® chemically competent TOP10 *E. coli* cells and incubated for 30 minutes on ice. After heat-shocking the cells for 30 seconds at 42°C, the tube was immediately transferred to ice. 250 µl of room temperature S.O.C. medium were added to a tube. The tube was capped tightly and shaken horizontally (140 rpm) at 37°C for 1 h.

50 µl from transformation was spreaded on a prewarmed (37°C) LB (Luria-Bertani) plate and incubated overnight at 37°C.

**2) Identification of bacterial colonies that contain recombinant plasmid**

The vectors in current use carry a short segment of *E. coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*Lac Z*). Embedded in this coding region is a polycloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of β-galactosidase. Vectors of this type are used in host cells that code for the carboxy-terminal portion of β-

galactosidase. Although neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to form an enzymatically active protein. This type of complementation, in which deletion mutants of the operator-proximal segment of the *Lac Z* gene are complemented by β-galactosidase-negative mutants that have an intact operator-proximal region, is called α-complementation. The $lac^+$ bacteria that result from α-complematation are easily recognized because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in the production of an amino-termianl fragment that is not capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white clonies. The development of this simple color test has greatly simplified the identifiction of recombinants constructed in plasmid vector of this type.

**3) The fastest way to screen bacterial colonies**

26 white clonies were picked with a a fresh toothpick and streaked onto a fresh replica LB agar plate using a numbered template to grow in samll–scale cultures overnight at 37°C. The rest of bacteria on the tip of toothpick was dipped into a PCR tube, which was labelled corresponding to the agar plate.

A 10 µl pre-mix for every PCR tube was set up as follows:

1 µl 10 x PCR buffer (containing $MgCl_2$ 15 mM)
0.1 µl M13 forward primer (100mM)
0.1 µl M13 reverse primer (100mM)
0.2 µl dNTP (10 mM)
8.5 µl sterile $H_2O$
0.1µl *Taq* Polymerase

PCR program is designed as follows:

| | |
|---|---|
| 94°C | 30 s |
| 55°C | 30 s |
| 72°C | 1 min |

After 24 cycles, the PCR fragments were separated on a 1% agarose gel. Likely candidate colonies have DNA fragments with appropriate length.

**4) *E. coli* cultivation and plasmid DNA isolation**

Candidate colonies were picked in 3 ml LB medium (Luria-Bertani) and incubated at 37°C by 200 rpm shaking overnight. For purification of plasmid DNA, QIAprep® Spin Miniprep Kits was used. The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane in the presence of high salt. The procedure consists of three basic steps:

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep membrance
- Washing and elution of plasmid DNA

The overnight cultures of *E. coli* were transferred to 2 ml tubes and centrifugated at 13000 rpm for 30 s. Pelleted bacterial cells were resuspended in 250 µl P1 thoroughly without any visible cell lumps. 250 µl buffer P2 were added and the tube was inverted 4-6 times gently to lysate bacterial cells. 350 µl buffer N3 were added and the tube was inverted 4-6 times gently again, the lysate was subsequently neutralized and adjusted to high-salt binding conditions. The lysate was cleared by centrifugation at maximun speed in a tabletop microcentrifuge for 10 min. The supernatants were applied to the QIAprip column by decanting. The silica-gel membrane in the column ensures that only DNA will be adsorbed, while RNA, cellular proteins and metabolites are not retained on the membrane. They were found in the flow-through after 30 s centrifugation and discarded. The QIAprep spin column was washed by adding 0.5 ml buffer PB, buffer PE following short centrifugation for 30 s, respectively. To elute DNA, 20-30 µl sterile water was added to the center of each QIAprep column and incubated for 1 min. The column was centrifuged for 1 min to collect the elution. After spectrophotometric determination of plasmid DNA, the samples were diluted to 100 ng/µl. Plasmids were sequenced by the ABI Prism 310 Genetic Analyzer.

### 2.8.7 Real-time PCR

Real-time reverse transcription (RT) PCR is currently the most sensitive method for the detection of low-abundance mRNAs. In contrast to classic techniques, such as Northern blot analysis and RNase protection assays, which require large amounts of total RNA, RT-PCR assay is capable of quantifying mRNA levels from samples as small as individual cells.

To compare the mRNA expression of plasma membrane $H^+$ ATPase and other enzymes in the development of proteoid roots under P deficiency, cDNAs of proteoid roots from emergence to 8 d were used as templates for real time PCR. All synthesized cDNAs corresponded to 100-200 ng total RNA. Gene specific primers were designed using Primer Express Software according to the software guidelines. All primers were purchased from Carl-Roth. The primers from reference gene and taget genes, which were reported by Uhde-Stone et al (2003), were summarized below.

| | | | |
|---|---|---|---|
| Ubiquitin | Forward Primer | GGCAAGACCATCACTCTCGA | 62°C |
| 227 bp | Reverse Primer | ACCTCAAGGGTGATGGTCT | 58°C |
| Plasma membrane $H^+$ ATPase | Forward Primer | CCTGAGCAGATCATGATCCT | 60°C |
| 310 bp | Reverse Primer | GAAGATGGATGCATATTCGT | 54°C |
| ATP-citrate lyase | Forward Primer | TTAGTCATGGCGACTGGACA | 60°C |
| 198 bp | Reverse Primer | TGGGATTGCAATTTCTTCCT | 60°C |
| Citrate synthase | Forward Primer | CCGTCGTCGTTTTCATTCTT | 60°C |
| 216 bp | Reverse Primer | TTGACACGTTCCTGGTGTTC | 60°C |
| Fructokinase | Forward Primer | CCAACCGTCTCTGGTGTCTC | 64°C |
| 293 bp | Reverse Primer | CATATCAGCACTCGGGTTCC | 62°C |
| PEP carboxylase 6E-48 | Forward Primer | TGACGTCAGGGATAGGCTCT | 60°C |
| 200 bp | Reverse Primer | CTTGCCTCAAGAAATCGAGAA | 60°C |
| Phosphoglucomutase 2E-36 | Forward Primer | GGTTTTGGCCTGGCTATCTA | 59°C |
| 196 bp | Reverse Primer | TGAGGACTGCAGTTTGACCA | 60°C |
| Malate dehydrogenase | Forward Primer | CCAAAAACCCAGTTCGTGTT | 60°C |
| 199 bp | Reverse Primer | CCTTTAAGAAGGGGGAATGC | 60°C |

For each 10 µl Mast Mix, the following reagents were mixed:

| | |
|---|---|
| 1 µl | 10xBuffer Bio Therm (containing 15 mM $MgCl_2$) |
| 6.2 µl | sterile Water |
| 0.2 µl | dNTP (10 mM) |
| 0.1 µl | *Taq* Polymerase Biotherm |
| 0.15 µl | sense primer (100µM) |
| 0.15 µl | antisense primer (100 µM) |
| 0.2 µl | Sybr Green (1:2000) |
| 2 µl | diluted cDNA |

Real-time PCR assays for all specific genes were performed on the Rotor-Gene 2000/3000 real-Time Amplification Thermal Cycling System.

To analyze quantitative data, the relative quantitation method was used. This method can determine the changes in steady-state mRNA levels of a gene across multiple samples and gives a result relative to the levels of an endogenous control RNA. This reference gene is often a house-keeping gene, which is not influenced by the treatment example. In this experiment, ubiquitin is selected as house-keeping gene in accordance with Uhde-Stone et al. (2003). Therefore, relative quantification does not require standards with known concentrations. It is based on the expression levels of a target gene versus a reference gene and in many experiments is adequate for investigating physiological changes in gene expression levels.

In this model, a standard curve is generated from a dilution series of cDNA. The units used to describe the dilution series are relative and based on the dilution factor of the standard curve. A sample has to be analyzed twice, firstly with the gene of interest and secondly with the endogenous control. Two standard curves have to be run to obtain two values (endogenous control and gene of interest) for the one sample. The relative values automatically calculated by using the standard curves are then divided by each other. The normalized data are obtained by dividing the gene of interest by the reference gene, which resulted in a normalized value. No unit is given to this value. The ratios of all other samples are obtained in the same way. All the values are

compared to a so-called calibrator sample. A calibrator sample in our expriment is the sample of 1 d-old proteoid roots, which was arbirtarily designated (abundance set to 1X) and the normalized values for the remaining samples are expressed as X-fold of 1X.

## 2.9 Chemicals

**Agar** (Agar Agar Kobe I): Serva 11392

**Ammonium-Molybdat** ($(NH_4)_6Mo_7O_{24}$): 82,3 % $MoO_3$; Sigma

**Anthracene-9-Carboxylic acid**: $C_{15}H_{10}O_2$, Sigma

**AO = Acridin Orange (3, 6-Bis[Dimethylamin] Acridin-Base)**: ca. 95% Farbstoffgehalt; Sigma

**L(+)-Ascorbinsäure**: mid. 99.7% Reinheit (idodometrisch); Merck

**Brij 58** (Polyoxyethylen-20-cetylether): Sigma

**Bromkresol Purpur Na-Salz** ($C_{12}H_{15}Br_{205}xNa$): reinst; Serva

**BSA** (Bovines Serumalbumin): fettsäurefrei, ≥ 96% Albumin; Sigma

**BTP** (1,3-Bis[tris(hydroxymethyl)methylamino]propan: mind. 99 % Reinheit (Titration); Sigma

**Coomassie Brilliant Blue G-250**: Calbiochem Corp., La Jolla

**Dextran T 500**: durchschnittliches Molekulargewicht = 485 000 g/mol; Sigma

**Dikaliumhydrogenphosphat** ($K_2HPO_4$): p.a.; Merck

**DTT** (DL-Dithiothreitol): 99 % Reinheit (Titration); Sigma

**EGTA** (Ethylenglycol-bis(ß-aminoethylether)N,N,N`,N`-Tetraessigsäure): 97 % Reinheit; Sigma

**Fusicoccin from *Fusicoccum amygdali*** $\mathbf{C_{36}H_{56}O_{12}}$, approx. 90 %, Sigma

**Glycerin**: 99 + % Reinheit; Sigma

**Gramicidin D**: von Bacillus brevis, 1080 µg Gramicidin $mg^{-1}$; Sigma

**Kaliumchlorid** (KCl): p.a.; Fluka

**Kaliumdihydrogenphosphat** ($KH_2PO_4$): p.a.; Fluka

**Kaliumjodid** (KJ): ≥ 99.5% Reinheit (argentometrische Titration); Fluka

**Kaliumnitrat** ($KNO_3$): p.a.; Merck

**Kaliumsulfat** ($K_2SO_4$): p.a.; Fluka

**2-Mercaptoethanol**: mind. 99% Reinheit (GC); Serva

**MES** (2-[N-Morpholin]ethansulfonsäure): freie Säure, mind. 99,5% Reinheit (Titration).; Serva

**Magnesiumsulfat** ($MgSO_4$): p.a.; Fluka

**$Na_2$ATP** (adenosin 5´-Triphosphat, $Na_2$Salz): 98 % Reinheit; Merck

**Natriumazid** ($NaN_3$): p.a.; Merck

**Natriumcitrat-Dihydrat**: p.a.; Merck

**Natrium-Metaarsenit** ($NaAsO_2$): mind.99 % Reinheit; Sigma

**Natriummolybdat** ($Na_2MoO_4$): p.a.; Merck

**Natrium-Orthovanadat** ($Na_3VO_4$): mind. 95 % Reinheit; Sigma

**PEG 3350** (Polyethylenglycol): durchschnittliches Molekulargewicht = 3350 g/mol; Sigma

**PEP** (Phosphoenolpyruvat): Boehringer Mannheim GmbH

**PMSF** (Phenylmethylsulfonylfluorid): > 99 % Reinheit(GC) ; Sigma

**Pyruvat-Kinase**: 1 000 U; Sigma

**D(+)-Saccharose**: für biochemische & mikrobiologische Zwecke; Merck

**SDS** (Natriumdodecylsulfat): 99 % Reinheit; Sigma

**Valinomicin**: mid. 98% Reinheit (TCL); Sigma

**Kit**:

Citrate and Malate Test Kit, Boehringer, Mannheim

SuperScriptTM First-Strand Synthesis System for RT-PCR, Invitrogen

GeneRacer™ Kit, Invitrogen

TOPO TA Cloning® Kit, Invitrogen

One Shot® Top10 Chemically Competent E.coli, Invitrogen

QIAprep® Spin Miniprep Kit, Qiagen

## 2.10 Online-Data banks

Clustl W 1.82: Alignment: http://www2.ebi.ac.uk/clustlw/

BLAST Search: NCBI-Homology-Date bank: http://www.ncbi.nlm.nih.gov/blast/

ExPASY-Translate tool: http://expasy.cbr.nrc.ca/tools/dna.html

# 3. Results

## 3.1 Development and acidifying activity of proteoid roots of P-deficient white lupins

### 3.1.1 Development of proteoid roots under P deficiency

The operational definition of a proteoid root is a secondary root with densely clustered rootlets (Fig. 4), following the morphological description of previous authors (Purnell, 1960; Bardner et al., 1981; Dinkelaker et al., 1995, Watt and Evans, 1999b).

After about 10 d of cultivation, proteoid roots became visible in P-sufficient plants. In comparison with P-sufficient plants, the induction of proteoid roots in P-deficient plants occurred 1 to 2 d earlier. After 21 d, the average number of proteoid roots per plant was 61 ± 8 (means ± SE in four independent experiments) for P-deficient plants and 21 ± 2 for P-sufficient plants. Typically, P-sufficient plants produced only one long proteoid root on a primary lateral root, whereas P-deficient plants showed three or more sequentially clustered proteoid roots on a primary lateral root (Fig. 4). P deficiency resulted in an increase of root weight with a simultaneous decrease of shoot weight. After 21 d of cultivation, 7.4 ± 1.2 g $plant^{-1}$ of fresh root weight for control plants and 10.3 ± 2.1 g $plant^{-1}$ for P-deficient plants were determined. The shoot fresh weights were 8.2 ± 0.4 g $plant^{-1}$ for control plants and 6.3 ± 0.1 g $plant^{-1}$ for P-deficient plants. The P concentrations of dry shoot matter were 6.2 ± 0.3 mg $g^{-1}$ for control plants and 1.4 ± 0.2 mg $g^{-1}$ for P-deficient plants. Despite lower P concentration in shoots of P-deficient plants, no typical symptom of P deficiency was observed.

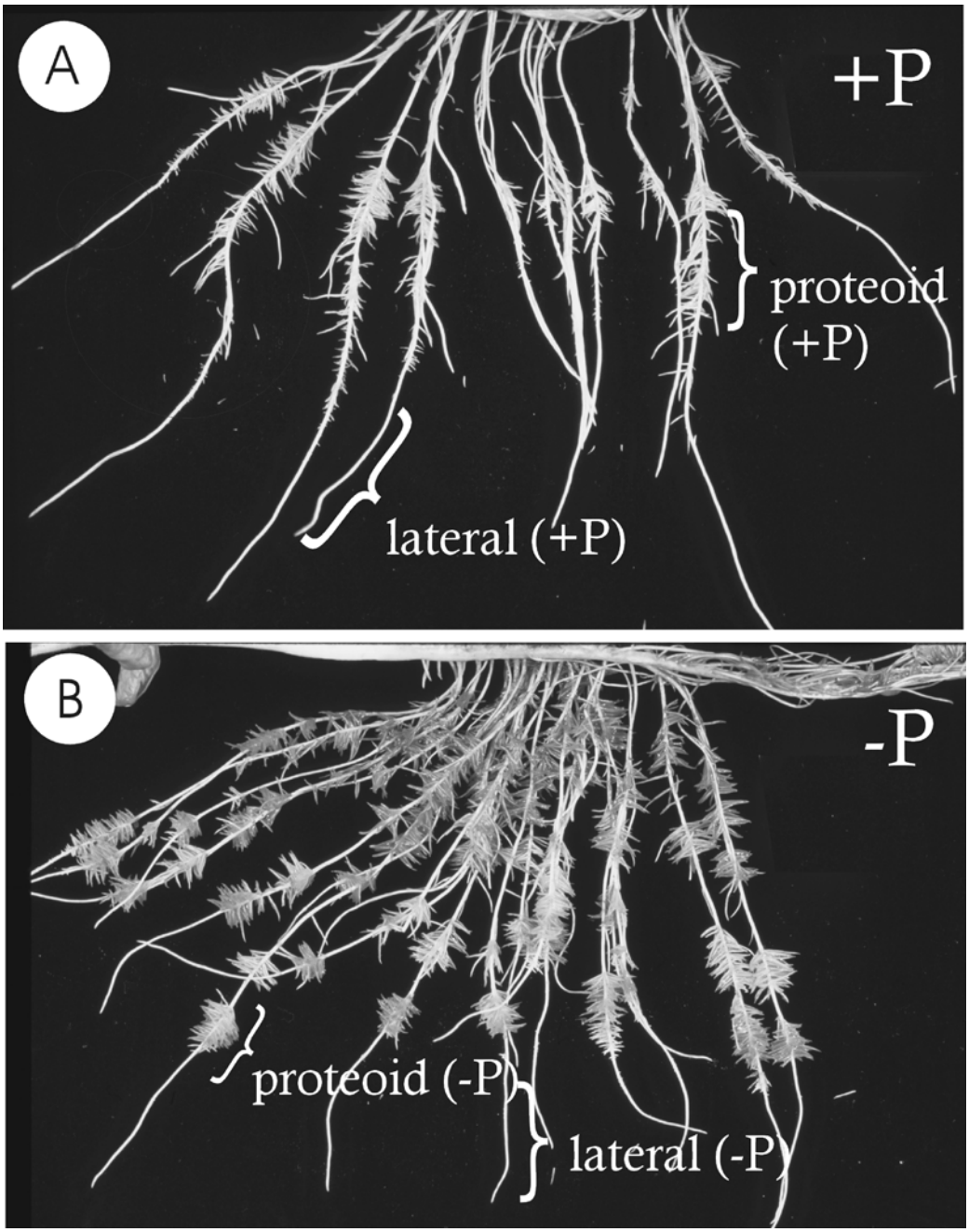


Figure 4. Proteoid roots produced by white lupin with (A) and without (B) phosphate. Plants were grown in a solution culture for 3 w. Plasma membrane was isolated from different types of roots: proteoid roots of P-sufficient plants; lateral roots of P-sufficient plants,; active proteoid roots (the youngest, fully developed proteoid root) of P-deficient plants; lateral roots of P-deficient plants.

Net $H^+$ release by plant roots during the whole cultivation period was recorded by means of a pH stat system. Up to 12 d, plants of both treatments showed comparable $H^+$ release. However, after 12 d of cultivation, P-sufficient plants showed increasing consumption of $H^+$, whereas P-deficient plants continued to release $H^+$ with a higher rate than before (Fig. 5). This concurred with an extensive production of proteoid roots by P-deficient plants during this period.

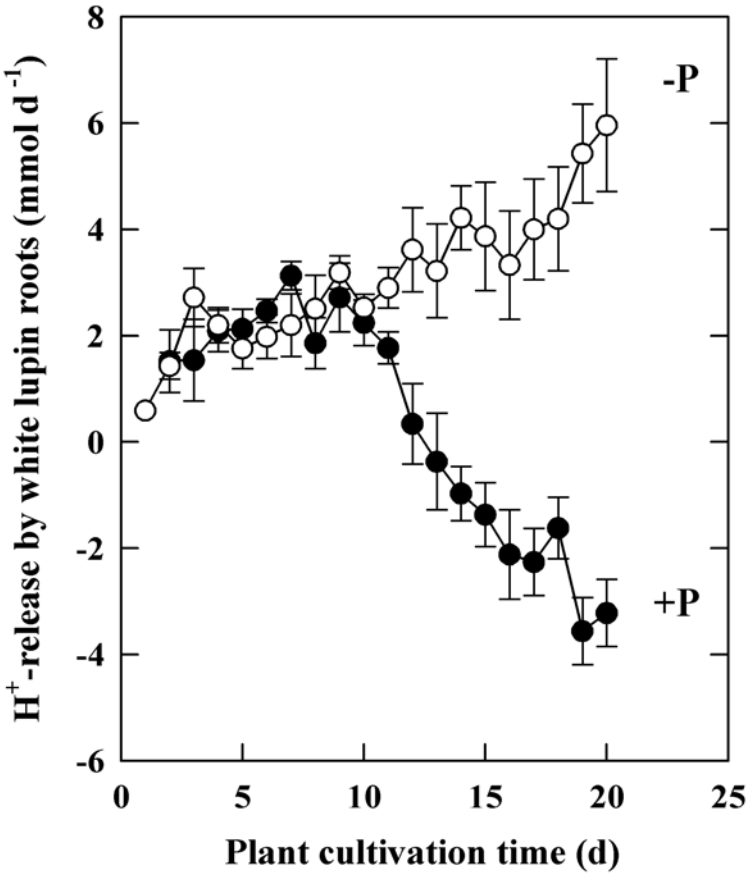


Figure 5. $H^+$ release by white lupin roots during a 3-week cultivation period. Plants were grown in nutrient solution, pH of which was kept constant at pH 6 by means of a pH stat system (Schott, Mainz, Germany). The amount of NaOH (or $H_2SO_4$) used for maintaining pH 6 was recorded daily and used for the calculation of net $H^+$ release. Values represent means ± SE of four independent experiments.

### 3.1.2 Acidification by proteoid roots under P deficiency

After 21 d cultivation in nutrient solution, the net $H^+$ release of proteoid roots was determined using an agar sheet with bromocresol purple as pH indicator. Only the fully developed proteoid roots showed strong rhizosphere acidification (Fig. 6, left) and therefore were defined as active proteoid roots. There was no acidification either by old proteoid roots by P-deficient plants or by the proteoid roots of P-sufficient plants (not shown). The acidification of active proteoid roots was completely inhibited by 1 mM vanadate (Fig. 6, right), indicating the involvement of active $H^+$ pumping of the plasma membrane $H^+$ ATPase *in vivo*.


Figure 6. Identification of active proteoid roots (left) and inhibitory effect of vanadate on rhizosphere acidification (right). Plants had been grown in nutrient solution without phosphate for 3 w. After washing with deionized water, roots were carefully spread on the surface of agar sheet ( 0.75% [w/v] agar, 0.006% [w/v] bromocresol purple, 1 mM $CaSO_4$, and 2.5 mM $K_2SO_4$, pH 6 ) and gently pressed into the agar sheet and incubated for 5 h under light in a growth chamber. For the study of the inhibitory effect of vanadate (right), 1 mM vanadate was included on the right side of the agar sheet.

## 3.2 Quatitative investigation of relationships between the net processes of citrate release and release of $H^+$ by active proteoid roots

### 3.2.1 Cations and anions exuded by active proteoid roots under P deficiency

*In situ*, active proteoid roots of white lupin adapted to P-deficiency were able to exudate not only $H^+$, citrate and malate, but also other ions such as $K^+$, $Na^+$, $Mg^{2+}$, $Ca^{2+}$, $SO_4^{2-}$ and $Cl^-$ (Table 1).

Table 1. Effect of incubation periods for root exudation on the releases rate of various ions by intact active proteoid roots of white lupin adapted to P deficiency. Plants had been cultivated for 3 w in nutrient solution without phosphate. After an overnight pre-incubation in a test solution (0.5mM $K_2SO_4$, $Na_2SO_4$, $CaSO_4$, pH 6), the root exudates were collected with 3 ml test solution in a special chamber. The values represent means ± SE of six independent experiments.

| Ions | Release rate [µmol (g FW)$^{-1}$ h$^{-1}$] | | | |
|---|---|---|---|---|
| | 0-15 min | 0-30 min | 0-60 min | 0-120 min |
| $H^+$ | 3.63 ± 0.61 | 2.70 ± 0.39 | 1.94 ± 0.36 | 1.41 ± 0.34 |
| $K^+$ | 7.11 ± 0.71 | 3.87 ± 0.57 | 2.44 ± 0.34 | 1.40 ± 0.09 |
| $Na^+$ | 5.30 ± 0.94 | 2.39 ± 0.37 | 1.43 ± 0.33 | 0.77 ± 0.13 |
| $Mg^{2+}$ | 1.46 ± 0.09 | 0.78 ± 0.06 | 0.50 ± 0.05 | 0.31 ± 0.03 |
| $Ca^{2+}$ | 0.30 ± 0.72 | 0.16 ± 0.33 | 0.52 ± 0.33 | 0.27 ± 0.15 |
| Citrate | 2.75 ± 0.34 | 1.66 ± 0.14 | 1.20 ± 0.14 | 0.77 ± 0.14 |
| Malate | 0.18 ± 0.01 | 0.15 ± 0.01 | 0.12 ± 0.00 | 0.09 ± 0.01 |
| $SO_4^{2-}$ | 2.59 ± 1.60 | 1.82 ± 0.61 | 1.13 ± 0.50 | 0.77 ± 0.25 |
| $Cl^-$ | 2.25 ± 0.40 | 0.44 ± 0.08 | 0.34 ± 0.06 | 0.14 ± 0.06 |

Under the same conditions there was, however, no detectable exudation of $NO_3^-$ and $HPO_4^{3-}$ by the active proteoid roots. With extending incubation time, there was a decline of exudation rates for all ions with an exception of $Ca^{2+}$. During the incubation time of 15 min the exudation rates of $K^+$ and $Na^+$ were markedly higher than that of the $H^+$. In addition, the exudation rates of $Ca^{2+}$ and $Mg^{2+}$ weres markedly lower than those of $H^+$, $K^+$ and $Na^+$. This difference in exudation rates among ions was changed with the extending of incubation time. When incubation time was extended to 120 min an exudation rate of 1.4 µmol (g FW)$^{-1}$ h$^{-1}$ for $H^+$ and $K^+$ was measured, while an exudation rate of $Na^+$ of 0.77 µmol (g FW)$^{-1}$ h$^{-1}$, approximately two times lower than that for $H^+$ and $K^+$, was determined. For the incubation time of 120 min, exudation rates of 0.31 and 0.27 µmol (g FW)$^{-1}$ h$^{-1}$ were measured for $Mg^{2+}$ and $Ca^{2+}$, respectively. Based on ion charges there was a change of contribution for each ion species to the total positive charges exudated by proteoid roots during the incubation period from 15 to 120 min. For the incubation time of 15 min the contribution to total positive charges of $H^+$, $K^+$, $Na^+$, $Mg^{2+}$, $Ca^{2+}$, was 19%, 36%, 27%, 15%, 3%, respectively, while for the incubation time of 120 min these were changed to 30%, 30%, 16%, 13%, 11%, respectively.

For anions, the exudation rate of citrate was from 2.75 to 0.77 µmol (g $^{-1}$ FW h$^{-1}$) for incubation time from 15 to 120 min. In addition, the citrate exudation rate was markedly higher than that of malate. In comparison with malate, citrate release rate was 15, 11, 10 and 8.5 times higher for the incubation time of 15, 30, 60 and 120 min, respectively. The exudation rates of $SO_4^{2-}$ and $Cl^-$ were comparable for the incubation time of 15 min. However, with extended incubation time, the exudation rate of $SO_4^{2-}$ was markedly higher than that of $Cl^-$. Throughout all incubation time periods, citrate made a contribution of more than 50% to total negative charges exudated by proteoid root cells, assuming each citrate ion had 3 negative charges ($pK_{S1}$ = 2.94, $pK_{S2}$ = 4.14, $pK_{S3}$ = 5.82 ). More than 30% total negative charges exudated by active proteoid roots were carried by $SO_4^{2-}$. The contribution of malate (with 2 negative charges, $pK_{S1}$ = 3.4, $pK_{S2}$ = 3.4) and $Cl^-$ to total negative charges exudated by proteoid roots was less significant. When total positive and negative charges were compared, total positive

charges were approximately 15% higher than total negative charge troughout all incubation periods.

### 3.2.2 Relationship between citrate exudation and exudation of other ions by active proteoid roots

Citrate exudation rate was closely correlated with the exudation rates of $H^+$, $K^+$, $Na^+$, and $Mg^{2+}$, respectively (Fig. 7 A,D). Equally close relationships were found also between malate exudation rate and the exudation rates of the cations (Fig. 7 E,H). However, there was no relationship between exudation rate of $Ca^{2+}$ and exudation rates of citrate or malate (data not shown). The relationships between exudation rates of different cations and $SO_4^{2-}$ or $Cl^-$ were also calculated. The determination coefficients ($r^2$) of these relationships were significantly lower ($r^2 < 0.5$, data not shown) than those found between citrate or malate and different cations.

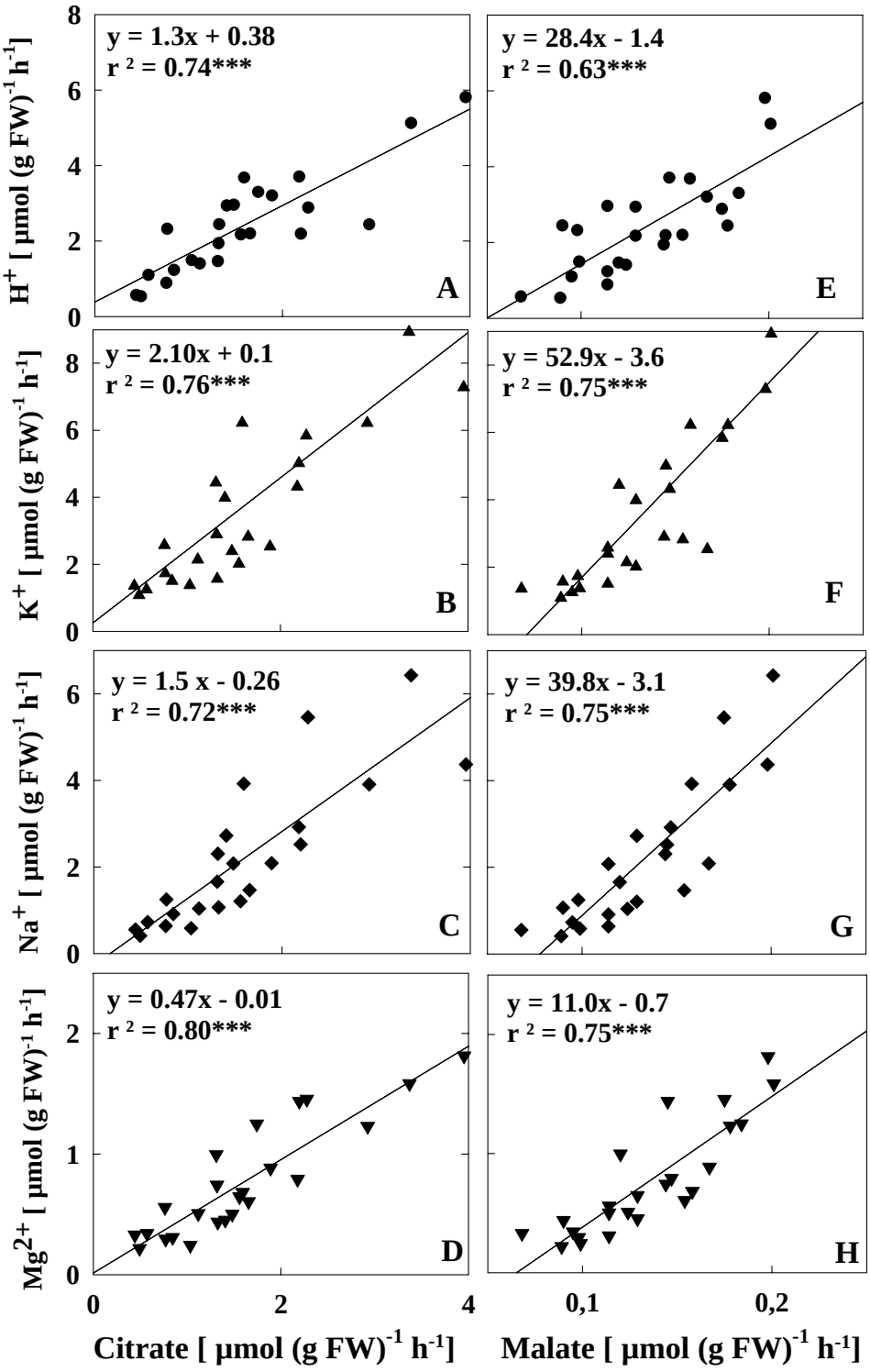


Figure 7. Relationships of proteoid root exudation rates of citrate and malate with exudation rates of $H^+$, $K^+$, $Na^+$ and $Mg^{2+}$. Plants had been grown in solution culture without phosphate for 3 w. Active proteoid roots were selected for the collection of root exudation. The time periods for collection of root exudation were 15, 30, 60 and 120 min, respectively. The data were when pooled from six independent experiments.

Furthermore, based on charge equivalents, for all analyzed ions there was a very close relationship for total charges between exudated cations and anions, assuming that citrate and malate had 3 and 2 negative charges, respectively (Fig 8). The regression slope of 0.84 indicated only a small deviation between total positive and negative charges, being higher for positive than for negative charges. This good charge balance between exudated cations and anions indicates a proper consideration and precise quantification of the relevant ions exudated by active proteoid roots of white lupins adapted to P-deficiency.

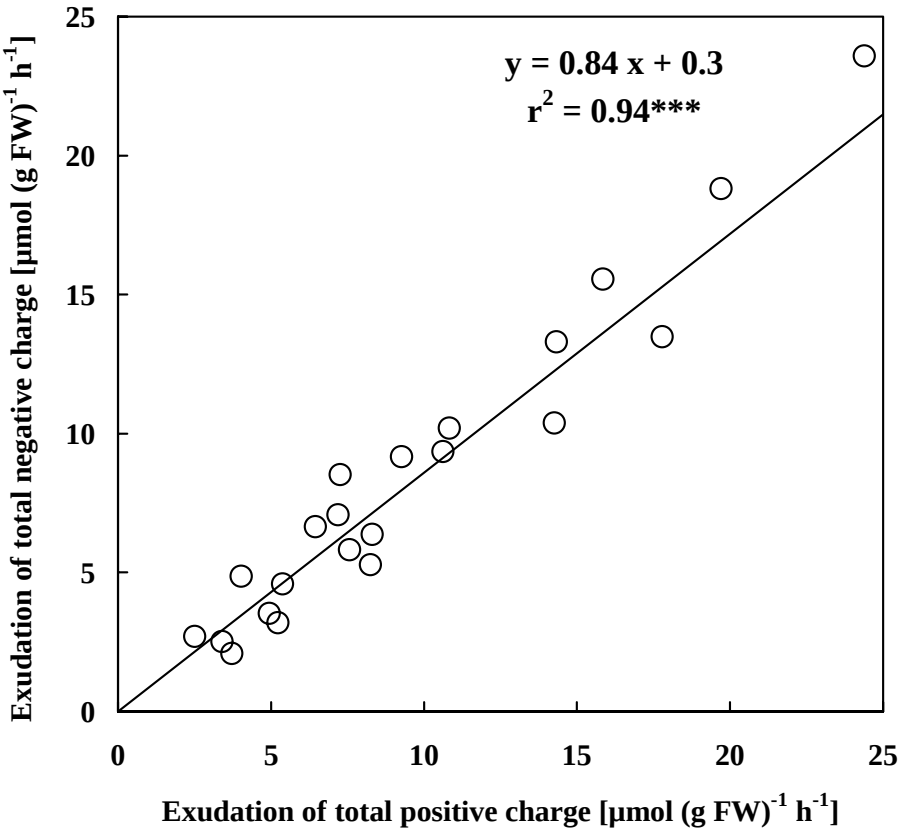


Figure 8. Relationship between total positive and negative charges exudated by active proteoid roots adapted to P deficiency. The plants had been grown in solution culture without phosphate for 3 w. Active proteoid roots were selected for the collection of root exudates. The total positive charges were calculated as sum of exuded $H^+$, $K^+$, $Na^+$, $Mg^{2+}$ and $Ca^{2+}$, whereas for anions exuded $Citrate^{3-}$, $Malate^{2-}$, $SO_4^{2-}$ and $Cl^-$ were summed. The data were pooled from six independent experiments with four collection periods (15, 30, 60 and 120 min) for each.

### 3.2.3 Effect of fusicoccin on the exudation of ions by active proteoid roots

Because the goal of the study was to investigate the relationship between different ions, especially between citrate and $H^+$, during the exudation of citric acid by proteoid root cells, the incubation time with various pharmacological drugs was limited from 15 to 60 min to minimize possible secondary effects of the drugs on the ion trasnport across plasma membrane.

Fusicoccin, a phytotoxin produced by *Fusicoccum amygdali*, is known as a stimulator of plasma membrane $H^+$ ATPase of plant cells and results in increased $H^+$ release (Marré, 1979; Sze et al., 1999). Throughout all three incubation times, 5 µM fusicoccin significantly increased $H^+$ exudation of active proteoid roots of white lupin adapted to P deficiency (Fig. 9A). In contrast, the exudation of $K^+$ by active proteoid roots was significantly decreased by fusicoccin (Fig. 9B). There was no significant effect of fusicoccin on the exudation of $Na^+$ and $Mg^{2+}$ (Fig. 9C, D). Fusicoccin significantly increased the exudation rate of citrate for both incubation times of 15 and 60 min but not at 30 min (Fig. 9E). In addition, for malate a significantly higher exudation rate relative to control was observed for the incubation time of 30 and 60 min (Fig. 9F). There was no effect of fusicoccin on the exudation of $SO_4^{2-}$ and $Cl^-$ (Fig. 9G, H).

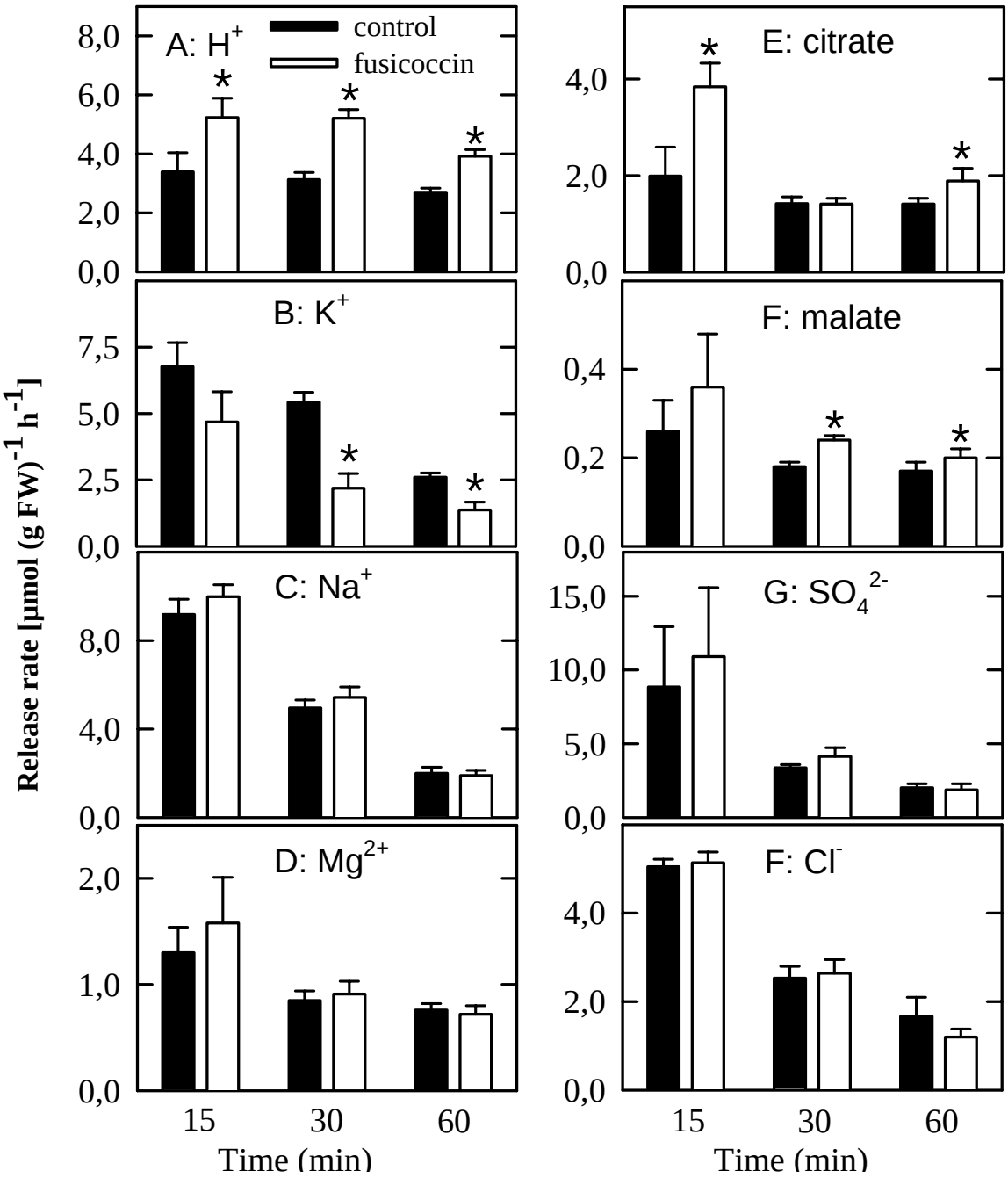


Figure 9. Effect of fusicoccin on the exudation rate of active proteoid roots. The plants had been grown in solution culture without phosphate for 3 w. Active proteoid roots were selected for the collection of root exudates. In one treatment, fusicoccin (5 µM) was applied to the solution for collection of root exudates. Each value represents the mean ± SE of four independent experiments. Significant differences ($P < 0.05$) between the control and fusicoccin treatment were calculated by paired *t*-test and is indicated by *.

### 3.2.4 Effect of vanadate on the exudation of ions by active proteoid roots

Vanadate is an inhibitor of all P-type ATPases, which forms an analog of the enzyme-phosphate (E-P) transition state and thus blocks the reaction cycle (Palmgren, 1998). To evaluate the contribution of plasma membrane $H^+$ ATPase to exudation of carboxylic anions by active proteoid roots of white lupin adapted to P deficiency, 350 µM vanadate were included in the incubation solution. Vanadate significantly reduced $H^+$ exudation by active proteoid roots throughout all three incubation periods (Fig. 10 A). In comparison with the control, a reduction of $H^+$ exudation rate by more than 50% was achieved by 350 µM vanadate. In addition, a significant decrease in $Mg^{2+}$ exudation was also detected for the incubation time of 30 min (Fig.10 D). In contrast, $Na^+$ exudation rate was significantly increased by vanadate for all incubation periods (Fig. 10 C), while a significant increase for $K^+$ exudation was only found for the incubation time of 30 min (Fig. 10 B).

Vanadate significantly decreased the exudation rate of malate throughout all three incubation time periods (Fig. 10 F). In contrast, the exudation of citrate was not affected by 350 µM vanadae irrespective of incubation periods (Fig. 10 E). A dramatic decrease in $Cl^-$ exudation by proteoid roots was evident in the treatment with vanadate during the incubation time of 15 min (Fig. 10 H). However, the inhibitory effect of vanadate on $Cl^-$ exudation declined with extended incubation time. For $SO_4^{2-}$, a significant increase in exudation rate was found for the incubation time of 30 min (Fig 10 G).

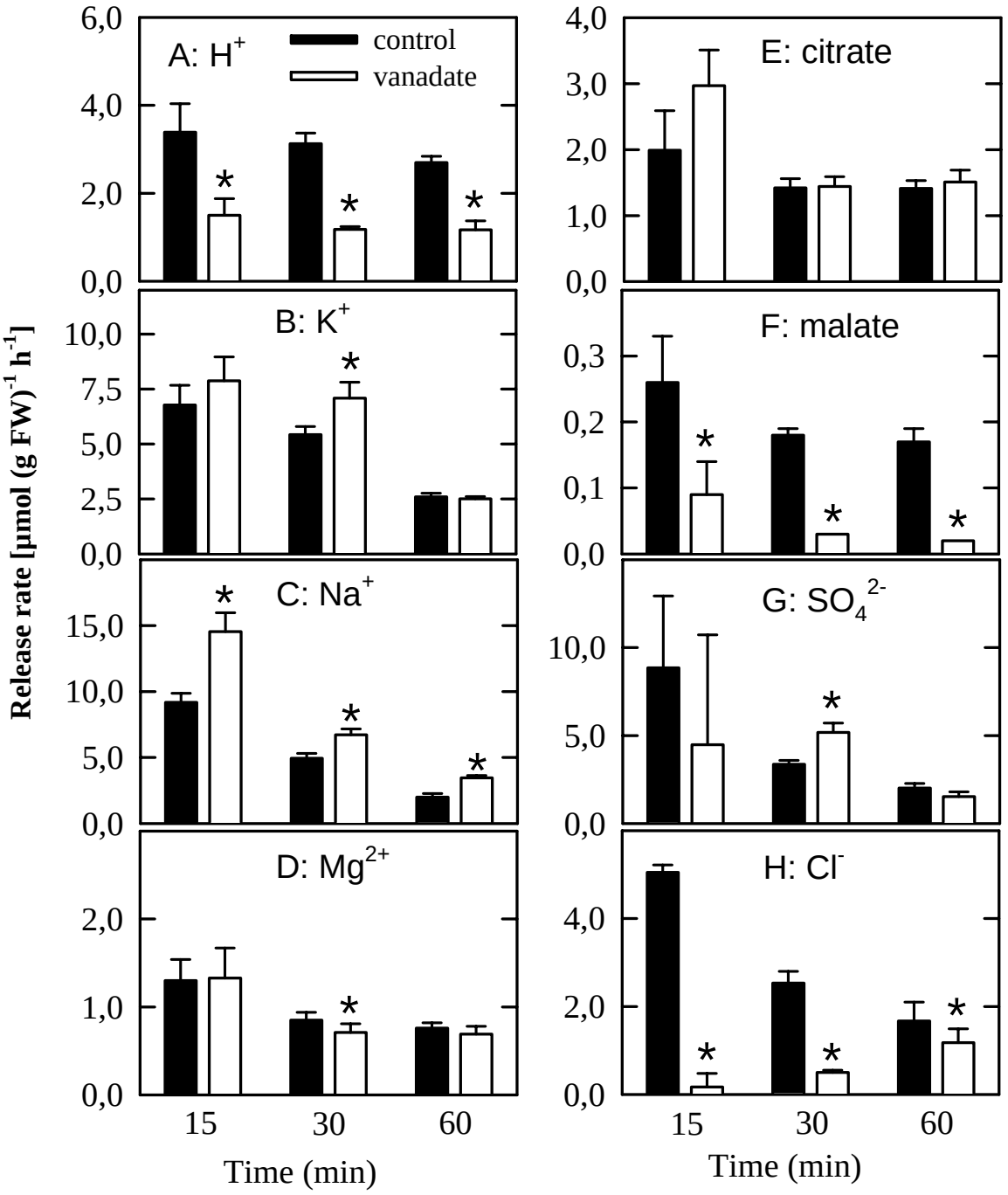


Figure 10. Effect of vanadate on the exudation rate of active proteoid roots. The plants had been grown in solution culture without phosphate for 3 w. Active proteoid roots were selected for the collection of root exudates. In one treatment, vanadate (350 µM) was applied to the solution for collection of root exudates. Each value represents the mean ± SE of four independent experiments. Significant differences ( $P<0.05$) between the control and vanadate treatment were calculated by paired *t*-test and is indicated by *.

### 3.2.5 Effect of anthracene on the exudation of ions by active proteoid roots

Anthracene is known as an anion-channel blocker for plant cells and showed an inhibitory effect on the citrate exudation by white lupin roots (Neumann et al., 1999). In the present study, 150 µM anthracene did not show any inhibitory effect on the exudation of citrate and malate by active proteoid roots of white lupin during the incubation time of 15 and 30 min (Fig. 11 E,F). A significant inhibitory effect of anthracene on the exudation of citrate and malate was only measured when the incubation time was extended to 60 min. However, anthracene acted much more quickly on the exudation of $Cl^-$. In comparison with control, a significant inhibitory effect of anthracene on $Cl^-$ exudation was observed throughout all three incubation periods (Fig. 11 H). For $SO_4^{2-}$ exudation, anthracene did not show any inhibitory effect (Fig. 11 G). Among cations studied, there was a significant inhibitory effect of anthracene on $H^+$ exudation when the incubation time extended to 60 min (Fig. 11 A). For $K^+$ exudation, the inhibitory effect of anthracene was evident for the incubation periods of 15 and 60 min (Fig. 11 B). There was no effect of anthracene on the exudation of $Na^+$ and $Mg^{2+}$ (Fig. 11 C, D).

To clarify the inhibitory effect of anthracene on $H^+$ exudation by intact proteoid roots we studied the effect of anthracene on $H^+$-pumping activity with plasma membrane vesicles. *In vitro*, anthracene showed no inhibitory effect on $H^+$-pumping activity of plasma membrane $H^+$ ATPase (data not shown).

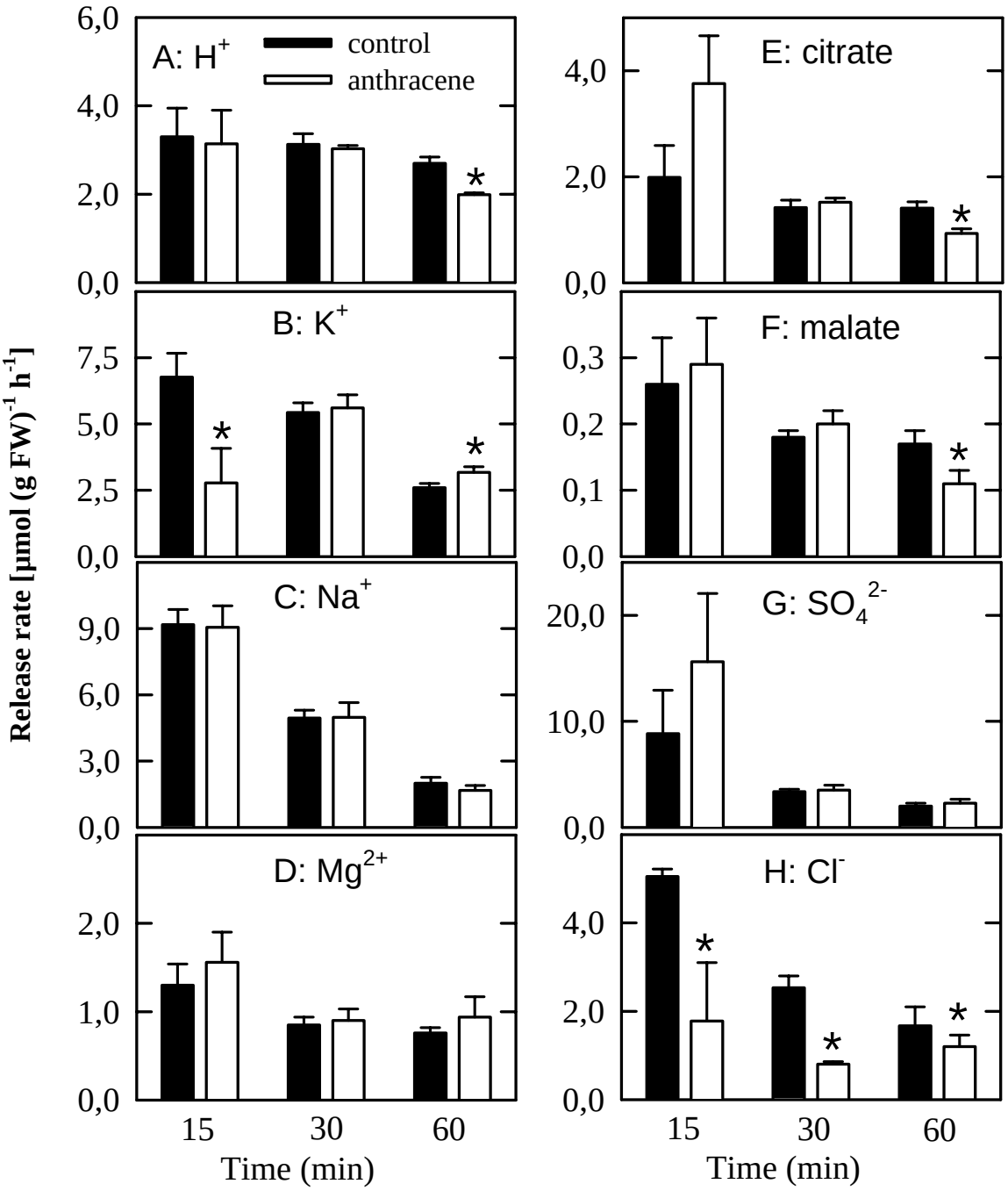


Figure 11. Effect of anthracene on the exudation rate of active proteoid roots. The plants had been grown in solution culture without phosphate for 3 w. Active proteoid roots were selected for the collection of root exudates. In one treatment, anthracene (150 µM) was applied to the solution for collection of root exudates. Each value represents the mean ± SE of four independent experiments. Significant difference ( $P<0.05$) between the control and anthracene treatment was calculated by paired *t*-test and is indicated by *.

### 3.2.6 Effect of pharmacological drugs on citrate and malate concentrations of active proteoid roots

The concentrations of citrate and malate of active proteoid roots were analyzed after exudate solution collection. The concentration of citrate in the active proteoid roots ranged from 29 to 39 µmol (g FW)$^{-1}$ (Table 2). The concentration of malate ranged from 19 to 24 µmol (g FW)$^{-1}$ and was approximately 2/3 of the concentration of citrate. There was no significant effect of the various drugs on the concentrations of citrate and malate in active proteoid roots of white lupin during the incubation periods from 15 to 60 min.

Table 2. Citrate and malate concentrations in the proteoid roots after treatment with 350 µM vanadate, 5 µM fusicoccin or 150 µM anthracene for 15, 30 and 60 min. Each value represents the mean ± SE of four replicate samples.

| Treatment | Citrate [µmol (g FW)$^{-1}$] | | | Malate [µmol (g FW)$^{-1}$] | | |
|---|---|---|---|---|---|---|
| | 15 min | 30min | 60 min | 15 min | 30min | 60 min |
| Control | 31.77 ± 1.56 | 31.31 ± 1.44 | 39.27 ± 4.05 | 21.74 ± 2.19 | 22.29 ± 0.89 | 24.39 ± 3.27 |
| Vanadate | 31.49 ± 1.10 | 32.64 ± 2.83 | 33.30 ± 2.13 | 22.00 ± 0.23 | 21.80 ± 0.59 | 21.68 ± 2.65 |
| Fusicoccin | 31.31 ± 1.39 | 31.10 ± 3.51 | 30.71 ± 2.20 | 21.29 ± 2.69 | 19.69 ± 0.61 | 22.20 ± 1.01 |
| Anthracene | 32.51 ± 2.24 | 28.86 ± 2.76 | 35.11 ± 2.44 | 24.47 ± 3.07 | 22.61 ± 1.19 | 19.36 ± 1.59 |

So far, the study with phamarcological drugs indicated a close linkage between the citrate and $H^+$ release from intact active proteoid roots *in situ*, although the vanadate did not inhibit citrate release. Anyway, the inhibition of $H^+$ release by vanadate *in vivo* indicated the involvement of plasma membrane $H^+$ ATPase in the adaptation to the P deficiency.

## 3.3 Adaptation of plasma membrane $H^+$ ATPase of proteoid roots to P deficiency

In order to investigate the adaptation of plasma membrane $H^+$ ATPase of proteoid roots to P deficiency, plasma membrane was isolated from four types of roots (Fig. 4): active proteoid roots from P-deficient plants (the youngest, fully developed, acidifying proteoid roots), lateral roots from P-deficient plants (4 – 5 cm apical zone of lateral roots), proteoid roots from P-sufficient plants, and lateral roots from P-sufficient plants (4 –5 m apical zone of lateral roots).

### 3.3.1 Effect of different root types of white lupin on the isolation of plasma membrane

A summary of ATPase-specific activities associated with phase-partitioned plasma membrane from different root types of white lupin is presented in Table 3. To avoid an underestimation of contamination by endoplasmic membranes, we analyzed azide- and nitrate-sensitive ATPase activity at pH 8.0. The inhibitor-sensitive ATPase hydrolytic activity of each membrane fraction was calculated by subtracting the ATPase hydrolytic activity in the presence of the inhibitor from the activity of the control at each assay pH. The ATPase activity of obtained membrane fractions from different types of white lupin roots showed a negligible sensitivity to azide and nitrate. On the other hand, about 95% of ATPase activity was sensitive to 0.1 mM vanadate. However, the membrane fractions showed a molybdate-sensitive ATP hydrolase activity, which indicates the presence of unspecific acid phosphatases (Widell and Larsson, 1990). This unspecific acid phosphatase activity was higher in roots from P-deficient plants than those from P-sufficient plants. Several attempts including homogenizing root tissues with 0.25 M KI and freezing-thawing the isolated membrane vesicles with following washing proved to be not effective to eliminate the unspecific acid phosphatase activity in the membrane fractions (data not shown). Therefore, in all analyses for ATPase activity, besides 1 mM azide and 50 mM nitrate, 1 mM molybdate was included to suppress the unspecific acid phosphatase activity. This assay medium warrants the determination of plasma membrane $H^+$ ATPase activity and a direct comparison of its activity between membrane fractions derived from different types of roots of white lupin.

Table 3. Inhibitor-sensitive ATPase hydrolytic activity associated with plasma membranes of different types of white lupin roots. Membranes were isolated from different root types of 3 w-old white lupin: active proteoid roots of P-deficient plants, lateral roots of P-deficient plants, proteoid roots of P-sufficient plants, and lateral roots of P-sufficient plants. Assays were conducted at 30°C. The inhibitor-sensitive activity was calculated by substrating the ATP hydrolytic activity in the presence of inhibitor from the activity of the control. The values represent means ± SE (percentage relative to control) of four independent experiments.

| Treatments | ATPase activity [µmol Pi (mg min)$^{-1}$] | | | |
|---|---|---|---|---|
| | Proteoid (-P) | Lateral (-P) | Proteoid (+P) | Lateral (+P) |
| Control (pH 6.5) | 2.01 ± 0.10 (100%) | 0.83 ± 0.08 (100%) | 1.00 ± 0.07 (100%) | 0.80 ± 0.06 (100%) |
| Vanadate-sensitive (0.1 mM, pH6.5) | 1.93 ± 0.08 (96%) | 0.78 ± 0.08 (95%) | 0.96 ± 0.07 (96%) | 0.74 ± 0.06 (92%) |
| Molybdate-sensitive (1 mM, pH6.5) | 0.50 ± 0.03 (25%) | 0.25 ± 0.02 (30%) | 0.19 ± 0.03 (19%) | 0.12 ± 0.02 (15%) |
| Azide-sensitive (1 mM, pH8.0) | 0.02 ± 0.03 (1%) | 0.02 ± 0.01 (2%) | 0.01 ± 0.02 (1%) | 0.04 ± 0.01 (4%) |
| Nitrate-sensitive (50 mM, pH8.0) | 0.09 ± 0.02 (4%) | 0.06 ± 0.01 (7%) | 0.06 ± 0.01 (6%) | 0.10 ± 0.00 (12%) |

### 3.3.2 Increase of plasma membrane $H^+$ ATPase activity in active proteoid roots

In an assay (pH range 5.6-7.0), the plasma membrane derived from active proteoid roots of P-deficient plants showed a more than 2 times higher ATPase activity than that from other types of roots. At its optimum pH (pH 6), a 3 times higher ATPase activity was determined in the plasma membrane of active proteoid roots in comparison with those of other membrane fractions (Fig. 12). On the contrary, the plasma membrane of lateral roots of P-deficient plants showed lower ATPase activity as compared with those of plasma membranes of P-sufficient plants. In addition, there was no difference in ATPase activity between proteoid roots and lateral roots when plants had been grown with 0.25 mM P. Figure 12 also indicates a variation of pH optimum for ATPase activity in the active proteoid roots. Plasma membrane ATPase of active proteoid roots showed a narrow pH optimum at pH 6.0, whereas a broader pH optimum between 6.2 and 6.4 was evident for ATPase activity of other types of roots. The pH optimum around 6.5 was reported for plasma membrane ATPase of maize (*Zea mays*) roots (Yan et al., 1998), oat (Avena sativa; Palmgren and Sommarin, 1989), and *Arabidopsis* (Luo et al., 1999).

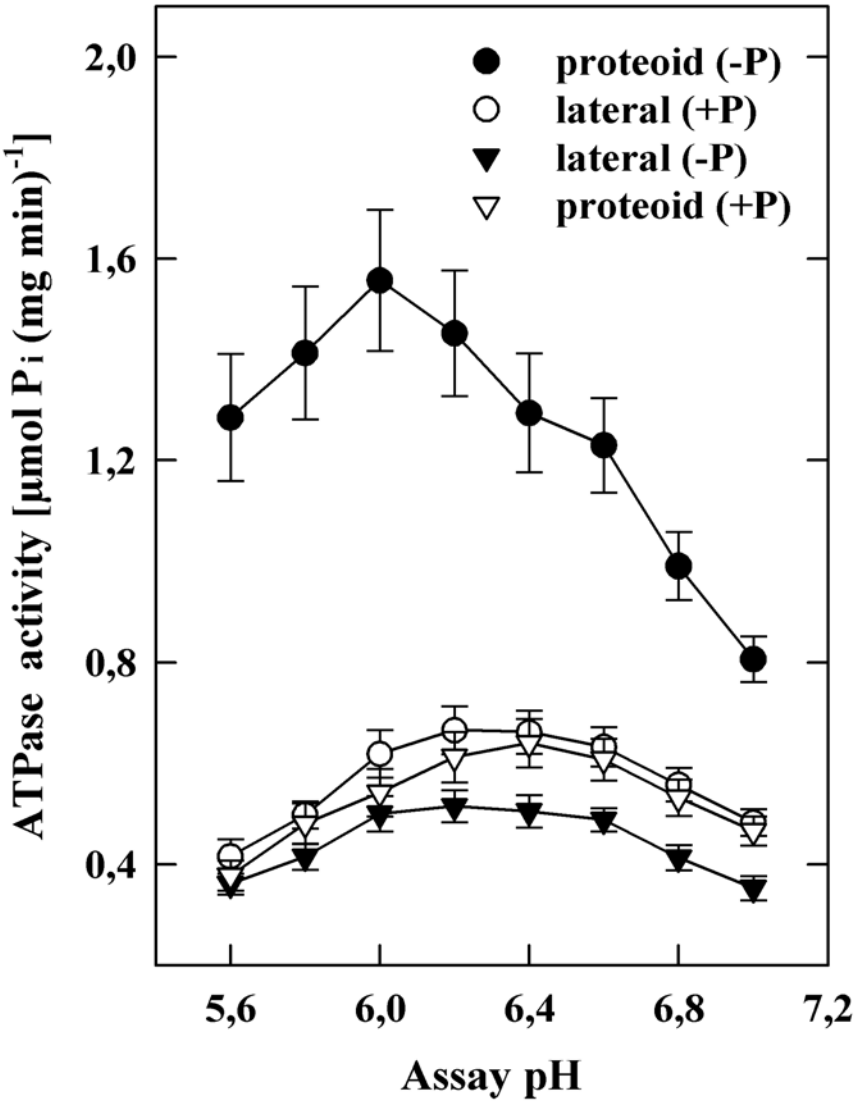


Figure 12. Comparison of ATPase activity of plasma membranes derived from different types of white lupin roots. Plants had been grown in nutrient solution at pH 6 for 3 weeks. Plasma membrane was isolated from proteoid roots of P-sufficient plants, lateral roots of P-sufficient plants, active proteoid roots (the youngest, fully developed proteoid root) of P-deficient plants, and lateral roots of P-deficient plants. Plasma membrane ATPase activity was analyzed in the presence of 1 mM molybdate, 1 mM azide, and 50 mM nitrate at 30°C. Values represent means ± SE of four independent experiments.

### 3.3.3 Increase in $V_{max}$ , $K_m$ , and vanadate sensitivity of plasma membrane $H^+$ ATPase from active proteoid roots

To gain a deeper insight into the enzyme properties of plasma membrane ATPase of active proteoid roots, we analyzed the kinetic characteristics of ATPase at ATP concentrations from 50 to 4,000 µM with an ATP regenerating system (Sekler and Pick, 1993). In this concentration range, plasma membrane ATPase revealed typical Michaelis-Menten kinetics for all membrane fractions (Fig. 13A), as found for maize (Yan et al., 1998) and *Arabidopsis* (Palmgren and Christensen, 1994).

Undoubtedly the most popular technique for calculating kinetic parameters is the Lineweaver-Burk, or double reciprocal, plot. But it does have some real drawbacks in dealing with data containing significant experimental error, particularly at the right hand side of the graph which represents the lower substrate concentrations. This plot has exaggerated the error at low substrate levels where measurements are often less accurate anyway as velocities are slower. In fact the error with the Eadie-Hofstee Plot is not so severe as with the Lineweaver-Burk plot, thus it was adopted in this study.
After transformation of data according to Eadie-Hofstee, a linear relationship ($r > 0.98$) was found between ATPase activity and the ratio of ATPase activity to ATP concentration (Fig. 13B). $V_{max}$ and $K_m$ were obtained from the intercept and slope of the straight line, respectively. At assay pH 6.0 and 6.5, the ATPase of active proteoid roots showed a significantly higher $V_{max}$ than that of other three root types (Table 4). In addition, the $V_{max}$ of plasma membrane ATPase from lateral roots of P-sufficient plants was significantly higher than that of lateral roots of P-deficient plants or the proteoid roots of P-sufficient plants. For active proteoid roots, there was also a significant increase in $K_m$ in comparison with other three types of roots, indicating a significant decrease in substrate affinity of the plasma membrane ATPase. Among lateral roots of P-deficient plants and lateral roots or proteoid roots of P-sufficient plants, there was no difference for $K_m$ at assay pH 6.0. When assay pH was changed from 6.0 to 6.5, an increase in $K_m$ by 2, 1.5, 1.9, and 1.8 times was recorded for active proteoid roots and lateral roots from P-deficient plants and proteoid roots and lateral roots from P-sufficient plants, respectively (Table 4).

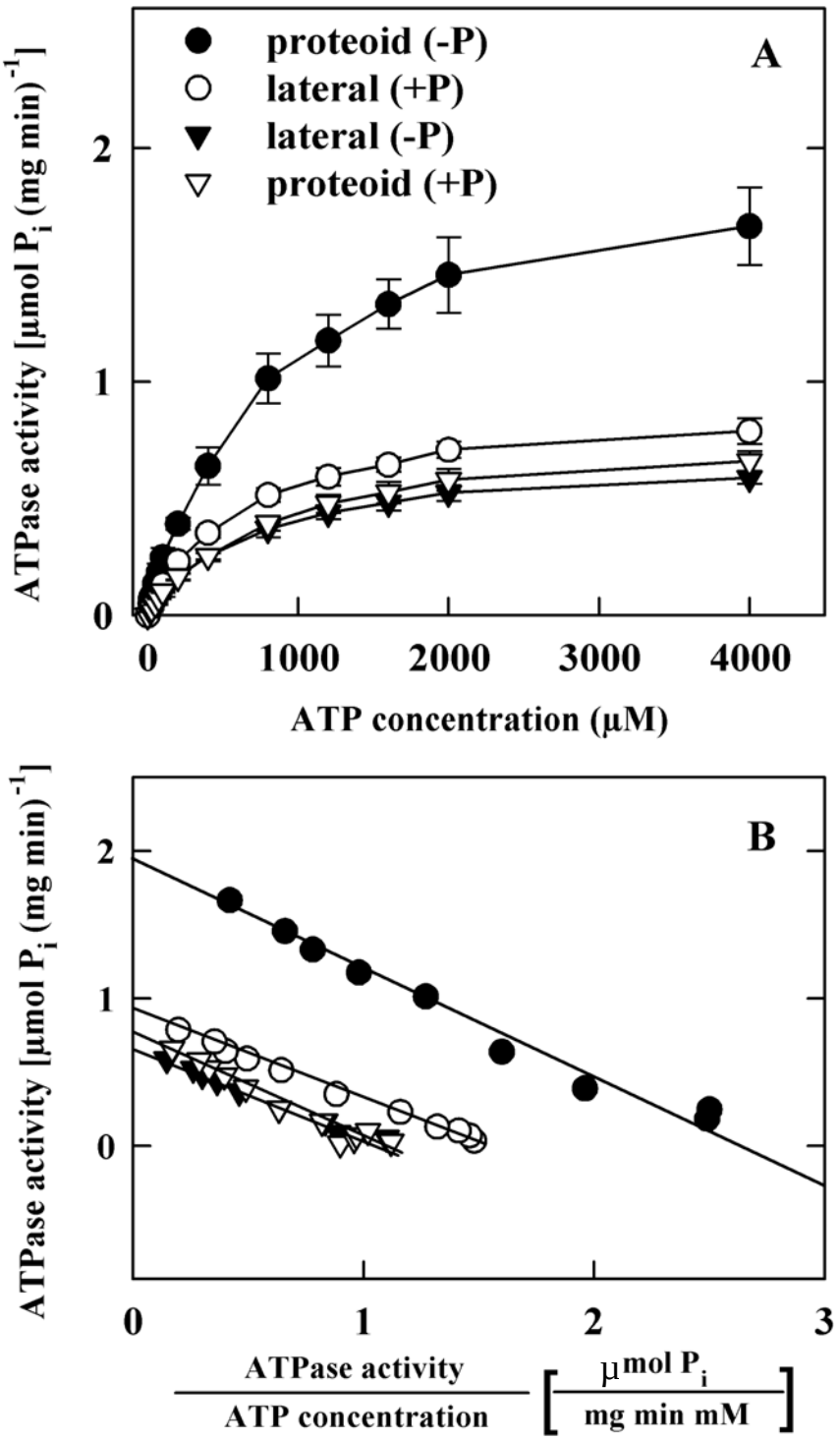


Figure 13. Comparison of the kinetic characteristics of plasma membrane ATPase from different types of white lupin roots (see legend of Fig. 5). A, Dependence of ATPase activity on ATP concentration. Plants were grown in nutrient solution at pH 6 for 3 weeks. Plasma membrane ATPase activity was analyzed in the presence of 1 mM molybdate, 1 mM azide, and 50 mM nitrate at 30°C. The concentration of ATP was kept constant in the range of 50 to

4,000 µM. Values represent means ± SE of four independent experiments. B, Eadie-Hofstee plot of the data presented in A ($r > 0.98$).

Table 4. Effect of assay pH on the kinetic characteristics and vanadate sensitivity of plasma membrane ATPase of different white lupin roots. Membranes were isolated from different root types of 3 w-old white lupin: active proteoid roots of P-deficient plants, lateral roots of P-deficient plants, proteoid roots of P-sufficient plants, and lateral roots of P-sufficient plants. $V_{max}$ and $K_m$ were determined using ATP concentrations from 50 to 4000 µM at 30°C. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentrations. The sensitivity of ATPase activity to vanadate was determined within a concentration range of 0.5 to 500 µM. The values represent means ± SE of four independent experiments. Significant differences (P < 5%) between treatments are indicated by different letters.

| | $K_m$ (mM) | | $V_{max}$ (µmol Pi $mg^{-1}$ $min^{-1}$) | | $I_{50}$ (µM) | |
|---|---|---|---|---|---|---|
| Assay pH | 6.0 | 6.5 | 6.0 | 6.5 | 6.0 | 6.5 |
| Proteoid (-P) | 0.45 ± 0.03b | 0.91 ± 0.05c | 2.14 ± 0.14c | 2.00 ± 0.18c | 9.38 ±1.17c | 4.61 ± 0.51b |
| Lateral (-P) | 0.34 ± 0.02a | 0.63 ± 0.05ab | 0.61 ± 0.04a | 0.67 ± 0.03a | 6.36 ± 0.21b | 6.56 ± 0.62c |
| Proteoid (+P) | 0.36 ± 0.02a | 0.69 ± 0.03b | 0.55 ± 0.03a | 0.74 ± 0.05a | 4.27 ± 0.39a | 2.99 ± 0.40a |
| Lateral (+P) | 0.33 ± 0.03a | 0.51 ± 0.02a | 0.74 ± 0.03b | 0.86 ± 0.05b | 4.79 ± 0.11a | 3.04 ± 0.23a |

For plasma membrane $H^+$ ATPase, besides kinetic parameters such as $K_m$ and $V_{max}$, another important parameter is the sensitivity of ATPase to vanadate. There is considerable variation for vanadate sensitivity among various plasma membrane ATPases (Regenberg et al., 1995; Morsomme et al., 1996). As for the determination of $K_m$ and $V_{max}$, the inhibition of ATPase activity by vanadate was determined in a vanadate concentration range from 0.5 to 500 µM. After transformation of data according to Eadie-Hofstee, the concentration of vanadate for 50% inhibition of ATPase activity ($I_{50}$) was estimated in a similar way as for $K_m$. At assay pH 6, a significantly higher $I_{50}$ was estimated for the ATPase in the plasma membranes derived from P-deficient plants in comparison with those from P-sufficient plants (Table 4). This indicates an induction of lower vanadate sensitivity of plasma membrane ATPase in white lupin roots by phosphate deficiency. In addition, at assay pH 6.0, the sensitivity of ATPase to vanadate was significantly lower for active proteoid roots than for lateral roots of P-deficient plants. It is interesting to note that the pH dependence of $I_{50}$ was totally different as for $K_m$. When assay pH was increased

from 6.0 to 6.5, there was a decrease of $I_{50}$ for plasma membrane ATPase by 2-fold for active proteoid roots of P-deficient plants and 1.4 and 1.6-fold for proteoid roots and lateral roots of P-sufficient plants, respectively. Conversely, there was no change for lateral roots of P-deficient plants (Table 4).

### 3.3.4 Increase of $H^+$ ATPase enzyme concentration in the plasma membrane of active proteoid roots

Although an increase in plasma membrane $H^+$ ATPase activity has been reported under a number of environmental conditions, in most cases it was not clear whether the observed changes in $H^+$ ATPase activity reflect modulation of the amount or the turnover rate of hydrolysis of the enzyme (Serrano, 1989). Identifying these two different components is a prerequisite for understanding plant response to environmental conditions on a molecular basis. In the present study, we solved this problem by determining the activation energy of ATP hydrolysis and measuring ATPase enzyme concentration in plasma membrane by means of western-blot analysis using a specific antibody for plasma membrane $H^+$ ATPase.

For the determination of activation energy of ATPase, which is, according to Arrhenius, related to the turnover rate of hydrolysis of the enzyme, we analyzed the $V_{max}$ at two different assay temperatures at optimum pH level for each membrane fraction (Yan et al., 1998). For all four membrane fractions, about 1.8 times increase in $V_{max}$ was caused by increasing assay temperature from 25°C to 30°C, from which a comparable activation energy of 92 kJ $mol^{-1}$ was calculated for all four membrane fractions analyzed. This indicates that the ATPase in different membrane fractions has a comparable turnover rate of ATP hydrolysis. As a consequence, the higher hydrolytic activity of ATPase found in the plasma membrane of active proteoid roots must be attributed to a higher ATPase enzyme concentration. ATPase enzyme concentration in plasma membrane was analyzed by means of western-blot technique. Membrane proteins (4 µg) were separated by SDS-PAGE on 10% (w/v) acrylamide gel. At a molecular mass of 97 kD, the band of plasma membrane derived from active proteoid roots of P-deficient plants was more intensive than that from proteoid roots of

P-sufficient plants or from lateral roots grown either with or without phosphate (Fig. 14). Immunoblotting with a polyclonal antibody specific for the central part of plant $H^+$ ATPase showed higher intensity for the plasma membrane of active proteoid roots of P-deficient plants (Fig. 14), as compared with the proteoid roots of P-sufficient plants or lateral roots from both P-sufficient and P-deficient plants. In addition to the strongly staining 97-kD region, there was also a weak cross-reaction with a minor band for the membrane fraction of proteoid roots from P-sufficient plants. For a quantitative comparison, the integrated evaluation of the intensity and area of signals was carried out by setting control (lateral roots of P-sufficient plants) as 100% in three independent experiments. In comparison with the control, the increase was 422% (±75%) for active proteoid roots of P-deficient plants, 142% (±12%) for the lateral roots from P-deficient roots, and 110% (±11%) for the proteoid roots of P-sufficient plants, respectively. These data reveal that the higher concentration of the plasma membrane $H^+$ ATPase in the active proteoid roots is responsible for higher ATPase hydrolytic activity. In addition, there was also a small increase in the ATPase enzyme concentration in the plasma membrane of lateral roots from P-deficient plants in comparison with that from P-sufficient plants, although the converse was true for ATPase hydrolytic activity and $V_{max}$ for these two membrane fractions (Figs. 12 and 13; Table 4).

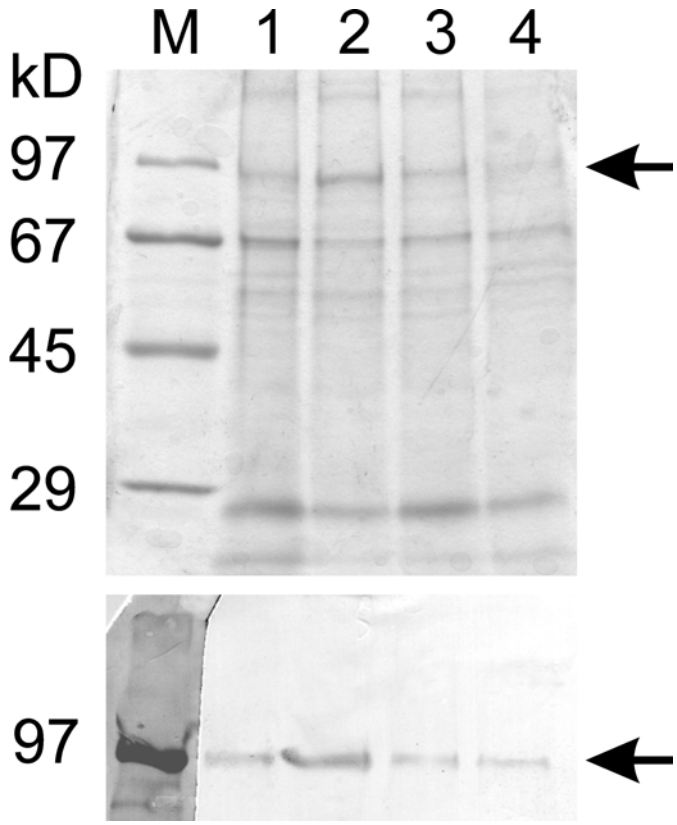


Figure 14. Separation of plasma membrane proteins by SDS-PAGE (above) and immunodetection of plasma membrane $H^+$ ATPase by western-blotting technique (below).
M, Standard markers for molecular mass (Sigma, St. Louis); 1, plasma membrane from lateral roots of P-sufficient plants; 2, plasma membrane from active proteoid roots of P-deficient plants; 3, plasma membrane from lateral roots of P-deficient plants; 4, plasma membrane from proteoid roots of P-sufficient plants. Arrows indicate plasma membrane $H^+$ ATPase. For separation of plasma membrane proteins, membrane vesicles (4 µg membrane proteins) were loaded onto polyacrylamide gel. After separation, the obtained gel was stained with Coomassie Brilliant Blue (above). For western-blot analysis (below), after separation on the gel the membrane proteins including molecular mass markers were transferred to polyvinylidene difluoride (PVDF) membrane filter (0.2 µm). For staining of the obtained blot, the lane of molecular mass markers was separated from other lanes of membrane proteins. The former was stained with Coomassie Brilliant Blue. The remaining blot with the lanes of plasma membrane proteins was incubated with a polyclonal antibody raised against the central portion of AHA2 (amino acids 340-650) and visualized with a secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma). After separate staining, the blot of standard marker for molecular mass and the blot of plasma membrane proteins were combined.

### 3.3.5 Increase in $H^+$-pumping activity of the plasma membrane of active proteoid roots

Plasma membrane $H^+$-pumping activity was monitored by $A_{492}$-quenching of acridine orange (AO). After initiation of $H^+$-pumping by addition of Mg-ATP, there was rapid quenching and it eventually reached a constant level. This established pH gradient was completely collapsed by 5 µM gramicidine (Fig. 15A). Furthermore, this $H^+$-pumping activity was completely inhibited by 100 µM vanadate (data not shown). Compared with lateral roots either grown with or without phosphate and the proteoid roots of P-sufficient plants, absorbance quenching of AO caused by plasma membrane vesicles from active proteoid roots of P-deficient plants was more rapid at the beginning and reached a higher level after 40 min. Despite comparable absorbance quenching of AO at the beginning, membrane vesicles from P-deficient lateral roots and from P-sufficient proteoid roots showed a higher level of quenching of AO after 40 min than those from P-sufficient lateral roots.

Two parameters, initial rate and maximum quenching (pH gradient), were used to characterize the plasma membrane $H^+$ pumping. The initial rate of $H^+$ pumping was determined according to the quenching rate within the 1st min, which reflects active $H^+$ influx into plasma membrane vesicles (Yan et al., 1998). Maximum quenching was measured 40 min after initiation of the $H^+$ pump. At this time, net $H^+$ transport across the plasma membrane was zero and $H^+$ influx due to active pumping and $H^+$ efflux because of leakage reached equilibrium. This parameter indicates the steepest pH gradient that can be created by $H^+$-pumping activity. At assay pH 6.5, the initial rate of $H^+$-pumping by plasma membrane ATPase from active proteoid roots of P-deficient plants was increased by about 3 times in comparison with that from the other three types of roots. There was no difference in initial rate of $H^+$ pumping among the other three membrane fractions (Table 5). Furthermore, the plasma membrane from active proteoid roots of P-deficient plants created a 1.4 to 1.8 times steeper pH gradient than proteoid and lateral roots of P-sufficient plants. For plants grown with phosphate, there was a significantly higher pH gradient for proteoid roots than that for lateral roots. However, the difference in pH gradient between active proteoid roots and lateral roots from P-deficient plants was less pronounced.

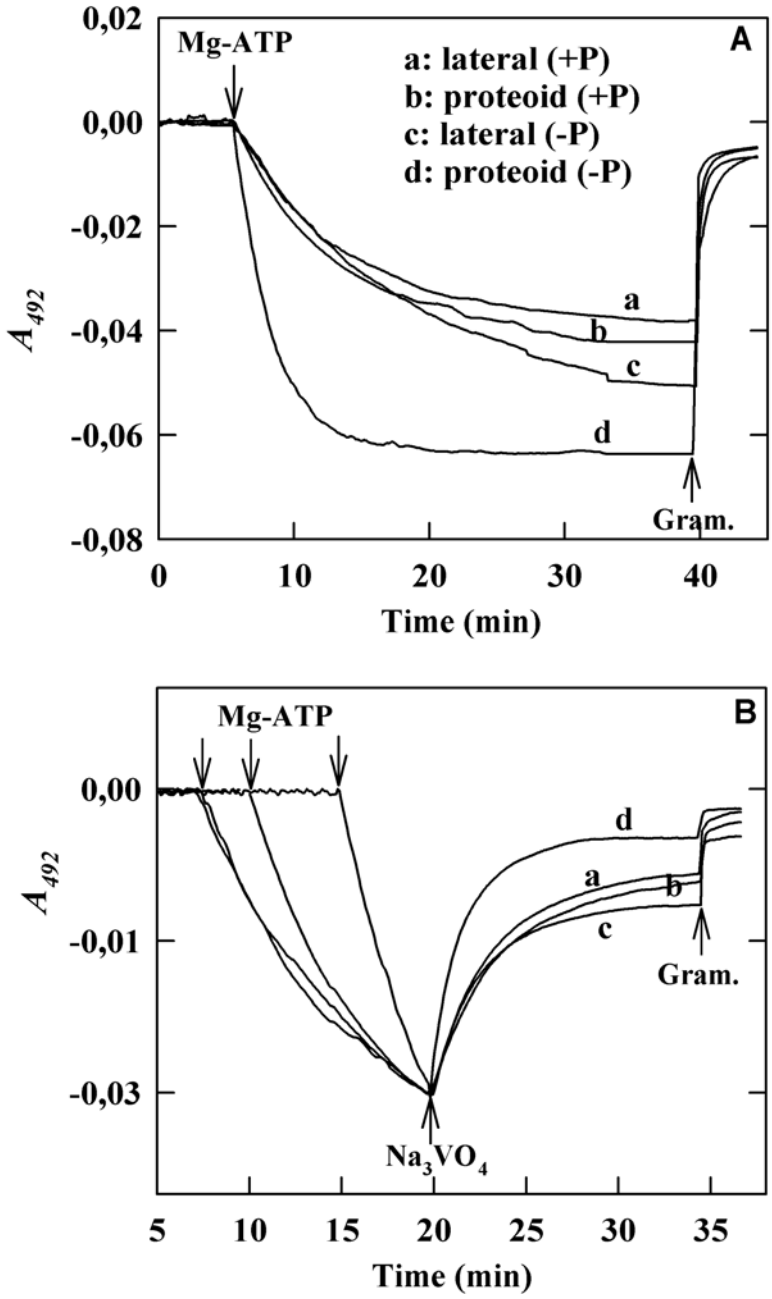


Figure 15. Comparison of active $H^+$ transport driven by plasma membrane $H^+$ ATPase (A) and passive $H^+$ transport by leakage (B) across the plasma membranes. Membrane vesicles were isolated from different types of roots of white lupin (see legend of Fig. 5). For the comparison of active $H^+$ transport (A), the pH gradient formation across vesicle membranes was monitored by $A_{492}$ of AO. At assay pH 6.5, intravesicular acidification was initiated by addition of 5 mM Mg-ATP. The established pH gradient was completely collapsed by 5 µM gramicidine (Gram.). For the comparison of passive $H^+$ transport (B), the intravesicular acidification was initiated by addition of 5 mM Mg-ATP to create a pH gradient across plasma membrane vesicles. For a reliable comparison, ATPase activity was stopped by addition of 500 µM vanadate after quenching had reached 0.0300 A units for all four membranes. The resting pH gradient was collapsed by gramicidine (Gram., 5 µM).

Table 5. Effect of different root types of white lupin on active $H^+$-pumping of ATPase and passive $H^+$ leakage of isolated plasma membrane vesicles. Membranes were isolated from different root types of 3 w-old white lupin: active proteoid roots of P-deficient plants, lateral roots of P-sufficient plants, and lateral roots of P-deficient plants. The assay was conducted at 25°C at pH 6.5 using 50 µg of membrane proteins. The values represent means ± SE of four independent experiments. Significant differences (P < 5%) between treatments are indicated by different letters.

| Root Types | Active $H^+$ transport | | Passive $H^+$ transport |
|---|---|---|---|
| | Initial Rate ($\Delta A_{492}$ $min^{-1}$) | pH Gradient($\Delta A_{492}$) | Initial Rate ($\Delta A_{492}$ $min^{-1}$) |
| Proteoid (-P) | 0.014 ± 0.003 b | 0.065 ± 0.009 c | 0.013 ±0.001 b |
| Lateral (-P) | 0.004 ± 0.001 a | 0.054 ± 0.009 bc | 0.007 ± 0.001 a |
| Proteoid (+P) | 0.005 ± 0.000 a | 0.045 ± 0.002 b | 0.006 ± 0.000 a |
| Lateral (+P) | 0.005 ± 0.001 a | 0.036 ± 0.003 a | 0.007 ± 0.002 a |

For inside-out vesicles, the establishment of a pH gradient is determined by both active influx (pumping) and passive efflux (leakage). The apparent discrepancy between the increase of $H^+$-pumping activity (3 times higher initial rate for vesicles from active proteoid roots than those from P-sufficient lateral roots) and the increase of pH gradient (1.8 times steeper pH gradient) is probably due to a difference in passive $H^+$ transport between the membrane fractions. To determine $H^+$ efflux from plasma membrane vesicles, we measured the degradation of the pH gradient after stopping $H^+$-pumping by addition of 500 µM vanadate. Because the degradation of pH gradient depends on the gradient itself, a comparison between treatments should be made at the same pH gradient. Therefore, we stopped $H^+$-pumping when the pH gradient of plasma membrane vesicles reached 0.0300 A units (Fig. 15B). After addition of vanadate, the established pH gradient was degraded quickly and then reached a relative constant level. This resting pH gradient was completely collapsed by gramicidine (Fig. 15B). It is evident from Figure 8B that the pH gradient degradation was faster for active proteoid roots than for the other three types of roots. The degradation rate within the 1st min after the addition of vanadate was measured as the initial rate to describe the passive $H^+$ permeability of the plasma membrane. Compared with the lateral roots and

proteoid roots of P-sufficient plants, the initial degradation rate for plasma membrane vesicles from active proteoid roots of P-deficient plants was approximately 2 times higher (Table 5). This indicates that the plasma membrane from active proteoid roots of P-deficient plants was more permeable for $H^+$ than those from proteoid roots of P-sufficient plants and lateral roots either from P-sufficient or -deficient plants. A similar result was reported for maize roots adapted to low solution pH (Yan et al., 1998). The difference in initial degradation rate among proteoid roots of P-sufficient plants and the lateral roots was less pronounced (Table 5).

## 3.4 Regulation of plasma membrane $H^+$ ATPase in different development stages of proteoid roots under P deficiency

Our data above demonstrated an up-regulation of plasma membrane $H^+$ ATPase in the active proteoid roots of white lupin adapted to P deficiency. In the present study, the regulation of plasma membrane $H^+$ ATPase of proteoid roots from different development stages was further investigated.

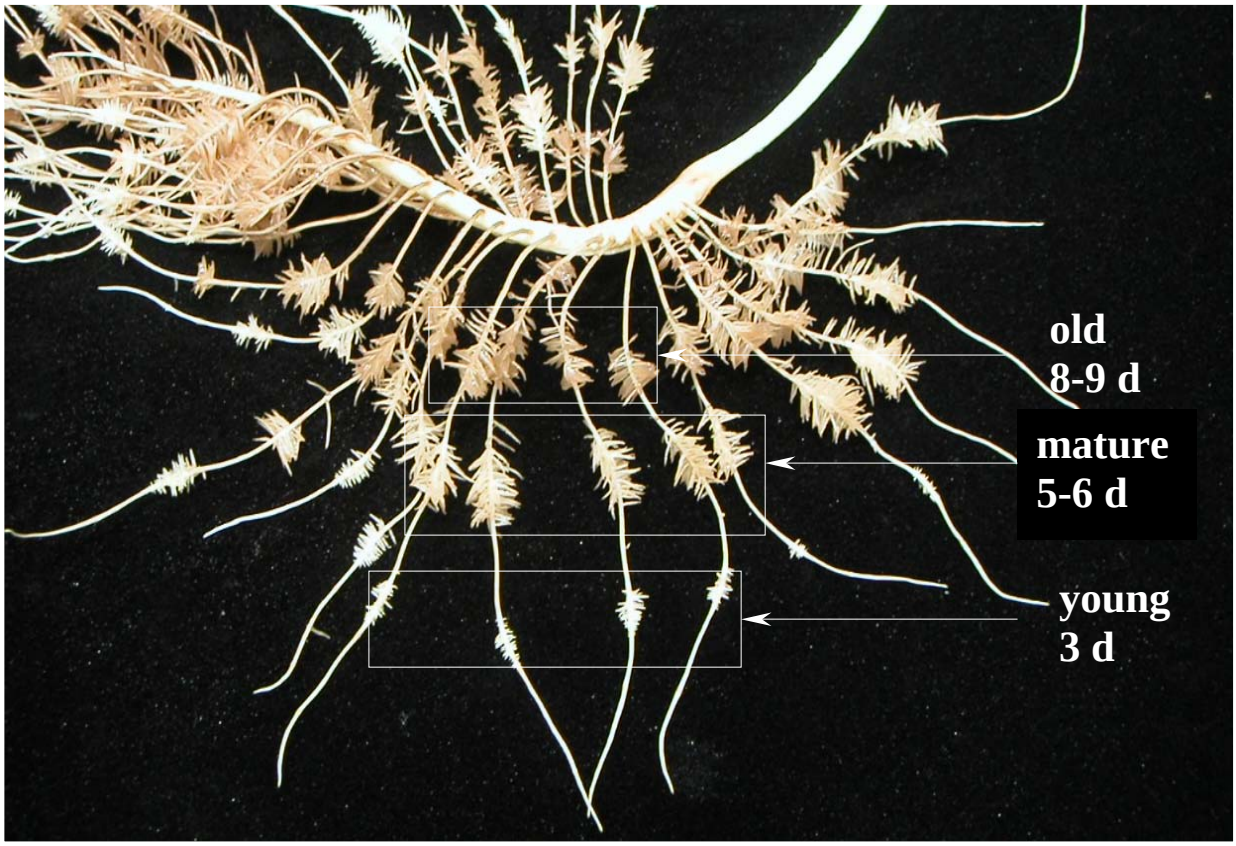


Figure 16. Proteoid roots at different developmental stages. Plants had been grown in solution culture without P for 24 d. Three types of proteoid roots at young (3 d), mature (5-6 d), and senescent (8-9 d) stages were used for plasma membrane isolation and exudation experiments.

### 3.4.1 Morphological description of proteoid roots in their different development stages under P deficiency

During about 23 d of hydroponic cultivation without P, plants formed three sequentially clustered proteoid roots on a primary lateral root (Fig.16). They were defined as young, mature and old proteoid roots according to their stages of development. Young proteoid roots were three days old after their emergence. They were white-colored and 2-3 cm from the root apex of the laterals. Mature proteoid roots were 5 to 6 d old and located 4-7 cm from the root tip. They were light brown-colored. Old proteoid roots were 8-9 d old and close to the lateral root base with a grey-brown color.

### 3.4.2 Increased plasma membrane $H^+$ ATPase activity in mature proteoid roots

Plasma membrane was isolated from young, mature and old proteoid roots as described. In an assay (pH range 5.6-7.0), the plasma membrane derived from mature proteoid roots of P-deficient plants showed the highest ATPase activity among all types of proteoid roots studied. At its optimum pH (pH 6), a 2 times higher ATPase activity was determined in the plasma membrane of mature proteoid roots in comparison with that of young proteoid roots (Fig. 17). The plasma membrane of old proteoid roots showed the lowest ATPase activity, which was less than a half of young proteoid roots. Fig. 17 also indicates different pH optimum for ATPase activity in different proteoid roots. Plasma membrane ATPase of mature proteoid roots showed a narrow pH optimum at pH 6.0, which was in agreement with the pH optimum for active proteoid roots. Young proteoid roots had a narrow pH optimum at pH 6.2, whereas a broader pH optimum between 6.0 and 6.6 was evident for ATPase activity of old proteoid roots.

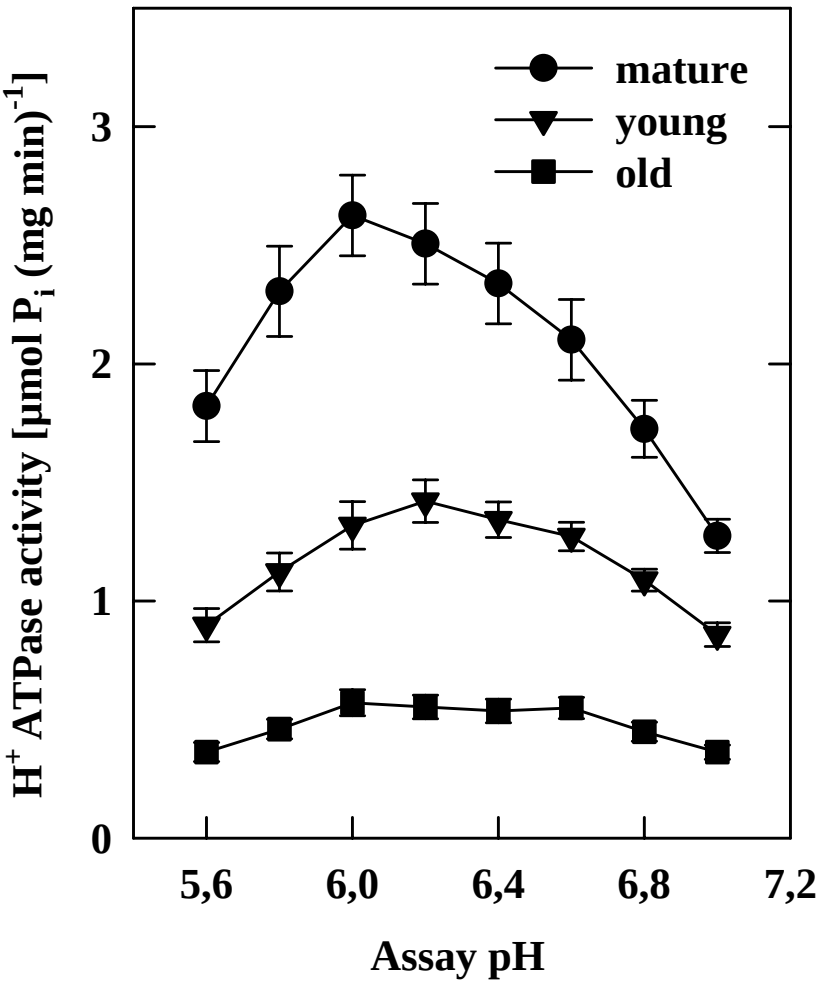


Figure 17. Comparison of ATPase activity of plasma membranes derived from different types of white lupin roots. Plants were grown in nutrient solution at pH 6 for 24 d. Plasma membrane was isolated from young proteoid roots, mature proteoid roots and old proteoid roots of P-deficient plants. Plasma membrane ATPase activity was analyzed in the presence of 1 mM molybdate, 1 mM azide, and 50 mM nitrate at 30°C. Values represent means ± SE of four independent experiments.

### 3.4.3 $V_{max}$ and $K_m$ of plasma membrane $H^+$ ATPase in mature, young and old proteoid roots

In an ATP concentration range from 50 to 4000 µM with an ATP regenerating system (Sekler and Pick, 1993), plasma membrane ATPase revealed typical Michaelis-Menten kinetics for all membrane fractions (Fig. 18A), as found for proteoid roots and lateral roots of white lupins with and without P (Yan et al., 2002). After transformation of data according to Eadie-Hofstee, a linear relationship ($r > 0.98$) was found between ATPase activity and the ratio of ATPase activity to ATP concentration (Fig. 18B). $V_{max}$ and $K_m$ were obtained from the intercept and slope of the regression, respectively. At assay pH 6.0, the ATPase of mature proteoid roots showed a significantly higher $V_{max}$ than those of young and old proteoid roots (Table 6). In addition, the $V_{max}$ of plasma membrane ATPase from young proteoid roots was significantly higher than that of old proteoid roots. For young and mature proteoid roots, a comparable $K_m$ was measured, while for old proteoid roots a significant lower $K_m$ was determined, indicating a higher affinity of the enzyme to its substrate in this membrane fraction (Table 6).

Table 6. Kinetic characteristics of plasma membrane $H^+$ ATPase of proteoid roots of different age

Membranes were isolated from different proteoid roots of 24 d-old white lupin: young proteoid roots, mature proteoid roots and old proteoid roots. $V_{max}$ and $K_m$ were determined using ATP concentrations from 50 to 4000 µM at 30°C. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep ATP concentrations constant. The values represent means ± SE of four independent experiments. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

| pH 6.0 | $K_m$ (mM) | $V_{max}$ (µmol Pi $mg^{-1}$ $min^{-1}$) |
|---|---|---|
| Young proteoid roots | 1.39 ± 0.10 b | 0.46 ± 0.02 b |
| Mature proteoid roots | 3.04 ± 0.11 c | 0.46 ± 0.05 b |
| Old proteoid roots | 0.49 ± 0.04 a | 0.26 ± 0.02 a |

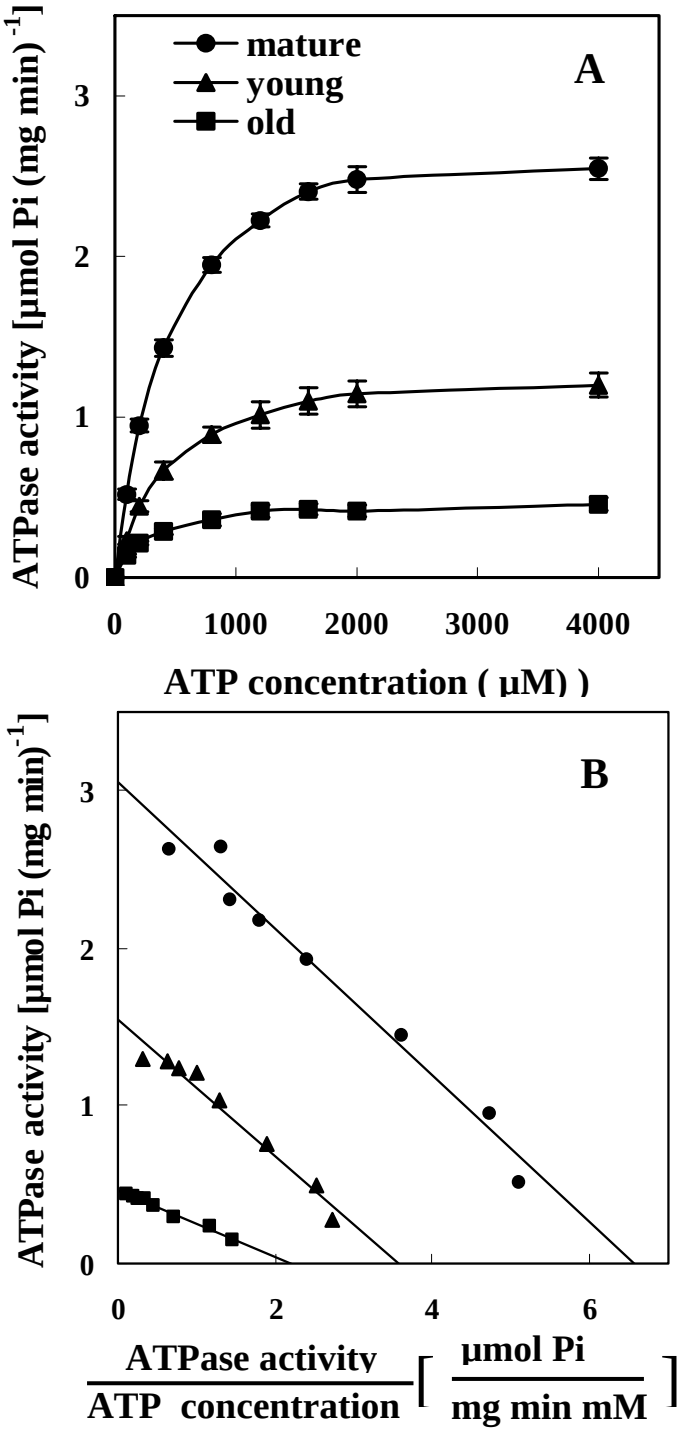


Figure 18. Comparison of the kinetic characteristics of plasma membrane $H^+$ ATPase from different types of white lupin roots (see legend of Fig. 5). A, Dependence of ATPase activity on ATP concentration. Plants had been grown in nutrient solution at pH 6 for 24 d. Plasma membrane $H^+$ ATPase activity was analyzed in the presence of 1 mM molybdate, 1 mM azide, and 50 mM nitrate at 30°C. The concentration of ATP was kept constant in the range of 50 to 4000 µM. Values represent means ± SE of four independent experiments. B, Eadie-Hofstee plot of the data presented in A ($r > 0.98$).

### 3.4.4 Change of $H^+$ ATPase enzyme concentration in the plasma membrane of proteoid roots

ATPase enzyme concentration in plasma membrane was analyzed by means of western-blot technique. Membrane proteins (4 µg) were separated by SDS-PAGE on 10% (w/v) acrylamide gel. At a molecular mass of 97 kD, the band of plasma membrane derived from mature proteoid roots was more intensive than that from young and old proteoid roots (Fig. 19). Immunoblotting with a polyclonal antibody specific for the central part of plant $H^+$ ATPase showed higher intensity for the plasma membrane of mature proteoid roots (Fig. 19), as compared with other proteoid roots. For a quantitative comparison, the integrated evaluation of the intensity and area of signals was carried out by setting mature proteoid roots as 100% in four independent experiments. In comparison with the mature proteoid roots, 76% (±6%) and 25% were measured for young and old proteoid roots, respectively. These data confirm that the higher concentration of the plasma membrane $H^+$ ATPase protein in the mature proteoid roots was responsible for its higher hydrolytic activity of the ATPase.

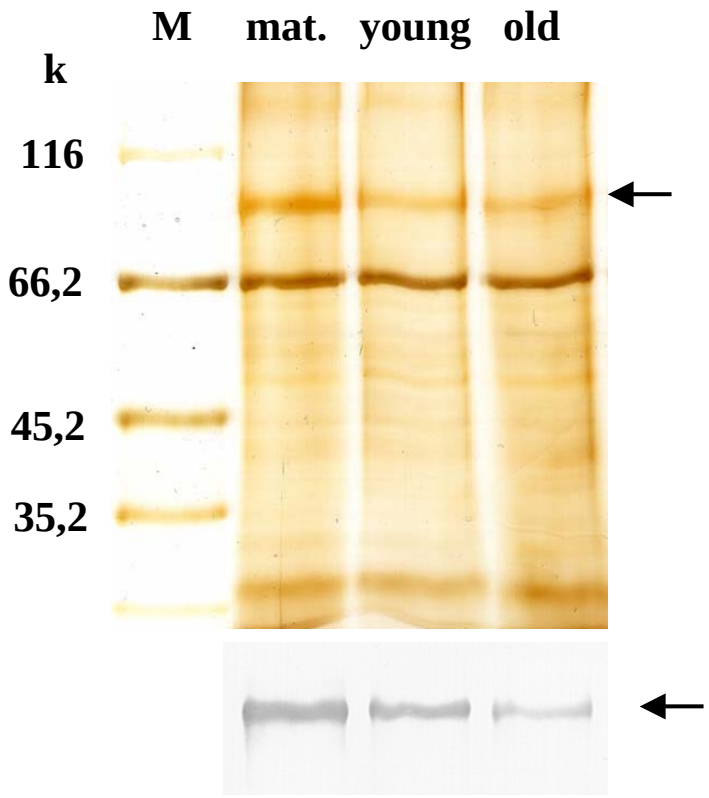


Figure 19. Separation of plasma membrane proteins by SDS-PAGE (above) and immunodetection of plasma membrane $H^+$ ATPase by western-blotting technique (below). M, Standard markers for molecular mass (Sigma, St. Louis); mat., plasma membrane from mature proteoid roots; young, plasma membrane from young proteoid roots; old, plasma membrane from old proteoid roots. Arrows indicate plasma membrane $H^+$ ATPase. For separation of plasma membrane proteins, membrane vesicles (4 µg membrane proteins) were loaded onto polyacrylamide gel. After separation, the obtained gel was stained with $AgNO_3$ (above). For western-blot analysis (below), after separation on the gel the membrane proteins including molecular mass markers were transferred to polyvinylidene difluoride (PVDF) membrane filter (0.2 µm). For staining of the obtained blot, the lane of molecular mass markers was separated from other lanes of membrane proteins. The former was stained with $AgNO_3$. The remaining blot with the lanes of plasma membrane proteins was incubated with a polyclonal antibody raised against the central portion of AHA2 (amino acids 340-650) and visualized with a secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma). After separate staining, the blot of standard marker for molecular mass and the blot of plasma membrane proteins were combined.

### 3.4.5 Change of $H^+$-Pumping Activity with the development of proteoid roots

Absorbance quenching of AO caused by plasma membrane vesicles from mature proteoid roots was more rapid at the beginning. At assay pH 6.5, the initial rate of $H^+$ pumping by plasma membrane ATPase from mature proteoid roots was 1.7 and 5 times than that of young and old proteoid roots, respectively (Table 7). Furthermore, the plasma membrane from mature proteoid roots of P-deficient plants created a 2 times steeper pH gradient than young and old proteiod roots. However, the difference in pH gradient between young and old proteoid roots was less pronounced.

Table 7. Effect of age of proteoid roots of white lupin on the active $H^+$-pumping of ATPase of plasma membrane Membranes were isolated from proteoid roots of different age of 24 d-old white lupin: young proteoid roots, mature proteoid roots and old proteoid roots. The assay was conducted at 25°C at pH 6.5 using 50 µg of membrane proteins. The values represent means ± SE of four independent experiments. Significant differences (P < 5%) between treatments are indicated by different letters.

| Root Types | Active $H^+$ transport | |
|---|---|---|
| | Initial Rate ($\Delta A_{492}$ $min^{-1}$) | pH Gradient($\Delta A_{492}$) |
| Young Proteoid root | 0.009 ± 0.001 b | 0.044 ± 0.009 ab |
| Mature Proteoid root | 0.015 ± 0.001 c | 0.070 ± 0.010 c |
| Old Proteoid root | 0.003 ± 0.000 a | 0.032 ± 0.002 a |

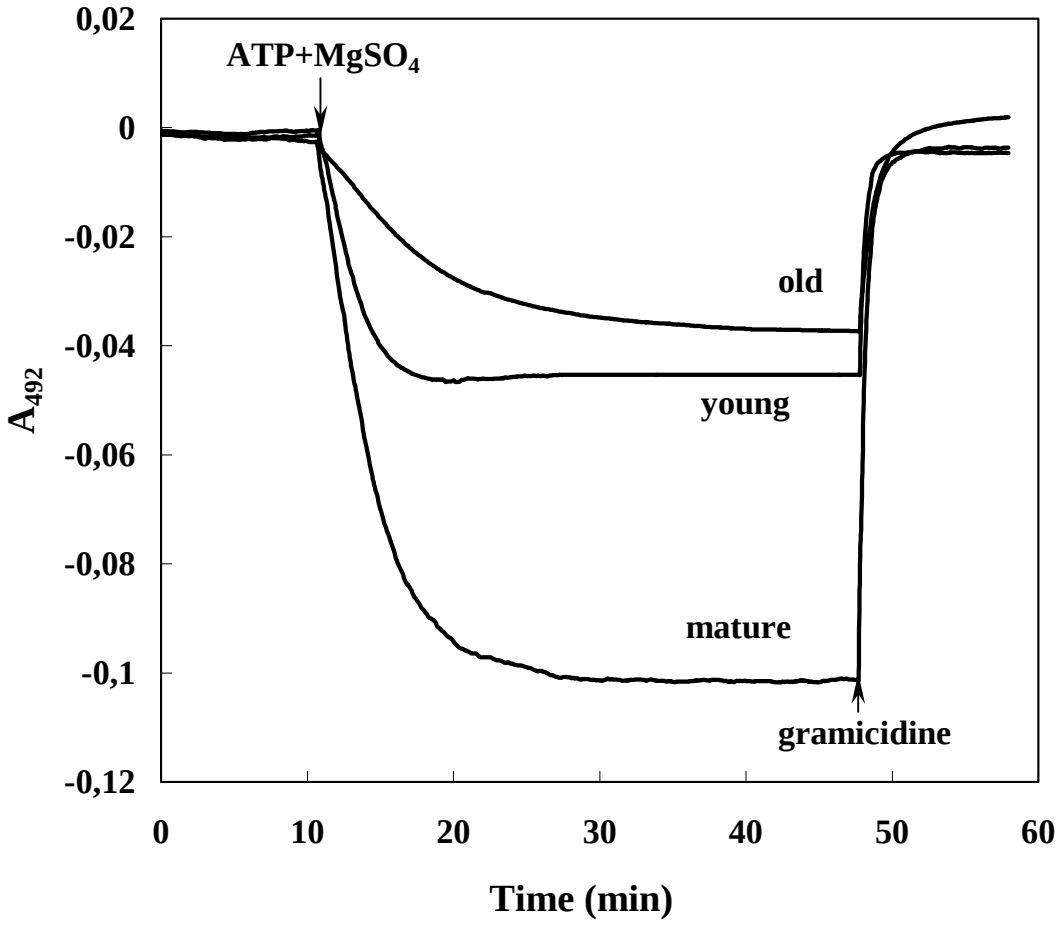


Figure 20. Comparison of active $H^+$ transport driven by plasma membrane $H^+$ ATPase across plasma membranes. Membrane vesicles were isolated from proteoid roots of different age of white lupin (see legend of Fig. 5). For the comparison of active $H^+$ transport, the pH gradient formation across vesicle membranes was monitored with $A_{492}$ of AO. At assay pH 6.5, intravesicular acidification was initiated by addition of 5 mM Mg-ATP. The established pH gradient was completely collapsed by 5 µM gramicidine.

## 3.5 Involvement of plasma membrane $H^+$ ATPase in citrate exudation and synthesis in the development of proteoid roots under P deficiency

### 3.5.1 Effect of proteid root development on the exudation of citrate and $H^+$

In agreement with the activity of plasma membrane $H^+$ ATPase, mature proteoid roots showed the highest $H^+$ exudation rate, being three times higher than that of young proteoid roots. Compared with young and mature proteoid roots, old proteoid roots had little $H^+$ exudation (Fig. 20 A). The release rate of $H^+$ coincided with citrate exudation from proteoid roots in different developmental stages. The citrate release rate of mature proteoid roots was 5 times higher than that of young proteoid roots, whereas old proteoid roots had nearly stopped to release citrate (Fig. 20 B). The results indicate that the citrate release from proteoid roots was closely related to the $H^+$ exudation caused by plasma membrane $H^+$ ATPase during the proteoid root development.

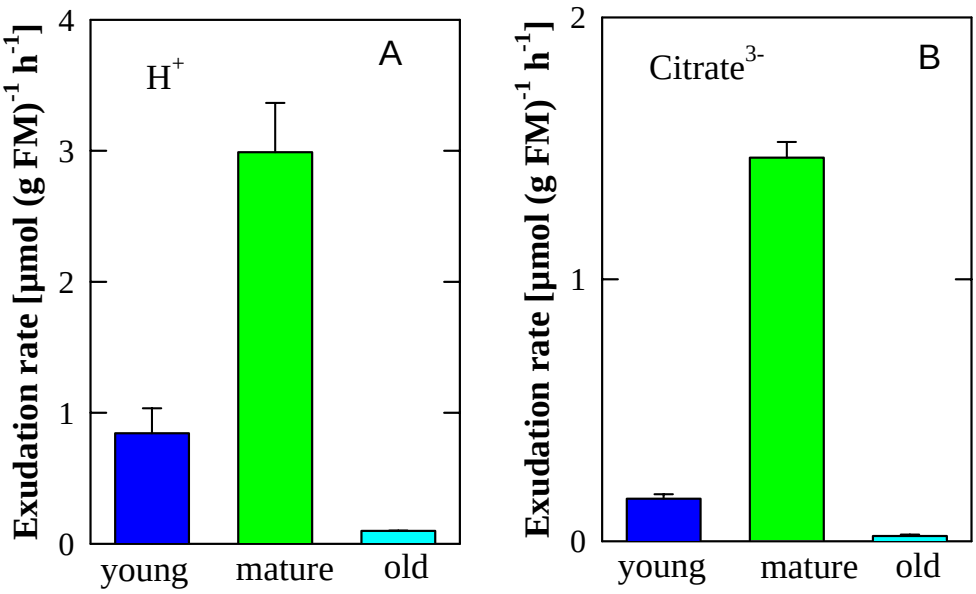


Figure 21. Effect of proteoid root development on the $H^+$ and citrate exudation rate. Proteoid roots in different development stages were incubated in the solution (containing 0.5 mM $K_2SO_4$, 0.5 mM $Na_2SO_4$ and 0.5 mM $CaSO_4$ ) for 60 min. Each value represents the mean ± SE of four replications.

### 3.5.2 Effect of proteid root development on the accumulation of citrate and malate in the root cells

Citrate accumulated at high levels mainly in mature and old proteoid roots, whereas malate was dominat in young proteoid roots and decreased with the development of proteid roots (Fig. 21). A peak of citrate exudation from mature proteoid roots occurred when citrate accumulation reached highest level of approx. 30 µmol/(g FW). In old proteoid roots, the citrate concentration was as high as in the mature proteoid roots (Fig. 21), although the citrate exudation was very low (Fig. 20). These data indicate that a high rate of citrate release in mature proteid roots is not simply leakage as a result of P deficiency-induced impairment of plasma membrane integrity (Ratnayake et al., 1978).

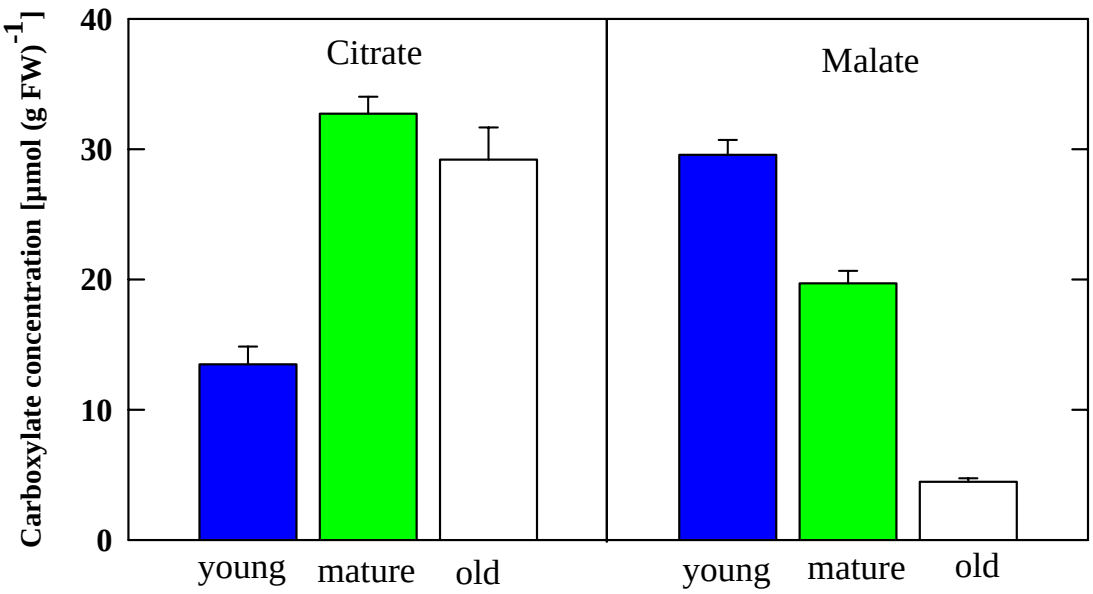


Figure 22. Tissue concentrations of citrate and malate in different developmental stages of proteoid roots of white lupins after 24-d culture without P. Each value represents the mean ± SE of four replications.

### 3.5.3 Relative expression of mRNA of various enzymes involved in the synthesis of citrate during proteoid root development

The highest activity of plasma membrane $H^+$ ATPase in mature proteoid roots coincided not only with the highest accumulation of citrate in root cells but also with highest citrate exudation rate from mature proteoid roots. Such results indicate that plasma membrane $H^+$ ATPase could contribute to both citrate synthesis and citrate release. In order to get a deeper insight into the contribution of plasma membrane $H^+$ ATPase in the develepment of proteoid roots under P deficiency, the relative mRNA expression rates of plasma membrane $H^+$ ATPase and enzymes related to citrate synthesis in proteoid roots during a development period of 8 d were compared.

The total RNA was extracted daily from proteoid roots during a developmental period from 1 to 8 d. The mRNA was reversely transcripted to cDNA. The gene expression of different enzymes was quantified by means of real-time PCR. The expression rate of mRNA at the first day was defined as 1 and expression rate of mRNA in the following days was compared with that at the first day and defined as relative expression rate.

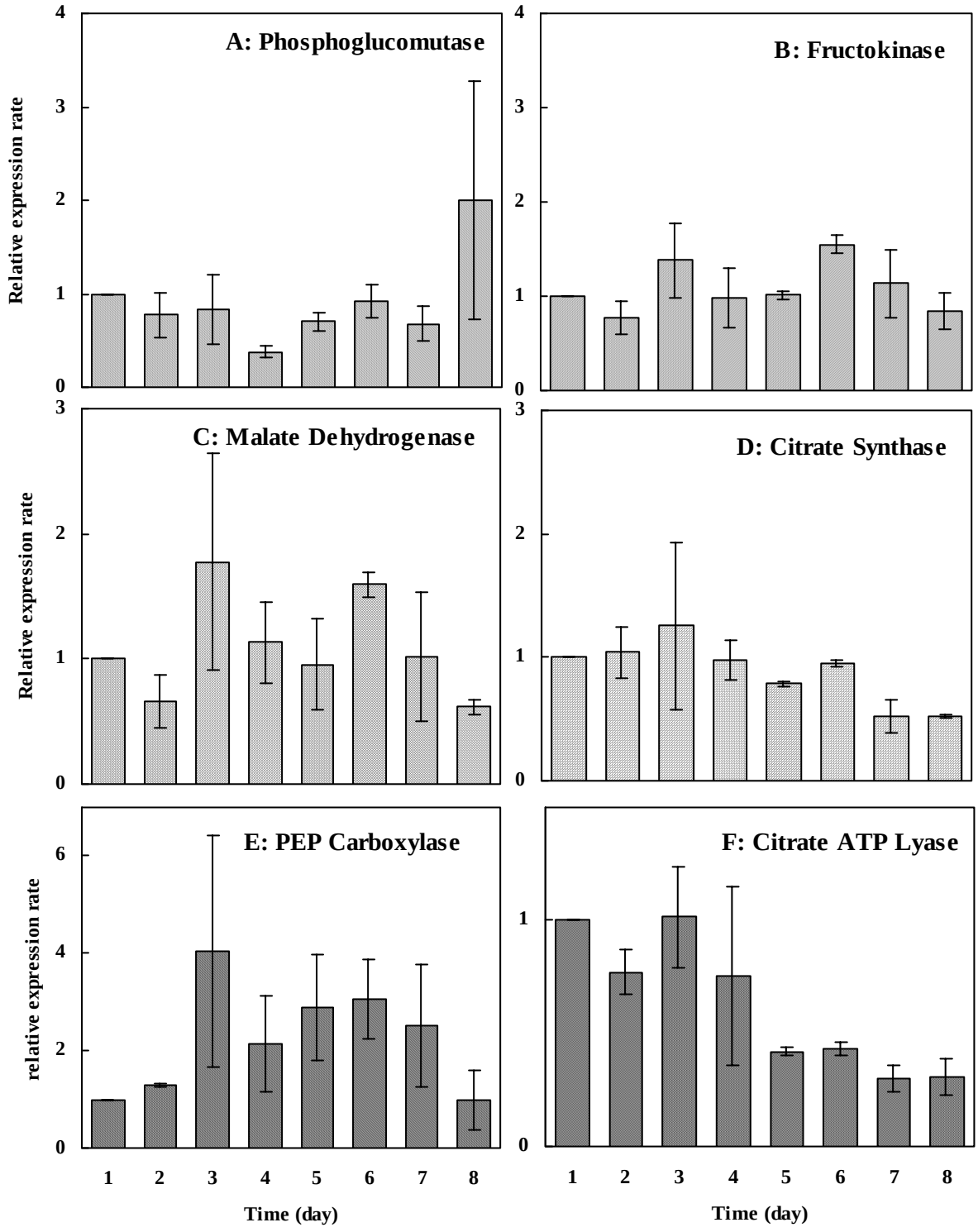


Figure 23. Comparison of mRNA of different enzymes in proteoid roots during the development period from 1 to 8 d. Plants were grown in nutrient solution at pH 6 for 3 w. Total RNA was isolated from proteoid roots. The relative expression rate was analyzed with real-time PCR. Values represent means ± SE of two independent experiments.

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), which includes glycolysis and citric acid cycle. Before glycolysis, one sucrose molecule is broken down into two hexoses: glucose or fructose. Phosphoglucomutase reversibly converts glucose 1-phosphate to glucose 6-phosphate. ATP-dependent phosphofructokinase irreversibly transforms fructose 6-phosphate to fructose 1,6-bisphosphate. Both glucose 6-phosphate and fructose 1,6-bisphosphate are then converted to pyruvate in glycolysis. In the experiments, the mRNA expression rate of phosphoglucomutase (EC 5.4.2.2) and phosphofructokinase (EC 2.7.1.4) did not show significant changes during a development period of 8 d (Fig. 23 A, B).

Pyruvate can be decarboxylated into acetyl-CoA, which links glycolysis (in cytosol) and citric cycle (in mitochondria). As the citric acid cycle begins, acetyl-CoA and OAA condense to citrate by citrate synthase. The mRNA expression rate of citrate synthase (EC 4.1.3.7) did not change significantly during the first 6 d of proteoid root development, and decreased slightly in the last 2 d (Fig.23 D).

In the cytosol of plants, the pyruvate production is not only directly determined by pyruvate kinase and PEP phosphatase, but also by PEP carboxylase. PEP carboxylase generates oxaloacetate (OAA) which is either transported directly into mitochondria or reduced to malate by malate dehydrogenase in cytosol. Malate can be transported into mitochondria and oxidized to OAA or decarboxylated to pyruvate. The mRNA expression of PEP carboxylase (EC 4.1.1.31) increased at the 2nd d and reached at the highest level at the 3rd d, being about 4 times higher than that of control, and remained at this high level until the 7th d (Fig. 23 E). It started to decrease at the 8th d. In contrast, the mRNA expression of malate dehydrogenase (EC 1.1.1.37) changed slightly during the development period of 8 d (Fig. 23 C).

The accumulation of citrate in proteoid roots during the development was linked not only directly to synthesis, but also to low degradation. In cytosol, citrate can be converted into acetyl-CoA and OAA by ATP-dependent citrate lyase. The results show

that mRNA expression of citrate lyase (EC 4.1.3.8) began to decrease at the 4th d and maintained about less than 50% of control for the last 4 d (Fig. 23 F).

On the other hand, the mRNA expression of plasma membrane $H^+$ ATPase increased 1.9 and 2.4 times on 2nd and 3rd d (Fig. 24). Expression was significantly accelerated from 4th to 6th d. The relative expression rates were 4.0, 6.0 and 8.7 times higher than that of the 1st d, respectively. In the last 2 d, the mRNA expression started to decrease.

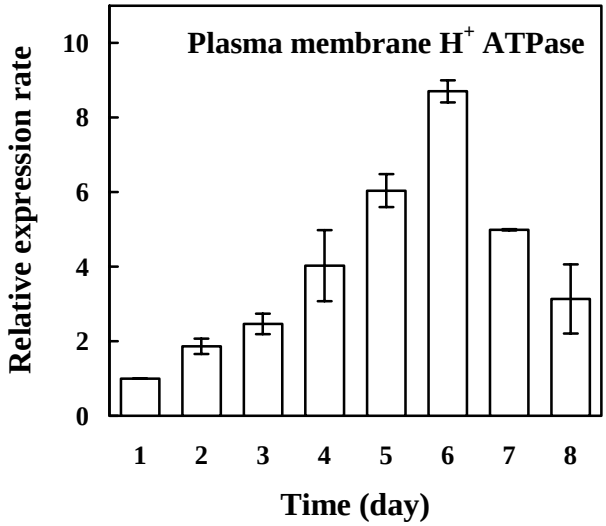


Figure 24. Change of mRNA expression of plasma membrane $H^+$ ATPase depending on the development of proteoid roots. Plants were grown in nutrient solution at pH 6 for 3 w. Total RNA was isolated from proteoid roots. The relative expression rate was analyzed with real-time PCR. Values represent means ± SE of two independent experiments.

A comparison of the time course of expression among plasma membrane $H^+$ ATPase, PEP carboxylase and ATP citrate lyase (Fig. 25) showed that both PEPC and plasmamembrane $H^+$ ATPase were up-regulated at the 2nd d. While expression rate of PEPC maintained at a relative higher level frome 3th to 7th d, expression rate of $H^+$ ATPase continued increasing and reached to a maximum on 6th day. Conversely, the expression of ATP citrate lyase was down-regulated 3 d after emergency and kept low throughout the rest of 4 d.

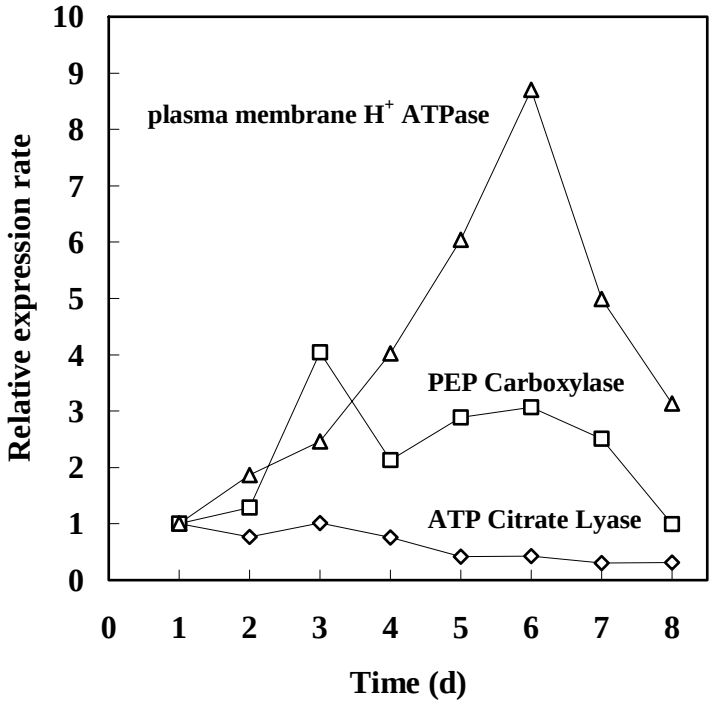


Figure 25. Comparison of mRNA expression of plasma membrane $H^+$ ATPase, PEP carboxylase and ATP citrate lyase in proteoid roos during 8 d development period. Plants were grown in nutrient solution at pH 6 for 3 w. The total RNA was isolated from proteoid roots. The relative expression rate was analyzed with real-time PCR.

# 4. Discussion

## 4.1 Relationship between citrate and $H^+$ exuded by intact proteoid roots of white lupin adapted to P deficiency

### 4.1.1 Validity of the methods used for the quantification of cations and anions exuded by active proteoid roots

A prerequisite for the study of relationships between citrate release and the release of $H^+$ and other ions by proteoid roots is the establishment of methods, which warrant a precise quantification of the mount of various ions exudated by proteoid roots. In literature there are a number of studies in which the citrate exudation rate was measured with whole root system (Neumann et al., 1999; Sas et al., 2001). One of the disadvantages of such measurement is that the contribution to citrate exudation is not homogenous throughout the whole root system. This proves to be a problem especially for the white lupin root system including proteoid roots. In fact, only part of proteoid roots contribute to citrate and $H^+$ exudation. Therefore, in the present study a special cuvette for the collection of exudates was developed. By means of this cuvette exudates from active proteoid roots can be collected. The small volume of the cuvette can increase the scale of the concentration change of ions being exuded. This makes it possible to precisely quantify the amount of ions exudated by active proteoid roots.

In addition, for each ion species, the differences of ion concentration were determined between the solutions before and after incubation experiment. According to the change of ion concentration and the solution volume used for incubation, the amount of ions exuded was quantified.

Regarding various ions, the quantification of $H^+$ exuded by protoeid roots is the most critical one. A precise quantification of $H^+$ exuded by plant roots is complicated by the fact that the proteoid roots exude large amounts citrate and malate, which buffer $H^+$. To optimize the quantification of $H^+$ in the root exudation solution we chose to titrate both solutions (solutions before and after incubation with proteoid roots) to pH 7. The

difference in consumed alkalinities was then equivalent to the $H^+$ exuded by proteoid roots. The choice for the end point of titration was based on the fact that for most plant cells the cytosolic pH is around 7 (Guern et al., 1993) at which citrate or malate are transported across plasma membrane. In the present study, the effect of $CO_2$ on the quantification of $H^+$ exudation was tested by titration the exdation solutions with and without vacuum treatment (data not shown), because vacuum can eliminate soluble $CO_2$ in the exudation solution. No strong effect of $CO_2$ on the quantification of $H^+$ exudation by proteoid roots was detected. This may be attributed to lower respiration rate of proteoid root cells (Neumann et al., 1999; Massonneau et al., 2001; Kania et al., 2003) and to the low solution pH (lower than pH 5.6) at which volatilization of $CO_2$ in water is not high.

The validity of the methods used for the quantification of various ions has been partly confirmed by a good correlation ($r^2 = 0.94$) between total charge equivalents of cations and anions with a slope of 0.84 (Fig. 8). The discrepancy of the estimated slope from the theoretical value of 1 may reflect either the sum of errors involved in the measurements of each ion species or the ion species considered. Despite of this small deviation between total positive and negative charges our data indicate that not only the quantity of ions but also the relevant ion species exuded by active proteoid root cells have been precisely determined.

An additional important aspect for the determination of root exudation by intact roots is the time period used for the collection of root exudates. This is especially critical for the incubation with pharmacological drugs. For the study of root exudation of white lupin adapted to P deficiency, the incubation time ranged from 1.5-2 h ( Sas et al., 2001; Peñaloza et al., 2002; Neumann et al., 1999) to 6-18 h (Watt and Evans 1999). Because the aim of the present study focused on the trans-plasma membrane transport of various ions, we studied ion exudation during different incubation periods ranging from 15 to 120 min. For ions such as citrate, malate, $Mg^{2+}$ and $Cl^-$ the exudation rate within 15 min is an approximate of efflux since the concentration of these ions in the incubation solution was very low and the contribution of uptake process to the concentration changes of the ions may not be significant. The shortcoming of the

exudation rate with the incubation time of 15 min relative to longer incubation time was the greater deviations (Table 1). Generally, the exudation rate of ions declined, though to different extent, with the extending incubation periods. This may be explained by the increasing contribution of ion uptake by proteoid root cells. For incubation time of 120 min, the exudation rate probably approached steady-state.

### 4.1.2 Quantitative relationship between citrate and $H^+$ exuded by intact proteoid roots of white lupin adapted to P deficiency

Quantitatively, active proteoid roots of white lupin exude citrate with a rate of 0.8-2.8 µmol $(g\ FW)^{-1}\ h^{-1}$ (Table 1). This is in good agreement with the findings of various authors. Using a similar small cuvette for root exudation experiment, citrate exudation rates of 1.1, 1.2 and 1.7-2.4 µmol $(g\ FW)^{-1}\ h^{-1}$ have been reported for active proteoid roots of white lupin adapted to P deficiency by Keerthisinghe et al. (1998), Neumann et al., (1999) and Peñaloza et al. (2002), respectively. Furthermore, malate exudation rate was approximately 8.5-15 times lower relative to citrate (Table 1), although the concentration ratio of citrate to malate in the active proteoid roots was about 1.5 (Table 2). This indicates that active proteoid roots have a much higher exudation capacity for citrate than for malate, which has been known as the typical characteristics of active proteoid roots of white lupin (Neumann and Martinoia 2002).

Over a wide range, citrate exudation rate is closely related to the $H^+$ exudation rate with a stoichiometry of 1:1.3 (Fig. 7 A). This means that proteoid root cells exude 1.3 $H^+$ ions for one citrate anion on average. Interestingly, an identical stoichiometry of 1:1.3 for exudation of citrate plus malate and $H^+$ was determined for symbiotically grown white lupin by Sas and co-authors (2001). It is evident that $H^+$ exudation alone is not sufficient to keep the charge balance for carboxylic anions by proteoid roots of white lupin adapted to P deficiency. $K^+$, $Na^+$ and $Mg^{2+}$ can function as additional counterions for citrate exudation since they all correlated well with citrate exudation (Fig. 7 B,D). Relative to citrate exudation, a stoichiometry of 1:2.1 and 1:1.5 was determined for $K^+$ and $Na^+$, respectively (Fig. 7 B,C). For $Mg^{2+}$, a stoichiometry of 1:0.94 was calculated by multiplying the slope of the line (Fig. 7 D) with 2.

Quantitatively, our data suggest an approximately equal importance of $H^+$, $K^+$, $Na^+$ and $Mg^{2+}$ as counterions for the citrate exudation out of proteoid root cells.

### 4.1.3 Linkage between $H^+$ release and citrate release by intact proteoid roots of white lupin adapted to P deficiency

To evaluate the significance of $H^+$, $K^+$, $Na^+$ and $Mg^{2+}$ for the exudation of citrate by proteoid roots various pharmacological drugs were applied. For such kind of experiments, caution should be taken for the interpretation of results, because secondary effects of drugs on metabolism cannot be ruled out. To minimize the interference of secondary effects of drugs, the incubation was limited for 15 to 60 min. During these time periods there was no effect of drugs on the concentrations of citrate and malate in proteoid roots (Table 2). Under the same condition, niflumic acid (anion-channel blocker) and tetraethylammonium (TEA, $K^+$-channel blocker) significantly reduced the citrate and malate concentrations of active proteoid roots by more than 50% (data not shown) and were, therefore, not considered further in this study.

Within 15 min, proteoid root cells responded to fusicoccin with a significant increase in $H^+$ exudation which was accompanied by a significant increase in citrate exudation (Fig. 9 A, F). During this time period there was no significant change in exudation for both $K^+$ and $Na^+$ (Fig. 9 B, C). It is well known that fusicoccin removes the auto-inhibitory domain of the plasma membrane $H^+$ ATPase and thus stimulates its activity, resulting in a higher $H^+$ pumping activity, and consequently a hyperpolarization of membrane potential (Johansson et al., 1993; Taylor and Assmann 2001). The concomitant stimulation of exudation of $H^+$ and citrate was also evident when the treatment was extended to 60 min, but not at 30 min. However, There was no such concomitant increase in exudation rate for $Na^+$ , $K^+$ or $Mg^{2+}$ (Fig. 9 B, C). In fact, $K^+$ exudation was decreased, while citrate exudation increased. The decrease in exudation rate of $K^+$ may be explained in terms of a stimulation of $K^+$ uptake by an increase of the membrane potential difference which results from stimulation of plasma membrane $H^+$ ATPase by fusicocccin (Romani et al., 1985). Our data with fusicoccin suggest that

$H^+$ exudation is more closely related to citrate exudation as compared with the exudation of $K^+$, $Na^+$ or $Mg^{2+}$.

This conclusion can be further supported by data with the anion channel blocker anthracene. A significant decrease in citrate exudation by anthracene was achieved after incubation for 60 min. A concomitant decrease in exudation rate was observed only for $H^+$, but not for $K^+$, $Na^+$ or $Mg^{2+}$ (Fig. 11 F, A, B, C). In addition, a direct inhibitory effect of anthracene-9-carboxylate on plasma membrane $H^+$ ATPase can be ruled out (data not shown). Therefore, among the cations studied so far, $H^+$ showed the closest relationship with the citrate exudation by proteoid roots of white lupin adapted to P deficiency.

In addition, we investigated the effect of proteoid roots development on the exudation of citrate and $H^+$. In a 60 min incubation period, the concomitant change of citrate and $H^+$ exudation was found in various development stages of proteoid roots under P deficiency (Fig. 21). The change of plasma membrane $H^+$ ATPase activity matched the change of $H^+$ exudation in the various development stages (Fig. 18). These results further indicate that the citrate release during the physiological development was closely related to $H^+$ released by plasma membrane $H^+$ ATPase. In this way, plasma membrane $H^+$ ATPase of active proteoid roots was involved in the adaptation to phosphate deficiency.

## 4.2 Adaptation of plasma membrane $H^+$ ATPase of proteoid roots to P deficiency

### 4.2.1 Isolation of plasma membrane

To investigate plasma membrane $H^+$ ATPase of proteoid roots adapted to P deficiency, it is necessary to isolate pure plasma membrane from proteoid roots. In our study, membrane fractions isolated from different types of white lupin roots showed a high sensitivity to vanadate (0.1 mM, pH 6.5) and negligible sensitivity to nitrate (50 mM, pH 8) and azide (1 mM, pH 8), indicating a plasma membrane fraction with low contamination by membranes of tonoplast and mitochondrial origins (Table 3). However, ATP hydrolysis by the obtained membrane fractions, especially those from P-deficient plants, showed a considerable sensitivity to molybdate (1 mM, pH 6.5), indicating an unspecific acid phosphatase activity (Widell and Larsson, 1990). Although the localization of unspecific acid phosphatase activity is in most cases uncertain, the fact that the ATPase hydrolytic activity in the obtained membrane fractions was not sensitive to nitrate suggests that the unspecific acid phosphatase activity was not related to the tonoplast.

In plant cells, most of the unspecific acid phosphatases exist in the vacuole as soluble proteins (Duff et al., 1994). These proteins can be trapped in membrane vesicles during homogenization of plant tissues. In addition, white lupin roots release a large amount of acid phosphatases in response to P deficiency (Li et al., 1997; Gilbert et al., 1999; Neumann et al., 2000; Wasaki et al., 2000; Miller et al., 2001). After synthesis, acid phosphatases are transported via secretory vesicles to plasma membranes and are released into apoplast. Isolated membrane was treated with freezing-thawing-cycling followed by washing. However, this treatment did not reduce the unspecific acid phosphatase activity. This result suggests a binding of phosphatases to plasma membrane. In white lupin, cDNA clones of two isoforms of acid phosphatases, lasap1 and lasap2, have been characterized (Wasaki et al., 1999, 2000). lasap2 is specifically expressed in roots exposed to P deficiency and is supposed to encode secretory acid phosphatase. The expression of lasap1 evidently not only occurs in roots but also in

leaves and responses to P deficiency. According to cDNA analysis, it was suggested that acid phosphatase encoded by lasap1 may be anchored to plasma membrane by a glycosyl-inositol-phospholipid (Wasaki et al., 2000). In conclusion, despite a considerable activity of unspecific acid phosphatases, the isolated membrane fractions can be considered as plasma membrane with low contamination of other membrane origins. To warrant a characterization of plasma membrane $H^+$ ATPase, 1 mM molybdate was included in all analyses to suppress acid phosphatase activity.

### 4.2.2 Quantitative adaptation of plasma membrane $H^+$ ATPase in active proteoid roots to P deficiency

The quantitative adaptation of plasma membrane $H^+$ ATPase of active proteoid roots to P deficiency is supported by the following results obtained *in vitro*: compared with lateral roots from both P-sufficient and P-deficient plants and proteoid roots of P-sufficient plants, (a) the hydrolytic activity of plasma membrane $H^+$ ATPase of active proteoid roots was about 3-fold higher (Fig. 12), (b) $V_{max}$ of $H^+$ ATPase in plasma membrane of active proteoid roots was about 3 times higher (Fig. 13; Table 4), (c) western blotting showed an about 4 times higher $H^+$ ATPase enzyme concentration in the plasma membrane of active proteoid roots (Fig. 14), (d) the initial rate of $H^+$-pumping activity of active proteoid roots was 3-fold higher (Fig. 15A; Table 5), (e) plasma membrane $H^+$ ATPase of active proteoid roots created and maintained a higher pH gradient (Fig. 15A; Table 5), and (f) this higher pH gradient was not explained in terms of reduced $H^+$ permeability of this membrane; rather, the plasma membrane of active proteoid roots showed about 2 times higher $H^+$ permeability (Fig. 15B; Table 5).

Of all characteristics for the quantitative adaptation, the high $H^+$ ATPase enzyme concentration in the plasma membrane is the fundamental one that is responsible for overall increase in hydrolytic ATPase activity and $H^+$-pumping activity recorded for active proteoid roots. In fact, for all membrane fractions analyzed, there was no difference in hydrolytic efficiency for $H^+$ ATPase, because for all four membrane fractions, about 1.8 times increase in $V_{max}$ was caused by increasing assay temperature from 25°C to 30°C, from which a comparable activation energy of 92 kJ $mol^{-1}$ was

calculated for all four membrane fractions analyzed. In addition, there was no difference in coupling efficiency between ATP hydrolysis and $H^+$ pumping for plasma membrane $H^+$ ATPase derived from different types of roots. This conclusion is supported by the fact that the increase of rates for both hydrolytic ATPase activity and $H^+$-pumping activity for active proteoid roots was similar (Figs. 12 and 15; Tables 4 and 5).

A higher enzyme concentration of $H^+$ ATPase in the plasma membrane of active proteoid roots can result either from a higher expression rate (transcriptional level) of genes encoding plasma membrane $H^+$ ATPase or an enhanced synthesis rate of the enzyme proteins (translational level), or from a reduced degradation rate of $H^+$ ATPase (Sze et al., 1999). So far, our results indicate that the up-regulation of mRNA expression is responsible for the higher $H^+$ ATPase enzyme concentration in the active proteoid roots and thereby for the adaptation of white lupin to P deficiency (Fig. 25). In maize coleoptile, auxin stimulated not only gene expression of plasma membrane $H^+$ ATPase but also the membrane flow from endoplasmic reticulum to plasma membrane, resulting in a higher steady-state $H^+$ ATPase concentration in the plasma membrane (Hager et al., 1991). In addition, this study also showed that the half-life time of plasma membrane $H^+$ ATPase was only 12 min. Therefore, if the degradation rate of the plasma membrane $H^+$ ATPase can be slowed down in active proteoid roots, this may also significantly contribute to the higher $H^+$ ATPase concentration in the plasma membrane. It is suggested that the translational regulation of $H^+$ ATPase controls the amount of the proteins only within a short range of concentration. A 2-fold variation in translation efficiency has been suggested for pma1 and pma3, the plasma membrane $H^+$ ATPases of *Nicotiana plumbaginifolia* (Lukaszewicz et al., 1998). If this holds true for white lupin, it implies that the 4-fold higher $H^+$ ATPase enzyme concentration in the plasma membrane of active proteoid roots can only be partly explained in terms of translational regulation.

### 4.2.3 Qualitative adaptation of plasma membrane $H^+$ ATPase in active proteoid roots to P deficiency

Besides quantitative adaptation, a qualitative adaptation of plasma membrane $H^+$ ATPase of active proteoid roots to P deficiency is also evident. This conclusion can be supported by following results: Compared with lateral roots from both P-sufficient and P-deficient plants and proteoid roots of P-sufficient plants, (a) plasma membrane $H^+$ ATPase of active proteoid roots showed a more acidic pH optimum (Fig. 12), (b) the $K_m$ value of the plasma membrane $H^+$ ATPase of active proteoid roots was significantly higher (Fig. 13; Table 4), and (c) at assay pH 6.0, the vanadate sensitivity of the plasma membrane $H^+$ ATPase of active proteoid roots was lower (higher $I_{50}$ for vanadate, Table 4).

At enzyme level, it is well established that a part of the C-terminal region of $H^+$ ATPase constitutes an auto-inhibitory domain. Removal of this domain by various effectors such as fusicoccin (a fungal toxin) *in vivo*, lysophospholipids, and trypsin *in vitro* can activate the enzyme (Palmgren et al., 1991; Johansson et al., 1993). The question arises whether this mechanism is involved in the regulation of plasma membrane $H^+$ ATPase of active proteoid roots and whether it is responsible for the qualitative adaptation to P deficiency. The removal of the auto-inhibitory domain of $H^+$ ATPase is characterized by (a) a higher degree of stimulation in $H^+$-pumping than in hydrolytic ATPase activity, (b) an increase in $V_{max}$ and a decrease in $K_m$, (c) a shift of pH optimum toward more alkaline values, and (d) sensitivity of the enzyme to vanadate remains unchanged (Palmgren et al., 1988; Palmgren and Sommarin, 1989). All our data determined for the $H^+$ ATPase in plasma membrane of active proteoid roots are not consistent with the above-mentioned changes in $H^+$ ATPase caused by removal of auto-inhibitory C-terminus. Compared with lateral roots and the proteoid roots of P-sufficient plants, the plasma membrane $H^+$ ATPase of active proteoid roots showed (a) the same degree of stimulation for both $H^+$-pumping and hydrolytic activity (Figs. 12 and 15; Table 5); (b) not only a higher $V_{max}$, but also a higher $K_m$ (Fig. 13; Table 4); (c) a more acidic pH optimum (Fig. 12); and (4) a lower sensitivity to vanadate at assay pH 6.0 (Table 4). In addition, the higher activity of $H^+$ ATPase

can be explained in terms of higher $H^+$ ATPase enzyme concentration in the plasma membrane of active proteoid roots (Fig. 14).

The qualitative changes of the enzyme may be explained in terms of differential expression of $H^+$ ATPase isoforms in the plasma membrane of active proteoid roots. In proteoid roots, we found two expressed sequence tags (data not show). One displayed 99% homology to that found in lateral roots from P-sufficient and P-deficient plants. The other revealed 77-79% homology to the first one, and the expression of this gene was up-regulated during the development of proteoid roots under P deficiency (Fig. 24).

It is well known that the plant plasma membrane $H^+$ ATPase is encoded by a multigene family (Michelet and Boutry, 1995). Using heterologous expression of functional plant plasma membrane $H^+$ ATPase in yeast (*Saccharomyces cerevisiae*), distinct differences in enzyme kinetics, pH optimum, and vanadate sensitivity have been reported for different isoforms of $H^+$ ATPase in *Arabidopsis* (Palmgren and Christensen, 1994). A 10-fold higher $K_m$ was found for AHA3 as compared with AHA1 or AHA2. In addition, AHA2 showed 3 times lower sensitivity to vanadate than AHA1 or AHA3 (Palmgren and Christensen, 1994). Such a tissue-, organ-, or cell-specific expression of isoforms of $H^+$ ATPase has been reported by a number of investigators (DeWitt et al., 1991; Michelet et al., 1994; Moriau et al., 1999) and summarized as a "tissue-specific expression model" in which enzymatically equivalent enzymes are expressed in different cells by promoters providing controlled expression levels ideally suited for a cell's particular needs (Palmgren and Harper, 1999). It is also true that in active proteoid roots, an enhanced expression of a special isoform of plasma membrane $H^+$ ATPase is induced to meet the high demand of the cells to extrude $H^+$ (Fig. 24 and Fig. 21).

### 4.2.4 Quantitative change of plasma membrane $H^+$ ATPase during proteoid root development

During the physiological development of proteoid roots under P deficiency, a quantitative change of plasma membrane $H^+$ ATPase could be deduced by the following results obtained *in vitro*: (a) the hydrolytic activity of plasma membrane $H^+$ ATPase of mature proteoid roots was about 2-fold higher than that of young proteoid roots and 5-fold higher than that of old proteoid roots (Fig. 17), (b) $V_{max}$ of $H^+$ ATPase in plasma membrane of mature proteoid roots was 2.3 times higher than that of young proteoid roots, and 6 times higher than that of old proteoid roots (Fig. 18; Table 6), (c) western blotting showed an about 1.3 times higher $H^+$ ATPase enzyme concentration in plasma membrane of mature proteoid roots than that of young proteoid roots and 4 times higher enzyme concentration than that of old proteoid roots (Fig. 19), (d) the initial rate of $H^+$-pumping activity of mature proteoid roots was 1.7-fold higher than that of young proteoid roots and 5-fold higher than that of old proteoid roots (Fig. 20; Table 7), (e) plasma membrane $H^+$ ATPase of mature proteoid roots created and maintained the highest pH gradient and plasma membrane $H^+$ ATPase of old proteoid roots created and maintained the lowest pH gradient (Fig. 20; Table 7).

All characteristics indicate that hydrolytic ATPase activity and $H^+$-pumping activity were contributed by the change of enzyme concentration of $H^+$ ATPase in the plasma membrane of proteoid roots during their development. This change of enzyme concentration match the timecourse of gene expression of plasma membrane $H^+$ ATPase during the development of proteoid roots (Fig. 24 and Fig. 19).

*in vivo,* the strong rhizosphere acidification was associated only with the active proteoid roots (mature proteoid roots) from P-deficient white lupin. On the contrary, neither old proteoid roots from P-deficient plants nor proteoid roots produced by P-sufficient plants showed rhizosphere acidification. In addition, the apical zone of lateral roots from P-deficient plants also failed to acidify rhizosphere significantly (Fig. 6). These *in vivo* data are closely related to the activity of plasma membrane $H^+$ ATPase *in vitro* (Fig. 12). The data suggest that the enhanced expression of $H^+$

ATPase in active proteoid roots of white lupin is related to a combination of root structures, P deficiency, and physiological development of proteoid roots. A similar conclusion for the release of citrate by white lupin roots was drawn by a number of investigators (Johnson et al., 1996 a, 1996b; Keerthisinghe et al., 1998; Neumann et al., 1999, 2000; Watt and Evans, 1999a; Massonneau et al., 2001).

## 4.3 Regulation of plasma membrane $H^+$ ATPase is involved in citrate synthesis and citrate releasing during the proteoid root development

During proteoid root development, P deficiency induces a shift in the metabolism of organic acids from predominant accumulation of malic acid in young proteoid roots to accumulation of citric acid in mature proteoid roots (Fig. 21) and causes more citrate to be released by mature proteoid roots than young proteoid roots (Fig. 20). This metabolic changes were associated with an increase in phosphoenolpyruvate carboxylase (PEPC) activity (Johnson et al., 1994, 1996; Neumann et al. 1999), and a reduction in ATP-citrate lyase activity (Neumann and Martinoia 2002). Because the regulation of gene expression of plasma membrane $H^+$ ATPase is related to the combination of root structures, P deficiency, and physiological development of proteoid roots as described in chapter 4.2. Correspondingly, it may be also true for the genes encoding for PEPC, ATP citrate lyase.

In the glycolytic bypass, non-photosynthetic $CO_2$ fixation mediated by the up-regulation of PEPC played an important role in the anaplerotic carbon supply for P deficiency-induced biosynthesis of organic acids in proteoid roots (Johnson et al. 1994, 1996a, b). Evidence on the involvement of PEPC in the stratery to withstand P stess in plants has been observed also in C3 species (Johnson et al. 1994). Metabolic studies of P-deficient algae indicated that during the malate production via PEPC and MDH from PEP, PEP may function to provide $P_i$ as well as carbon skeletons (Theodorou et al., 1991), which make this a favorable reaction under P-limited conditions. Increased PEPC mRNA expression (Fig. 23 E) during the development of proteoid roots in this study was directly related to an increase in PEPC protein and PEPC specific activity reported by many researchers (Hoffland et al. 1992; Pilbeam et al. 1993; Johnson et al. 1994, 1996a, b; Uhde-stone et al., 2003). This result suggests that PEPC is at least partially under transcriptional regulation and the development of P-deficient proteoid root altered its expression in a time-dependent fashion.

The up-regulation of PEPC mRNA was found early in the 3rd d (Fig. 23 E), but the highest rate of citrate releasing was detected at about 5-6 d (Fig. 21 B). The observation that maximal PEPC activity precedes citrate efflux was also reported by

other authors (Neumann et al., 1999), and explained by the increase of internal citrate concentrations prior to excretion.

The up-regulation of PEPC after emergence of proteoid roots causes more oxaloacetate to be produced, which is then reduced to malate. The comparison of radioactive label in citrate versus malate supports that malate produced via PEPC and MDH is converted to citrate in the proteoid roots of P-deficient lupin, where the ratio is about 1:1 (Johnson et al., 1994). Hence, malate/citrate exchange on the tricarboxylate carrier was involved. The tricarboxylate carrier, also named citrate carrier, is an intrinsic protein of the inner mitochondrial membrane, which exchanges cytoplasmic malate for citrate synthesized inside mitochondria (Genchi, et al., 1999). One malate molecule exchanges one citrate molecule with an associated $H^+$ out of the mitohondria. An alternative fate of oxaloacetate is that it could be directly transported into the mitochondria and converted into citrate. The exchange of oxaloacetate/citrate across the mitochondrion membrane can also happen and a $H^+$ will be introduced into the cytoplasm.

After citrate being exchanged into cytoplasm, it would be cleavaged by ATP citrate lyase and converted to malate again. ATP citrate lyase has higher activity in the young proteoid roots than in mature porteoid roots (Langlade et al., 2002), which is dependent on the expression of this enzyme (Fig 24 F). This result suggests that ATP citrate lyase is regulated at the transcriptional level. Accordingly, accumulation of malate is the dominating character in the young proteoid root (Fig. 22).

Although the citrate concentration in young proteoid roots was still low (Fig. 22), the concomitant introduction of $H^+$ into the cytosol by citrate carrier could begin to accumulate early in young proteoid roots. In order to keep the pH constant in cytosol, some important mechanisms are involved, such as consumpt ion of $H^+$ by malate enzyme (biochemical pH-Stat, Davies 1973), $H^+$ import in the vacuole by tonoplast $H^+$ ATPase and PPase, and $H^+$ export to the apoplast by plasma membrane $H^+$ ATPase (Schubert and Yan, 1997). In comparison with the biochemical pH-Stat and vacuole, the plasma membrane $H^+$ ATPase can continuously pump $H^+$ out from the cytoplasm with unlimited volume in apoplast. According to this scenario, the increased gene expression of plasma membrane $H^+$ ATPase during maturation may be promoted by cytosolic acidification which was caused by accumulation of more and more citrate

and protons in cytoplasm. Consequently, the activity of plasma membrane $H^+$ ATPase increased (Fig. 17) and caused more $H^+$ to be released from mature proteoid roots than young proteoid roots (Fig. 21). This speculation was supported by Kanja et al. (2003), who used propionate to lower the cytosolic pH in the proteoid roots of white lupin adapted to P deficiency and stimulated the transient release of protons and also citrate.

In this way, plasma membrane $H^+$ ATPase may play an important role in the activation of channels responsible for citrate exudation and simultaneously supply counter ions in large quantity required for the citrate efflux out of proteoid root cells of white lupin adapted to P deficiency. Several lines of evidence indicate a linkage between the regulation of plasma membrane $H^+$ ATPase and citrate exudation from plant cells. The quantitative change of plasma membrane $H^+$ ATPase during the physiological development of proteoid roots under P deficiency was closely linked to citrate exudation from proteoid root cells (Fig. 18, 21). *In situ*, a rapid response of exudation of both $H^+$ and citrate from proteoid roots to fusicoccin strengthens the physiological significance of plasma membrane $H^+$ ATPase for citrate exudation out of proteoid root cells (Fig. 9 A, F). A similar up-regulation of plasma membrane $H^+$ ATPase was reported for transgenic carrot cells whose citrate exudation was significantly increased by over-expression of citrate synthase (Ohno et al., 2003). All of these results indicate that the regulation of plasma membrane $H^+$ ATPase was involved in the transport of citrate from the proteoid root cells.

## 4.4 $H^+$ release is not a prerequisite for citrate release by intact proteoid roots, but is a superior strategy of white lupin adapted to P deficiency

Transporters responsible for the efflux of carboxylic anions out of proteoid roots remain to be fully elucidated. Based on the effect of anion-channel blockers on citrate exudation by intact complete white lupin root system, it is speculated that citrate exudation from proteoid roots could be mediated by anion channels located in plasma membrane (Neumann et al., 1999). This speculation has been confirmed in the present study by direct application of anthracene to intact active proteoid roots (Fig. 11 F, G, I). Recently, the citrate transport through plasma membrane of proteoid root cells of white lupin has been studied using patch-clamp technique and a citrate-permeable anion channel has been identified in both P-deficient proteoid root cells and P-sufficient non-proteoid root cells (Zhang et al., 2003). Furthermore, no distinct differences in channel characteristics have been found in the cells derived from two different root types. The authors concluded that the differences in citrate efflux from intact P-deficient proteoid root cells and P-sufficient non-protoeid root cells might lie in the factors regulating the channel activity (Zhang et al., 2003).

Anion channel activity of plant cells can be modified by different factors. It is well established that plasma membrane anion channels permeable to citrate or malate can be activated by exposure to $Al^{3+}$ (Kollmeier et al, 2001; Zhang et al., 2001). These Al-activated anion channels are likely to be ligand-gated because for activation they require $Al^{3+}$, possibly mediated through cytosolic intermediates such as protein kinase (Osawa and Matsumoto 2001). However, the question remains open, to which extent these Al-activated anion channels resemble those mediating citrate efflux from proteoid roots, especially in the channel gating. One distinct difference between Al-activated anion channels and those mediating citrate release from proteoid roots of white lupin adapted to P-deficiency is that the activation of latter is independent of exposure to $Al^{3+}$ or other ions. In addition, cytosolic pH seems to play a significant role for the activation of anion channels permeable to citrate and malate in proteoid root cells of white lupin (Kania et al., 2003).

For proteoid root cells, there seems to be differences between anion channels responsible for the release of citrate, malate and $Cl^-$, respectively. In response to addition of anion channel blocker, a significant suppression of $Cl^-$ efflux by anthracene was achieved within 15 min, whereas a similar exudation suppression of citrate and malate was not obtained until incubation time extended to 60 min (Fig. 10 F, G, I). Furthermore, malate exudation was always related to $H^+$ exudation, regardless of drugs and incubation time periods used (Fig. 7 and Fig. 9, 10, 11 A, G), whereas for the exudation of citrate this linkage could not be shown by vanadate (Fig. 10 A, F). Vanadate can suppress $H^+$-pumping activity, resulting in a depolarization of membrane potential (Beffagna & Romani, 1988; O'Neill & Spanswick, 1984). On the other hand, vanadate inhibits not only plasma membrane $H^+$-ATPase but also other P-type ATPase including plasma membrane $Ca^{2+}$-ATPase (Thomson et al., 1993). Therefore, vanadate may also affect cytosolic $Ca^{2+}$ homeostasis (probably causes an increase in cytosolic $Ca^{2+}$ activity) of the proteoid root cells, which is known as a second messenger of cells of different organisms (Sanders et al., 2002, White et al., 2003). A change in cytosolic $Ca^{2+}$ activity may be sensed by protein kinases which then may modify the citrate-permeable anion channel activies of proteoid root cells (Osawa and Matsumoto 2001). Therefore, the results of our study may reflect distinct anion channels with gating properties. The channel responsible for malate efflux may be strictly regulated by membrane potential, whereas that for citrate efflux may be less dependent on membrane potential. Instead, it may be controlled by cytosolic $Ca^{2+}$ activity, probably through protein kinase. Alternatively, our results may be interpreted by substrate dependence of channel gating properties (malate or citrate), as found for vacuolar anion channels of CAM plans (Cerana et al., 1995) and for stomatal guard cells (Hedrich and Marten, 1993).

The fact that vanadate substantially inhibited $H^+$ release without showing any inhibitory effect on citrate release indicates that even though $H^+$-pumping is important for citrate release, it is not the only one which determines the citrate export out of proteoid root cells adapted to P deficiency. Theoretically, for a plant cell, the charge balance for citrate exudation is not necessarily always achieved by $H^+$. Alternatively, it can be achieved either through release of other cations such as $K^+$ or $Na^+$ etc, or

through uptake of anions such as $NO^{3-,}$ $Cl^-$ etc. In the present study $Na^+$ seemed inferior to $K^+$ in the charge balance for citrate release when $H^+$ release was suppressed by vanadate (Fig. 8 A-C). This may also explain why there was no detectable inhibitory effect of vanadate on citrate exudation, even when $H^+$ exudation was strongly suppressed by vanadate (Fig. 8- A, F). In the proteoid roots of various species of the family *Proteaceae*, $K^+$ seems to be the dominant cation accompanying citrate release (Roelofs et al., 2001), a similar case as found for the Al-activated release of carboxylic anions (Ryan et al., 1993; Ma et al., 2001; Ryan et al., 2001). However, it must be borne in mind that $K^+$ needed for citrate release has to be taken up prior to exudation. In a long-term experiment (incubation for 10 h), vanadate did show a significant suppression of citrate release from transgenic carrot cells, which over-expressed citrate synthase (Ohno et al., 2003).

In a natural ecosystem of poor soil fertility, soil usually not only lack of P but also other nutrients such as nitrogen and potassium. The increase of $H^+$-pumping activity to maintain the charge balance for the release of citrate should be a superior strategy over that to release $K^+$, for in this way, supply of $H^+$ is independent of soil $K^+$ availability.

# 5. Conclusion

White lupin (*Lupinus albus* L.) is able to adapt to phosphorus deficiency by forming proteoid roots that release a huge amount of citrate and protons. In the present study, the mechanisms underlying citric acids release from proteoid roots of white lupin were studied at different levels, from intact roots *in situ*, enzyme characterization on membrane viscles *in vitro*, identification and quantification of $H^+$ ATPase at protein level, to the gene expression at transcription level. The data obtained support the following conclusions:

1. *In situ*,. $H^+$ release by active proteoid roots is related to citrate release with a stoichimetry of 1:1.3 (citrate : $H^+$). A comparable stoichimetry between citrate release and release of $K^+$, $Na^+$ and $Mg^{2+}$ was also determined.
2. Studies with different pharmacological drugs reveal a closely relationship between citrate release and $H^+$ release from proteoid roots. However, $H^+$ release is not the prerequisite for citrate release.
3. The activity of plasma membrane $H^+$ ATPase in active proteoid roots is significantly increased during the development of proteoid roots and reaches a maximum at mature stage (5-6 d after emergency), and declines rapidly at senescent stage (8 d after emergency). This matches the time course of $H^+$ and citrate exudation by intact proteoid roots.
4. Increased activity of plasma membrane $H^+$ ATPase is attributed to the enhanced enzyme concentration in the plasma membrane of active proteoid roots.
5. An up-regulation of the mRNA of plasma membrane $H^+$ ATPase is responsible for the higher enzyme concentration in active proteoid roots (proteoid roots at mature stage).
6. The data support the hypothesis that exudation of citric acid is attributed to transport processes across plasma membrane: $H^+$ export by $H^+$ ATPase, and citrate export by anion channel-like transporters.
7. According to gene expression, PEPC, ATP-dependent citrate lyase and plasma membrane $H^+$ ATPase are important enzymes, which are closely related to citric

acid release by proteoid roots, and therefore should be considered as target enzymes for a genetic approach to create P-efficient crops.

# 6. Zusammenfassung

Die Weißlupine hat bezüglich der Phosphat-Mobilisierung und der Nutzung spärlich vorhandener P-Vorkommen im Boden ein effizientes System entwickelt. Diese Effizienz wird auf die Ausbildung der Proteoidwurzeln zurückgeführt. Proteoidwurzeln stellen flaschenbürstenartige Wurzelcluster dar, deren Oberfläche durch eine große Clusterwurzel-Dichte und die Ausbildung feiner Wurzelhaare stark erhöht ist, was einen verstärkten lokalen Effekt auf das umgebende Wurzelmedium ermöglicht. Die Mobilisierung des Bodenphosphates wird durch die Abscheidung phenolischer Substanzen und organischer Säuren, insbesondere Citronen- und Äpfelsäure und die Erhöhung der Aktivität der Phosphatase ermöglicht (Dinkelaker et al. 1995; Gilbert et al. 2000; Neumann et al. 2000).

Bisher sind die Mechanismen der Abgabe organischer Säuren noch nicht vollständig aufgeklärt. Es wurde wiederholt beobachtet, dass die Ansäuerung und die Abgabe des Citrats zeitlich und räumlich zusammenhängt (Dinkelaker et al. 1989, Massonneau et al. 2001, Neumann et al. 1999; 2000; Keerthisinghe et al. 1998; Watt  Evans 1999; Johnson et al. 1996b). In diesem Fall dürften $H^+$-Abgabe und Citratabgabe durch Proteoidwurzeln miteinander eng zusammenhängen, wobei nicht bekannt ist, wie eng die beiden Prozesse koordiniert sind. Ein cytosolischer pH-Wert von 7,0 -7,5 bedingt jedoch eine Abgabe dieser Säuren in ihrer deprotonierten Form. Im Allgemeinen sind die im Plasmalemma befindlichen Anionenkanäle für den Export organischer Anionen wie Malat und Citrat aus pflanzlichen Zellen zuständig (Ryan et al. 2001; Ma et al. 2001).  Eine deutliche Hemmung der Citratabgabe durch einen Anionen-Kanalblocker, Anthracen, konnte an den Wurzeln der Weißlupine erzielt werden (Neumann et al. 1999). Daher muss das Vorhandensein eines zweiten Transportsystems für die Ausscheidung der Protonen postuliert werden.

Im Allgemeinen resultiert die Protonenabgabe aus Pflanzenzellen aus der Aktivität der Plasmalemma-$H^+$-ATPase (Sze et al. 1999). Der durch die Pumpaktivität der $H^+$-ATPase aufgebaute elektrische Gradient kann eine große Bedeutung für den

Citrattransport durch das Plasmalemma haben. Zum anderen produziert die durch P-Mangel ausgelöste Carbonsäuresynthese eine große Menge an Protonen, die durch die Plasmalemma $H^+$ ATPase aus dem Cytosol entfernt werden können. Insofern wäre es möglich, daß die Adaption der Plasmalemma-$H^+$-ATPase in Proteoidwurzel-membranen an Phosphatmangel eine Voraussetzung für die Synthese und Abgabe organischer Säuren darstellt.

Ziel der vorliegenden Arbeit war es, die quantitative Beziehung zwischen der $H^+$-Abgabe und der Abgabe von Citrat an aktiven und intakten Proteoidwurzeln *in situ* aufzuklären. Dabei sollte geklärt werden, wie die beiden Transportprozesse aufeinander abgestimmt sind. Ferner sollte herausgearbeitet werden, ob eine Beteiligung der Plasmalemma-$H^+$-ATPase von Proteoidwurzeln festzustellen ist, und ob es einen Adaptionsmechanismus des Enzyms an die P-Mangel-Situation gibt.

Um eine exaktere quantitative Untersuchung zur Exsudation an aktiven und intakten Proteoidwurzeln *in situ* durchführen zu können, wurden einzelne aktive Proteoidwurzeln in einer kleinen Küvette mit 3 ml Reaktionslösung unter Kontroll-bedingungen inkubiert. Zwischen den innerhalb von 2 Stunden gemessenen Ionen (Kationen und Anionen) besteht eine ausgeglichene Ladungsbilanz. Zwischen $H^+$ Abgabe und Citratabgabe an aktiven Proteoidwurzeln besteht eine gute Korrelation, deren Stöchiometrie 1:1,3 (Citrat : $H^+$) ist. Eine ebenfalls gute Beziehung besteht zwischen Citratabgabe und der Abgabe von $K^+$, $Na^+$ und $Mg^{2+}$.
Durch den Einsatz von Fusicoccin, einem Stimulator der $H^+$-ATPase, wurde eine Erhöhung der $H^+$-Abgabe und eine Stimulation der Citrat-Abgabe durch Proteoidwuzeln erreicht. Ein Anionenkanal-Blocker, Anthracen, führte zu einer Hemmung der Citrat-Abgabe, und ebenfalls zu einer Hemmung der $H^+$-Abgabe, obwohl Anthracen keinen direkten Effekt auf die Aktivität der $H^+$-Pumpe hatte. Außerdem zeigten die $H^+$-Abgabe und die Citratabgabe durch Proteoidwurzeln während ihrer Entwicklung einen engen Zusammenhang. Diese Ergebnisse *in situ* sprechen für eine enge Korrelation zwischen $H^+$- und Citratabgabe.

Mit Vanadat, einem Plasmalemma-$H^+$-ATPase-Inhibitor, konnte keine zwingende Korrelation zwischen $H^+$-Abgabe und Citratabgabe gezeigt werden. Dieses Ergebnis bekräftigt zum einen die Hypothese, dass $H^+$-Abgabe und Citratabgabe über unterschiedliche Transportproteine im Plasmalemma pflanzlicher Zellen erfolgt, zum anderen bedeutet dieses Ergebnis eine separate Regulierung der $H^+$-Abgabe und der Carboxylatabgabe von aktiven Proteoidwurzeln.

Mit Hilfe einer Agar-Technik, welche Bromkresol-Purpur als pH-Indikator enthielt, ließ sich *in vivo* eine Mitwirkung der $H^+$-ATPase an der unter P-Mangel stattfindenden Ansäuerung der Rhizosphäre durch aktiv exsudierende Proteoidwurzeln nachweisen. Dieses wurde unter *in vitro*-Bedingungen an Plasmalemma-Membranvesikeln überprüft. Im Vergleich zu Kontrollwurzeln (Apikalzonen der Seitenwurzeln von den mit oder ohne Phosphat ernährten Pflanzen, Proteoidwurzeln von den mit Phosphat ernährten Pflanzen) zeigte die Plasmalemma $H^+$-ATPase der aktiven Proteoidwurzeln folgende Unterschiede: (1) Zunahme der hydrolytischen ATPase-Aktivität, (2) Erhöhung von $V_{max}$ und $K_m$, (3) Steigerung der Enzymkonzentration der $H^+$-ATPase, (4) höhere $H^+$-Pumpaktivität, (5) höherer pH-Gradient über die Membran der Plasmalemmavesikel, (6) höhere passive Permeabilität des Plasmalemmas. Prinzipiell kann diese Erhöhung entweder auf eine transkriptionale Regulierung oder auf eine translationale Regulierung der $H^+$-ATPase zurückgehen. Nicht nur eine mengenmäßige Zunahme, sondern auch eine qualitative Veränderung der $H^+$-ATPase konnte im Plasmalemma aktiver Proteoidwurzeln festgestellt werden. Im Vergleich zu Kontrollwurzeln (Apikalzonen der Seitenwurzeln von den mit oder ohne Phosphat ernährten Pflanzen, Proteoidwurzeln von den mit Phosphat ernährten Pflanzen) zeigte die Plasmalemma $H^+$-ATPase der aktiven Proteoidwurzeln (1) eine deutliche Verschiebung des pH-Optimums in den sauren Bereich; (2) eine signifikante Erhöhung des $K_m$-Wertes und (3) eine signifikant niedrigere Empfindlichkeit (ein höherer $I_{50}$) gegenüber Vanadat. Diese Daten sprechen für eine erhöhte Exprimierung einer gewebespezifischen Isoform der Plasmalemma- $H^+$-ATPase für die Anpassung an den Phosphatmagel. Es wurde kürzlich auf molekularbiologischer Ebene festgestellt, dass eine höhe Expression einer spezifischen Isoform in aktiven Proteoidwurzeln der Weißlupine für die Anpassung an Phosphatmangel mitverantwortlich ist.

Es konnte eine höhere Plasmalemma-$H^+$-ATPase-Aktivität nur bei den vollständig entwickelten Proteoidwurzeln bei jenen Pflanzen, die ohne Phosphat angezogen worden waren, festgestellt werden, während apikale Seitenwurzeln der gleichen Pflanzen keine große Änderung im Vergleich zur Kontrolle zeigten. Die bei den Kontrollpflanzen (mit Phosphat) gebildeten Proteoidwurzeln wiesen keine Erhöhung der $H^+$- ATPase im Plasmalemma auf. Die Aktivität der Plasmalemma-$H^+$-ATPase in älteren Proteoidwurzeln war viel niedriger als die in den jungen oder vollständig entwickelten Proteoidwurzeln. Die *in vitro* erfasste Aktivität der Plasmalemma-$H^+$-ATPase stimmte mit der *in vivo* gemessenen $H^+$-Nettoabgabe durch verschiedene Wurzeln überein. Das bedeutet, dass eine Erhöhung der Plasmalemma-$H^+$-ATPase-Aktivität in aktiven Proteoidwurzeln nicht nur von der Wurzelstruktur, sondern auch von der Phosphaternährung und besonders vom physiologischen Stadium der Proteoidwurzeln abhängt.

Eine höhere Aktivität der Plasmalemma-$H^+$-ATPase kann mindestens auf zwei Ebenen für aktiv Citronensäure exsudierende Porteoidwurzeln der Weißlupine von Bedeutung sein: Citronensäuresynthese und Citronensäuretransport durch das Plasmalemma in den Apoplasten.

Zum einen produziert die durch Phosphatmangel ausgelöste Carbonsäuresynthese eine große Menge an Protonen, die durch die Plasmalemma-$H^+$-ATPase aus dem Cytosol entfernt werden können. Diese Hypothese wurde durch einen Versuch zur Expression der Plasmalemma-$H^+$-ATPase und einiger wichtiger Schlüsselenzyme für andere Stoffwechselvorgänge in Proteoidwurzeln überprüft. Im Verlauf einer 8-tägigen Entwicklung der Proteoidwurzeln wurde die Expression der PEP-Carboxylase und der Plasmalemma-$H^+$-ATPase zunächst hochreguliert. Die Expression der ATP-Citrat-Lyase wurde anschließend herunterreguliert. Es lässt sich vermuten, dass diese drei Enzyme während der Anpassung an P-Mangel die wichtigste Rolle gespielt haben. Die PEP-Carboxylase katalysiert die Bindung von Bicarbonat an PEP. Danach kommt es zur Umwandlung des entstandenen Oxalacetats durch die Malat-Dehydrogenase. Das gebildete Malat wird durch einen Tricarboxylat-Translokator im Gegentausch mit Citrat plus Proton in die Mitochondrien importiert. Gleichzeitig können die ins Cytosol eingebrachten Protonen eine Absenkung des cytosolischen pH-Wertes induzieren.

Wegen der großen Bedeutung des pH-Wertes für die enzymatischen Reaktionen im Stoffwechsel wird der cytosolische pH-Wert durch verschiedene Mechanismen sehr gut reguliert. Die wichtigsten Mechanismen zur Regulation des cytosolischen pH-Wertes sind (1) $H^+$ durch biochemische Reaktionen (biochemischer pH-Stat) zu eliminieren, (2) $H^+$ in die Vakuole durch die Tonoplasten-$H^+$-ATPase und Tonoplasten-PPase zu pumpen und dort zu speichern, (3) $H^+$ durch die Plasmalemma-$H^+$-ATPase aus dem Cyosol in den Apoplasten zu pumpen. Im Vergleich zum biochemischen pH-Stat oder zur Vakuole wird die Pumpaktivität der Plasmalemma-$H^+$-ATPase nicht zeitlich oder räumlich limitiert, und bietet einen langfristigen Mechanismus zur Aufrechterhaltung des cytosolischen pH-Wertes, solange die Zelle mit Energie versorgt werden kann. Dies gilt wahrscheinlich auch für Proteoidwurzeln der Weißlupine, die große Mengen an organischen Säuren synthetisieren und abgeben. Die erhöhte Expression der Plasmalemma-$H^+$-ATPase in aktiven Proteoidwurzeln kann als eine spezifische Adaption an die durch den Phosphatmangel induzierte Säureproduktion betrachtet werden. Zum Anderen kann der durch die Pumpaktivität der Plasmalemma-$H^+$-ATPase aufgebaute elektrische Gradient Bedeutung für den Citrattransport durch das Plasmalemma haben. In diesem Fall dürften $H^+$-Abgabe und Citratabgabe durch Proteoidwurzeln miteinander eng zusammenhängen.

Die vorliegenden Untersuchungen erlauben folgende Schlussfolgerungen:

1. *In situ* steht die $H^+$-Abgabe durch aktive Proteoidwurzeln in Beziehung zur Citratabgabe (Stöchiometrie 1:1,3 – Citrat : $H^+$). Eine vergleichbares Verhältnis besteht auch zwischen Citratabgabe und Abgabe von $K^+$, $Na^+$ und $Mg^{2+}$.

2. Studien mit verschiedenen Inbibitoren *in situ* zeigten eine engere Beziehung zwischen $H^+$-Abgabe und Citratabgabe durch Proteoidwurzeln. Jedoch ist die $H^+$-Abgabe nicht die Voraussetzung für die Citratabgabe.

3. Die Aktivität der Plasmalemma-$H^+$-ATPase in aktiven Proteoidwurzeln erhöht sich während der Proteoidwurzelnentwicklung signifikant, und erreicht ihr Maximum im reifen Stadium (5-6 Tag nach Einsatz). Im senescenten Stadium (8 Tage nach Einsatz)

erfolgt ein rascher Rückgang der Aktivität. Dieses stimmt zeitlich überein mit der $H^+$- und Citratexsudation durch intakte Proteoidwurzeln.

4. Eine höhere Aktivität der Plasmalemma-$H^+$-ATPase ist auf eine höhere Enzymkonzentration der $H^+$-ATPase im Plasmalemma zurückzuführen.

5. Eine höhere Enzymkonzentration der Plasmalemma $H^+$-ATPase ist auf eine hochregulierte Expression der mRNA der $H^+$-ATPase zurückzuführen

6. Die Ergebnisse bekräftigen die Hypothese, dass $H^+$-Abgabe und Citratabgabe über unterschiedliche Transportproteine im Plasmalemma pflanzlicher Zellen erfolgen: die die $H^+$-Abgabe über die Plasmalemma-$H^+$-ATPase und Citrat über Anionenkanäle.

7. In Bezug auf Gen-Expression sind PEPC, ATP-Citrat-Lyase und Plasmalemma $H^+$ ATPase wichtige Enzyme, die in enger Beziehung zur Citrate-Abgabe durch aktive Proteoidwurzeln stehen. Daher sollten sie in der Gentechnik/Pflanzenzüchtung als Ansatzpunkt herangezogen werden, um P-effiziente Pflanzen zu züchten.

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