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A physiological and molecular approach to study organic acid exudation and development of cluster roots in *Lupinus albus* L

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By Nicolas Bernard Langlade

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Summary

Cluster roots (or proteoid roots) are bottlebrush-like structures, developed by hundreds of plant species (mainly of *Proteaceae* family, but also the legume *Lupinus albus* L.) induced by nutrient stresses, especially P. These structures are characterized by a strong capacity to exude citrate (up to 7 µmol $.h^{-1}$ $.g^{-1}$) and malate in the rhizosphere and thus to increase the availability of P, Fe and Mn. Furthermore, a wide range of P deficiency responses (acidification, acidic phosphatase induction, root surface increase) which allow the mobilization of inorganic phosphate (P_i) bound to Ca, Al/Fe rocks, humic complexes and other organic compounds, are found in this structures.

Different stages of cluster root development under P deficiency were defined in white lupin and precisely described in order to investigate their development and physiology. At the beginning of this work, a cDNA-AFLP (Amplified Fragment Length Polymorphism) approach allowed us to isolate ESTs (Expressed Sequence Tags) corresponding to genes putatively differentially expressed in different developmental stages. Among the 60 clones identified, sucrose synthase (SuSy) and other glycolysis-related enzymes were shown to be induced at the mRNA and protein levels in mature cluster roots. This creates a high sink strength, which explains partially the origin of C skeletons exuded via citrate.

Metabolic alterations underlying organic acids exudation, were investigated more extensively. Part of genes differentially expressed during the AFLP screen, the ATP citrate lyase (ACL) has been cloned for the first time in plants. ACL is constituted by to different subunits, both necessary for activity in yeast expression system. In juvenile cluster roots, ACL is strongly enhanced and is likely to produce the acetyl-CoA required for lipid synthesis. ACL also produces malate from citrate and consequently determines the nature of the organic acid preferentially exuded in cluster roots, which constitute a novel function for this enzyme.

Furthermore, the accurate discrimination of developmental steps was crucial for the understanding of the energy, cytosolic pH and phosphate status in the different types of rootlets within a cluster and the regulatory elements leading to the exudative burst in mature stage. During their expansion rootlets need large amounts of Pi (notably for nucleotides). The important pools present at the juvenile stage are reallocated following to growth stop. Consequently, P deficiency responses are observed, such as respiration inhibition, decrease of ATP and Pi contents and increase of organic acid contents. Combined with the overflow of carboxylate production, we assume this produces a strong acidification of the cytosol in mature stage, which induces rhizosphere acidification and activates citrate exudation.

A detailed knowledge of the complete root system allowed us to perform a successful RDA (Representational Difference Analysis), which compares tissues in more stringent conditions compared to AFLP. Using this technique, we identified a novel gene (*LaTCP1*) likely to be involved in the specific development of cluster roots and its evolutional apparition. Expression analysis by RT-PCR and *in situ* hybridization showed that the transcription of *LaTCP1* is restricted to meristems of emerging cluster rootlets. In addition messenger and auxin contents analysis in different tissues allowed us to link La TCP1 to auxin, which has been shown to induce cluster roots formation. This second facet of the present work constitutes a first result in the molecular comprehension of cluster root structure development and evolutionary apparition as an adaptation to very poor soils.

Résumé

Les racines protéoïdes sont des structures en écouvillon, développées par des centaines d'espèces végétales (principalement de la famille des Protéacées, mais aussi de la légumineuse *Lupinus albus*, L.). Induites par des stress nutritifs, spécialement le phosphore, ces structures sont caractérisées par une forte capacité d'exsuder le citrate (jusqu'à 7 µmol .h-1 .g-1) et le malate dans la rhizosphère et donc d'augmenter la disponibilité du P, Fe et Mn. De plus, une grande variété de réponses à la carence en P (acidification, induction d'acide phosphatase, augmentation de la surface racinaire) qui mobilisent le phosphate inorganique (P_i) lié aux minéraux Ca, Al/Fe, aux complexes humiques et aux composés organiques se trouvent dans ces structures.

Différents stades de développement des racines protéoïdes développées en déficience en P, ont été définies chez le lupin blanc et précisément décrits afin d'étudier leur développement et leur physiologie. Initiant ce projet, une approche par cDNA-AFLP (Amplified Fragments Length Polymorphism) a permis d'isoler des EST (Expressed Sequence Tags) correspondant à des gènes possiblement différemment exprimés dans les différents stades de développement. Parmi les 60 clones identifiés, la sucrose synthase (SuSy) et d'autres enzymes reliées à la glycolyse sont induites aux niveaux du transcrit et de l'activité dans les racines protéoïdes matures. Cela crée un fort puits, qui explique partiellement l'origine des squelettes carbonés exsudés sous forme de citrate.

Les altérations métaboliques sous-jacentes de l'exsudation d'acides organiques, ont été étudiées plus précisément. Faisant partie des gènes différemment exprimés lors du criblage de l'AFLP, l'ATP citrate lyase (ACL) a été clonée pour la première fois chez les plantes. L'ACL est constituée de deux sous-unités, toutes deux nécessaires pour l'activité dans la levure comme système d'expression. Dans les racines protéoïdes juvéniles, l'ACL est fortement induite certainement pour produire l'Acetyl-CoA nécessaire à la synthèse lipidique. L'ACL transforme parallèlement le citrate en malate et en conséquence détermine la nature de l'acide organique préférentiellement exsudé dans les racines protéoïdes, ce qui constitue une nouvelle fonction pour cette enzyme.

De plus, la discrimination précise des étapes du développement a été cruciale pour la compréhension des états énergétique, du pH cytosolique et du phosphate dans les différents types de racines protéoïdes, et des éléments régulant la bouffée exsudative au stade mature. Durant leur croissance les racines requièrent de grandes quantités de P_i (notamment pour les nucléotides). Les pools importants présents au stade juvénile sont réalloués après l'arrêt de la croissance. Par la suite, des réponses au manque de P, telles l'inhibition de la respiration, la diminution des contenus en ATP et P_i et l'augmentation des concentrations d'acides organiquessont donc observées. Combinées au surplus de production de carboxylates, nous assumons que cela entraine une forte acidification du cytosol au stade mature, ce qui induit l'acidification de la rhizosphère et active l'exsudation de citrate, principales caractéristiques des racines protéoïdes.

Une connaissance détaillée du système racinaire entier nous a permis de réaliser avec succès une RDA (Representational Difference Analysis) pour comparer les tissus de façon plus stringente

que l’AFLP. En utilisant cette technique, nous avons identifié un nouveau gène (*LaTCP1*) certainement impliqué dans le développement spécifique des racines protéoïdes et leur apparition au cours de l’évolution. L’analyse de son expression par RT-PCR et l’hybridation in situ a montré que la présence de LaTCP1 est restreinte aux méristèmes des racines protéoïdes émergentes. De plus, l’analyse de la séquence du messager et des contenus tissulaires en auxine a permis de relier LaTCP1 à l’auxine, qui a été montrée comme induisant la formation des racines protéoïdes. Cette seconde facette de ce travail constitue un premier résultat dans la compréhension moléculaire du développement structurel des racines protéoïdes et de leur apparition au cours de l’évolution comme adaptation aux sols très pauvres.

INTRODUCTION

General considerations

A cluster root is composed of tightly grouped rootlets along a parent root. The rootlets are size-determinate and show a bottlebrush-like morphology. Some authors consider a cluster root (also called proteoid roots because of its wide occurrence in the Proteaceae family) as a single cluster of rootlets (Skene 2001, Purnell 1960, Lamont 1982, Dinkelaker *et al.* 1995, Marschner 1995). However, another definition is also employed by Watt & Evans (1999) and in this work. A cluster root is defined as the whole of groupings along a parent root, since a grouping is developmentally and functionally related to others and thus the whole constitute the physiological unit.

Indeed a limited number of plants develop cluster roots, mainly *Proteaceae* (27 genera, more than 200 species) and some members of *Betulaceae, Casuarinaceae, Eleagnaceae, Leguminosae, Moraceae* and *Myricaceae. Proteaceae* are a significant component of the biodiversity and biomass of the flora of South Africa and sand plains of southwest Australia (Fig 1).

Fig. 1: Forrest in south-western Australia (around Perth). The great diversity of flora is illustrated by cluster-rooted plants: *Hakea* (foreground) and *Banksia* (background) family genus belonging to *Proteaceae* family

These soils (leached sands, sandstones and laterites; see fig 2) are characterized by extremely low contents of available nutrients, in particular phosphate and micronutrients. Cluster roots have been functionally related to a very efficient mobilization of phosphate. They constitute indeed an adaptation, which allows cluster-rooted plants to desorb insoluble P_i from Fe-P, Al-P and Fe/Al-P by exudating protons, organic acids (mainly citrate and malate), phenolics, and in addition organic phosphate is efficiently converted to inorganic phosphate by the action of phosphohydrolases released in high amounts at the level of cluster roots.

Fig 2: Soil profile in southwestern Australia (around Perth). The upper organic layer (dark) is reduced to only a few centimeters. Subjacent sands appear leached (indicated by the colour gradient).

Research has been increasing through the last ten years into the physiology and the biochemistry of clusters roots and more particularly since 1998. It is illustrated in the recent review of Neumann and Martinoia (in press), which refers to 19 articles before 1998 and the beginning of this work, 6 in 1998, 6 in 1999, 15 in 2000 and 13 in 2001 in the domain. Despite the apparent niche of the subject, cluster roots are becoming more and more known due to a chapter in Annals of Botany (in 2000), a special issue of Plant and Soil (in 2002), cluster roots conferences (St-Louis 1999 and Perth 2001), a chapter in Plant Physiology and Molecular Biology (published by the ASPB in 2001) or article in La Recherche (june 2001).

Two main aspects of cluster roots can be distinguished (Fig 3). First, their structure and morphological development; cluster roots are defined as morphological structures and follow a precise development program, this structure is an important prerequisite for their role in plant nutrition. Secondly, their physiological adaptation, which leads to a transient and specific stage of maturity, when an exudative burst occurs and all P mobilization and acquisition mechanisms are concomitantly induced, leading to mobilization of previously unavailable P sources.

Morphology and morphogenesis of cluster roots

Cluster roots are bottlebrush-like clusters of rootlets with limited growth, arising from the pericycle opposite protoxylem poles. There are some principal differences between plant species in root cluster morphology, such as length of axis, length and density of rootlets and root hairs, and whether they are simple or compound. All these attributes depend also on plant age and environmental factors. A classification distinguishing between simple (unbranched) and compound (branched) proteoid roots (Purnell, 1960) has been adopted for cluster roots in general (Dinkelaker *et al*., 1995). A compound cluster root is thus defined as “a group of three or more clusters of rootlets borne on three or more parent roots” (Purnell, 1960). This can produce a real net, since up to 24 simple cluster roots are formed per cluster in *Leucadrendron laureolum* (Lamont *et al.* 1984). Compound proteoid roots are formed by Banksia, Dryandra and most of South African Proteaceae, whereas simple cluster roots are formed by most of Australian *Proteaceae* and non-*Proteaceae* species such as *Lupinus*, our model in this work. The proportion of clusters of the total root system generally increases with age and may reach 40% to 50% of the fresh root mass and even 80% in *Leucandendron argenteum* (Lamont 1972b and 1983a). The axis length may range from 10 to 200 mm of one grouping and the rootlet length from 5 to 50 mm.

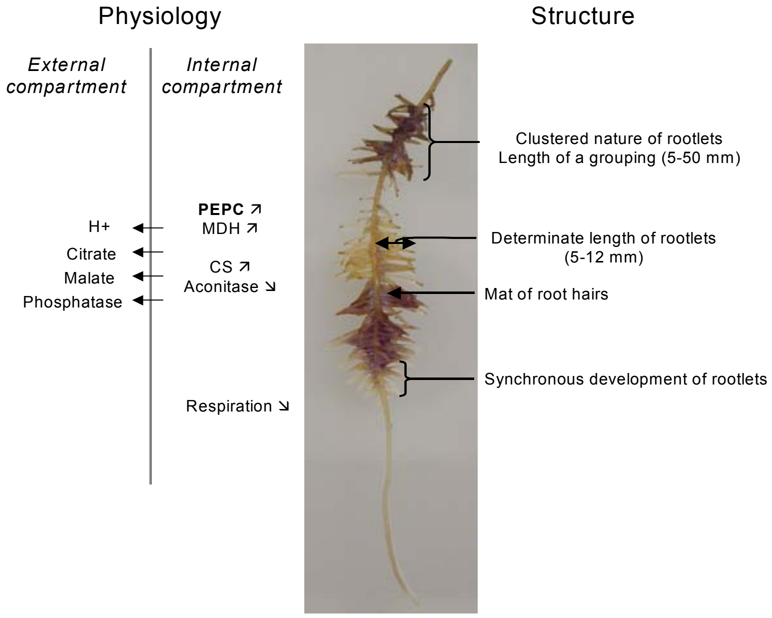

Fig. 3: Schematic presentation of the two main aspects of cluster roots and their respective properties

With a few exceptions in waterlogged soils, high root hair densities often exceeding those of normal roots usually characterize root clusters. The length of root hairs differs from one species to the other but particularly long root hairs are present in *Lupinus albus*. These two factors markedly increase root surface area of the parent root from 16 to 25 times depending of the species (Lamont *et al.* 1984)

Formation of cluster roots appears to be mainly induced by shortage of P and, in some species by Fe deficiency. Experiments with foliar applications and split root systems but also with phosphonic acid (an analogue of phosphate which cannot be metabolised) demonstrated a key regulatory function of the internal P_i status. On the other hand, cluster roots formation seems to be stimulated in nutrient richer patches of soil (Purnell, 1960; Skene, 1998), in the upper soil layers rich in organic matter and in deeper part of the soil where leached nutrients have precipitated (Lamont, 1973). This suggests the involvement of additional external factors, triggering the proliferation of lateral roots along which cluster roots develop. Additionally certain constituents of dissolved organic matter, the pH of the soil (Kerley 2000) or the growth medium, as well as microbial factors may exert some stimulatory effects, but these are not obligatory for the induction of cluster roots.

Some studies have investigated the role of hormones, mainly auxins and cytokinins, in cluster root formation (chapter 1 and 5, Gilbert *et al*., 2000). They show that auxin promotes the formation of cluster roots when applied in the culture medium or on foliage. Contrarily, auxin transport inhibitors and cytokinins inhibit their formation. In accordance, characterization of cluster root expressed sequence tags revealed a down-regulation of a homologue of IAA (indolacetic acid) glycosyl transferase, involved in IAA inactivation, and an up-regulation of a zeatin glycosyl transferase, which inactivates zeatin. Moreover, contradictory results about ethylene involvement in cluster roots morphogenesis let the question open whether this hormone is implicated. Uhde-Stone *et al.* (2000) have recently observed enhanced expression of a homologue of ACC oxidase in cluster roots. On the other hand, ethylene inhibitors did not affect cluster root initiation; its role could thus be the development of the mat of root hairs (Borch *et al.*, 1999). However, the induction and termination of clustered rootlets is thought to be the consequence of a complex and highly coordinated interaction of different hormones, involving a central role of IAA.

The special morphology of cluster roots, characterized by a very large number of closely spaced lateral root tips (50 to 1000 rootlets per cm of axial root), densely covered with root hairs may contribute to the high level accumulation of P mobilizing exudates in the rhizosphere by a cumulative effect due to the increased secreting root surface.

The specific cluster root structure allows to embed tightly a restricted rhizosphere soil compartment, and to dramatically change its chemical properties (pH, redox potential, concentration of organic chelators) thanks to an exudative burst which can last up to three days and the high absorption capacities even longer. This is not the case in apical zones of 'normal' roots. Assuming root growth rates up to 1.5-2.5 cm per day, in most cases, this time period seem to be too short to allow a significant accumulation of P-mobilizing exudates in the rhizosphere and the following nutrient acquisition.

Physiology of cluster roots developed by* Lupinus albus *under P deficiency

Lupinus albus cluster roots developed under P deficiency have been extensively studied in order to understand their particular physiology. They constitute a very powerful model to understand P mobilizing strategies such as acidification of the rhizosphere, organic acids exudation, including the modification of the metabolism allowing their synthesis and the mechanism of their release, organic P mobilization, and P_i acquisition.

Cluster roots are specialized organs allowing P acquisition from soils with sparingly available phosphate. Their physiological development is in consequence adapted to their function. To better understand the physiological adaptations, the comprehension of P mobilization mechanisms in the soil is essential.

P availability is one of the major constraints to plant growth. Plants can suffer from P deficiency even though the total content of the soil appears more than adequate. The reason for this apparent discrepancy is that the concentration of soluble P is often very low (<5µM) compared to the total amount of P bound to soil minerals and charged sites or fixed into organic forms that are inaccessible to the plants (Ryan and Delhaize, 2001). Therefore plants have evolved different mechanisms to replenish the only pool available to plants, soluble P_i. Most of the inorganic phosphate is indeed chelated to soil particles mainly as Ca phosphate or P_i bound to Fe/Al-humic acid complexes. While acidification, which is often observed in roots, is sufficient to release P_i from Ca-P, this is not the case for Al/Fe-P complexes. Cluster roots are able to solubilize these fractions with a concomitant release of Fe, Mn, Zn, Al, which could explain also the induction of cluster roots under Fe deficiency and the reinforcement of induction by Zn under other deficiencies (Dinkelaker *et al.,* 1995). This property is due to a coordinated acidification of the rhizosphere and a release of very large amounts of organic acids, mainly citrate and malate. Protons solubilize P from Ca-P fraction and via an anion exchange on sorption sites, citrate and malate take place of P_i in the soil matrix. They can desorbe P_i from oxides or humic Fe/Al complexes, chelate the bridging metal cations from organic matter and suppress readsorption and precipitation of P_i (Gerke *et al.*, 1994 and Braum, 1995). The last organic pool, which can be the most important (30 to 80% of the total P, of which is approximately one half in the form of phytin or its derivates), is mobilized by activities of acid phosphatases (Gilbert *et al.*, 1999; Neumann *et al.*, 1999 and Miller *et al.*, 2001) and phytases (Li *et al.*, 1997 and Richardson *et al.*, 2001) secreted in the rhizosphere.

Cluster root development and metabolism are highly coordinated, to allow a maximal P_i solubilisation effect of rhizosphere acidification and organic acids exudation. First studies linking cluster roots physiology and metabolism, which precede this work, considered clusters as a whole and limited their investigations to young plants (between 9 and 14 day-old plants). With this basic model, Vance and his group showed alterations of metabolism linked to citrate synthesis. Citrate Synthase (CS), Malate Dehydrogenase (MDH) and Phospho*enol*pyruvate Carboxylase (PEPC) specific activities were increased in cluster roots by 25% (CS and MDH) and 60% (PEPC) (Johnson *et al.*, 1994). In accordance to this, a 4-fold higher non-photosynthetic ^{14}C fixation was observed in P-deficient roots, and contributed to 25 and 34% of citrate and malate exuded by cluster roots, respectively (Johnson *et al.*, 1996a). Johnson and co-workers also demonstrated PEPC is induced at the mRNA and protein level and correlated this upregulation with a concomitant increase of citrate, malate and succinate exudation (Johnson *et al.*, 1996b). These data suggest PEPC and metabolism alterations are parts of the development of cluster roots developed under P deficiency and anapleurotic carbon fixation is an important origin of the C exudated as organic acids.

The following major advance in the comprehension of cluster root physiology occurred concomitantly with the beginning of this work. At this time, Neumann *et al.* (1999) and Watt & Evans (1999 a,b) defined a mature stage, when cluster roots exhibit most of their characteristics (organic acid exudation, acid phosphatase secretion, rhizosphere acidification, PEPC activity, P_i absorption). The mature stage is restricted in time (about one day) and in space (between 5 to 30 mm along the axis). This point emphasizes the previous results obtained with all clusters of the root system and soundly shows P-mobilization properties occur as a burst to rapidly acquire phosphorus.

Neumann and co-workers (1999) also showed anion channels inhibitors such as anthracene-9-carboxylic acid and ethacrynic acid partially inhibit citrate efflux in *Lupinus albus* cluster roots. The author confirmed this result limiting it to mature cluster roots. Citrate exudation appears thus to be partially supported by anions channels. However, no plant organic acids channels have been cloned yet and the release mechanisms are largely unknown although they constitute a very promising area for improvement of plant P-deficiency tolerance.

Once root exudates have solubilized phosphorus in the soil, P_i must be actively transported into the root cells. Despite energy limitation, mature and even senescent cluster roots of *Lupinus albus* are able to take up significant amounts of P_i with higher affinity than normal roots and to counteract a huge chemical gradient (chapter 1 and Neumann *et al.*, 1999).

What is common between all cluster roots?

White lupine cluster roots, developed under P deficiency, appear to be extremely specialized root structures, directed towards an active stage restricted in time and space. However, all these physiological characteristics have not been tested on other plants (except organic acids exudation) and cannot be simply transposed to other species developing cluster roots. In fact, evolutionary questions are raised considering the apparition of this adaptation, which occurred in a wide range of distantly related families. Indeed, neither every genus in the pre-cited families, nor every species in these genuses, produce cluster roots, but all cluster-rooted plants belong to a very limited number of ecotypes. In the case of *Lupinus* genus, phylogeny has been related to cluster root occurrence, pointing towards a single origin leading to cluster root formation ability in only some species of the 12 Old World ones. This suggest that the ability to develop cluster roots is not directly related to metabolic background, which is too complex to arise with a single origin, but that it constitutes in itself an important adaptation, conserved during evolution. Accordingly, K. R. Skene (2000) reminded that cluster roots developed among the whole plant kingdom should not be identical in respect to their two key aspects, physiology and morphology.

Presentation of this work

Comprehension of cluster root physiology made important steps, during the last decade. In regard to these findings and based on an accurate description of white lupin cluster roots, this works aims to precise the properties of each developmental stage, concentrating on the exudative burst occurring at the mature stage and the metabolic changes during root development.

The second goal of this work was to identify genes responsible for cluster root formation. As for the metabolic approach, we used lupin and compared root apices with emerging cluster rootlets. More particularly we focused on the initiation of cluster root formation, making the assumption there is a molecular difference between normal roots occurring in the initiation events of cluster root formation.

REFERENCES

Borch, K, Bouma, TJ, Lynch, JP and Brown, KM 1999 Ethylene: a regulator of root architectural responses to soil phosphorus availability. *Plant, Cell and Environment* **22**: 425-431.

Braum SM and Helmke PA 1995 White lupin utilizes soil phosphorus that is unavailable to soybean. *Plant Soil* **176**: 95-100.

Dinkelaker B, Hengeler C, Marschner H 1995 Distribution and function of proteoid roots and other root clusters. *Botanica Acta* **108**: 183-200.

Gerke J, Röhmer W, Jungk A 1994 The excretion of citric and malic acid by proteoid roots of Lupinus albus L.: effects on soil solution concentrations of phosphate, iron, and aluminium in the proteoid rhizosphere samples of an oxisol and a luvisol. *Zeitschrift für Pflanzenernährung und Bodenkunde* **157**: 289-294.

Gilbert GA, Knight JD, Vance CP, Allan DL 1999 Acid phosphatases in phosphorus-deficient white lupin roots. *Plant, Cell and Environment* **22**: 801-810.

Gilbert GA, Knight JA, Vance CP and Allan DL 2000 Proteoid root development of phosphorus defiecient lupin is mimicked by auxin and phosphonate. *Plant and Soil* **85**: 921-928.

Johnson JF, Allan DL, Vance CP 1994 Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus* L. *Plant Physiology* **104**: 657-665.

Johnson JF, Vance CP, Allan DL 1996a. Phosphorus deficiency in Lupinus albus: altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant Physiology* **112**: 31-41.

Johnson JF, Allan DL, Vance CP, Weiblen G 1996b Root carbon dioxide fixation by phosphorus deficient Lupinus albus. Contri- bution to organic acid exudation by proteoid roots. *Plant Physiology* **112**: 19-30.

Kerley SJ 2000 Changes in root morphology of white lupin (Lupinus albus L.) and its adaptation to soils with heterogenous alkaline/acid profiles. *Plant and Soil* **218:** 197-205

Lamont B 1972 The morphology and anatomy of proteoid roots in the genus *Hakea*. *Australian Journal Botany* **20:** 155-174

Lamont B 1973 Factors of affecting the distribution of proteoid roots within the root system of two Hakea species. *Aust. J. Bot.* **21**: 165-187.

Lamont B 1982 Mechanisms for enhancing nutrient uptake in plants, with particular reference to Mediterranean South Africa and Western Australia. *The Botanical Review* **48**: 597-689.

Lamont B 1983 Proteoid roots in the South Africa Proteaceae. *South African Journal of Botany* **49**:103-123.

Lamont BB, Brown G and Mitchell DT 1984 Structure, environmental effects on their formation, and function of proteoid roots in *Leucadendron laureolum* (Proteaceae). *New Phytologist* **97**: 381-390.

Li M, Shinano T, Tadano T 1997 Distribution of exudates of lupin roots in the rhizosphere under phosphorus deficient conditions. *Soil Science and Plant Nutrition* **43**: 237-245.

Marschner H 1995. Mineral nutrition of higher plants, 2nd edn. London: Academic Press.

Miller SS, Liu J, Allan DL, Menzhuber CJ, Fedorova M, and Vance CP 2001 Molecular control of acid phosphatase secretion into the rhizosphere of proteoid roots from phosphorus-stressed white lupin. *Plant Physiology* **127**: 594-606.

Neumann G, Massonneau A, Martinoia E and Römheld V 1999 Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* **208**: 373-382.

Neumann, G. and Martinoia, E 2002 Proteoid roots – an underground adaptation for survival in extreme environments. *Trends in Plant Sciences* (in press)

Purnell HM 1960 Studies of the family Proteaceae. I. Anatomy and morphology of the roots of some Victorian species. *Australian Journal of Botany* **8**: 38-50.

Richardson AE, Hadobas PA and Hayes JE 2001 Extracellular secretion of Aspergillus phytase from Arabidopsis roots enables plants to obtain phosphorus from phytate. *Plant J* **25**: 641-649.

Ryan PR and Delhaize E 2001 Function and mechanism of organic anion exudation from plant roots. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **52*:*** 527-560.

Skene KR 1998 Cluster roots: some ecological considerations. *Journal of Ecology* **86**: 1062-1066.

Skene KR 2000 Pattern formation in cluster roots: some developmental and evolutionary considerations. *Annals of Botany* **85**: 901-908.

Skene KR 2001 Cluster roots: model experimental tools for key biological problems. *Journal of Experimental Botany* **52**: 479-485.

Skene KR and James WM 2000 A comparison of the effects of auxin on cluster root initiation and development in Grevillea robusta Cunn. Ex R. Br. (Proteaceae) and in the genus Lupinus (Leguminosae) *Plant and Soil* **219**: 221-229

Uhde-Stone *et al.* 2000 Isolation and characterization of white lupin proteoid root expressed sequence tags (ESTs) associated with plant hormones. In plant Nutrition. Food security and sustainability of agro-ecosystems throught basic and applied research (Horst WJ et al. eds), pp 578-579, Kluwer.

Watt M and Evans J 1999a Linking development and determinacy with organic acid e.ux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO2 concentration. *Plant Physiology* **120**: 705-716.

Watt M and Evans J 1999b Proteoid roots. Physiology and development. *Plant Physiology* **121**: 317-323.

CHAPTER I

PHYSIOLOGICAL ASPECTS OF CLUSTER ROOT FUNCTION AND DEVELOPMENT IN PHOSPHORUS-DEFICIENT WHITE LUPIN (*LUPINUS ALBUS* L.)

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Dédié à la mémoire du Prof. Dr Dres h. c. Horst Marschner qui a initié et inspiré nos activités de recherche. Nous voulons aussi remercier Prof. Dr F. Bangerth pour l’analyse des cytokinines.

Summary

Cluster root formation in white lupin (*Lupinus albus* L.) is induced mainly by phosphorus (P) starvation, and seems to be regulated by the endogenous P status of the plant. Increased formation of cluster roots, when indole acetic acid is supplied to the growth medium of P sufficient plants, and inhibitory effects of kinetin application suggest the involvement of endogenous phytohormones (auxins and cytokinins), which may act in an antagonistic manner in the P-starvation response. Phosphorus deficiency-induced adaptations of white lupin, involved in P acquisition and mobilization of sparingly available P sources, are predominantly confined to the cluster roots, and moreover to distinct stages during their development. Increased accumulation and exudation of citrate and a concomitant release of protons were found to be mainly restricted to mature root clusters after prolonged culture (3±4 weeks) under P-deficient conditions. Inhibition of citrate exudation by exogenous application of anion channel antagonists such as ethacrynic- and anthracene-9-carboxylic acids may indicate involvement of an anion channel. Phosphorus deficiency-induced accumulation and subsequent exudation of citric acid seems to be a consequence of both enhanced biosynthesis and reduced turnover of citric acid in the cluster root tissue, indicated by enhanced expression of sucrose synthase, fructokinase, phosphoglucomutase, phosphoenol-pyruvate carboxylase, but reduced activity of aconitase and slower root respiration. The release of acid phosphatase and of phenolic compounds (isoflavonoids) as well as the induction of a putative high-affinity P uptake system were more highly expressed in juvenile, mature and even senescent cluster regions than in apical zones of non-proteoid roots. An Amplified Fragments Length Polymorphism (AFLP) cDNA library for cluster root-specific gene expression was constructed to assist in the identification of further genes involved in cluster root development.

Résumé

La formation de racines protéoïdes chez le lupin blanc (*Lupinus albus*, L.) est principalement induite par la carence en phosphore, et semble être régulée par le statut endogène de la plante en Phosphore (P). La formation accrue de racines protéoïdes, quand l'acide indole acétique est appliqué dans le milieu de croissance de plantes non-carencées et les effets inhibiteurs de l'application de kinétine suggèrent l'implication de phytohormones endogènes (auxines et cytokinines), qui peuvent agir de façon antagoniste dans la réponse à la carence en phosphate. Les adaptations induites par la carence en phosphate du lupin blanc, incluant l'acquisition de P et la mobilisation de sources très faiblement accessibles, sont principalement confinées aux racines protéoïdes et plus particulièrement à des étapes précises de leur développement. L'accumulation accrue, l'exsudation de citrate et la relâche concomitante de protons se trouvent principalement restreintes aux racines protéoïdes matures après une culture prolongée (3 ou 4 semaines) en absence de P. L'inhibition de l'exsudation de citrate par application exogène d'antagonistes de canaux à anions comme les acides éthacrynique et anthracène-9-carboxylique indiquerait l'implication d'un canal à anion. L'accumulation induite par la carence en phosphore et la subséquente exsudation d'acide citrique semblent être la conséquence à la fois d'une augmentation de la biosynthèse et d'un renouvellement réduit de l'acide citrique dans les tissus des racines protéoïdes, indiqués par une augmentation de l'expression de la sucrose synthase, la fructokinase, la phosphoglucomutase, la phosphoénol-pyruvate carboylase, une activité réduite de l'aconitase et une respiration racinaire moindre. La relâche de phosphatase acide et de composés phénoliques (isoflavonoïdes) ainsi que l'induction d'un possible système de transport à haute affinité sont plus exprimées dans les régions protéoïdes juvénile, mature et même sénescente que dans les régions apicales des racines non-protéoïdes. Une banque Amplified Fragment Lenght Polymorphism (AFLP) cDNA de gènes spécifiques des racines protéoïdes a été constituée pour aider l'identification prochaine de gènes impliqués dans le développement des racines protéoïdes.

Introduction

White lupin (*Lupinus albus* L.) is highly efficient with respect to P uptake and the utilization of sparingly available sources of soil phosphorus. This efficiency cannot be attributed to a high rate of root growth or to mycorrhizal associations. However, white lupin has developed effective mechanisms for the chemical mobilization of sparingly available P sources in the rhizosphere, involving the formation of cluster roots. During the last decade, our work on white lupin and other cluster-rooted species in the genus *Hakea* has focused on the chemical effects of cluster roots on mineral nutrient acquisition in the rhizosphere, and on the related changes in root physiology which occur during cluster root formation. The present work summarizes the results of our research activities on adaptations to P deficiency in white lupin.

Mobilization of mineral nutrients in the rhizosphere

When exposed to P starvation, white lupin and other cluster-rooted plant species excrete large amounts of citric and malic acids from closely-spaced lateral rootlets arranged in clusters along first order lateral roots, so-called proteoid roots. These root clusters exhibit limited growth, reaching an average length of 5 mm, and are densely covered with root hairs (Dinkelaker et al., 1989, 1995, 1997). Accumulation of 50±90 μmol of citric acid per g soil has been reported in the rhizosphere soil of cluster roots in white lupin (Dinkelaker *et al*., 1989; Gerke *et al*., 1994; Li *et al*., 1997). This concentration level was sufficient to mediate a significant desorption of phosphorus from sparingly soluble Ca-, Al- and Fe-P and from P-adsorbing Fe/Al humic acid complexes, mainly by mechanisms of ligand exchange, and dissolution of P sorption sites in the soil matrix (Gardner *et al*., 1983; Gerke *et al*., 1994). Citrate excretion from cluster roots of white lupin was also associated with a marked acidification of the rhizosphere to pH 4-5 (Fig. 1), which was detectable even in well-buffered calcareous soil with pH 7.5, and may dissolve acid-soluble Ca-P (Table 1; Dinkelaker *et al*., 1989). High stability constants for the formation of metal complexes with citrate (Jones, 1998) account for the intense mobilization of various metal ions reported in the rhizosphere of cluster roots (Table 1; Gardner *et al*., 1983; Dinkelaker *et al*., 1989, 1997; Gerke *et al*., 1994). A marked reduction of iron and manganese is detectable at the rhizoplane of cluster rootlets by application of agar gels or filter papers containing metal-specific redox indicators (Marschner *et al*., 1987; Dinkelaker *et al*., 1993a,b, 1995). This may be attributed to an increased activity of membrane-bound reductases, stimulated by low pH levels in the rhizosphere of cluster roots, but also to the excretion of large amounts of reducing compounds such as malate, citrate and phenolics (Fig. 2A), identified mainly as isoflavonoids (Neumann, unpubl. res.; Wojtaszek *et al.*, 1993). Iron solubilization, mediated by reduction of FeIII bound in Fe-P, might thereby also increase the availability of phosphorus. Antibiotic properties of isoflavonoids in root exudates (Rao, 1990) may not only counteract infection by root pathogens but also prevent the microbial degradation of organic exudate compounds involved in mobilization of mineral nutrients (Dinkelaker *et al.*, 1995).

Fig. 1: Root system of white lupin grown in hydroponic culture for 28 d without P supply. Spatial variation of pH along the root surface of cluster roots in different developmental stages detected by application of agar sheets containing bromocresol purple as a pH indicator: pH 6.5-7.0 = purple, pH 4-5 = yellow.

Table 1: Soil pH and concentrations of citrate, phosphate and micronutrients in bulk and proteoid rhizosphere soil of white lupin grown in a P-deficient calcareous soil

	Bulk soil	Rhizosphere soil (cluster roots)
pH (H_2O)	7.5	4.8
Citrate (μ mol g^{-1} soil)	1.1±0.2	47.7±7.2
Phosphorus (μ mol g^{-1} soil)		
H_2O-extractable	178±28	61±7
CAL	904±80	581±76
Olsen	581±93	484±68
DTPA-extractable (μ mol kg^{-1} soil)		
Fe	34±6	251±43
Mn	44±8	222±23
Zn	2.8±0.4	16.8±2.4

Means ± s.d. of at least three independent replicates. Source: Dinkelaker *et al.*., 1989.

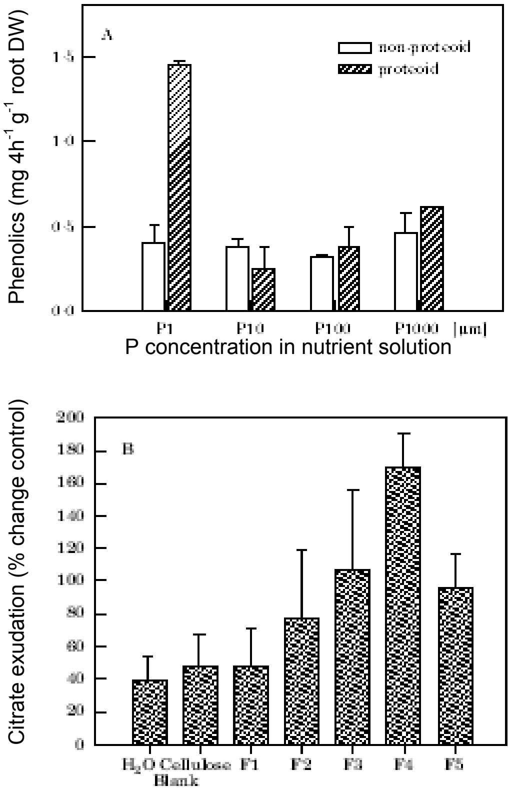


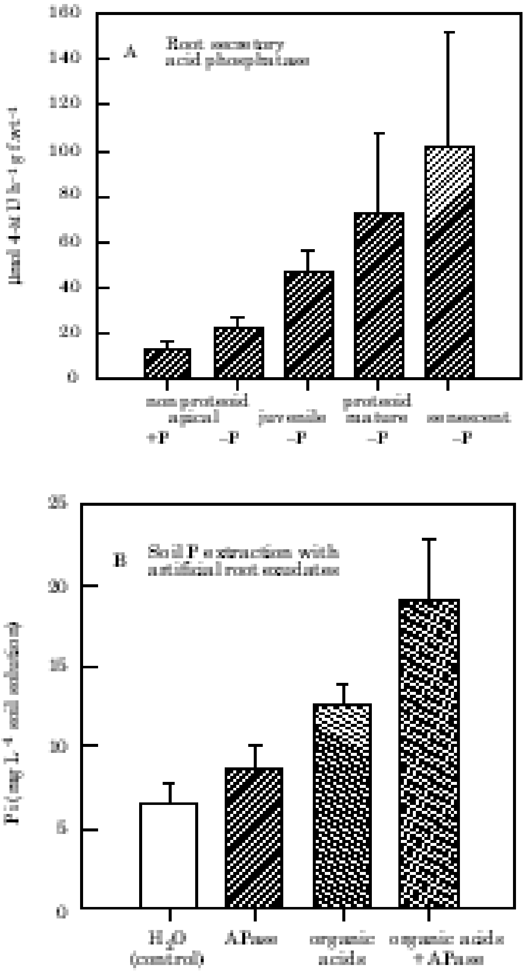
Fig 2: A, Exudation of phenolic compounds in different root zones of hydroponically-grown white lupin in response to variations of P supply in the nutrient solution. Determination of total phenolics with Folin- Ciocalteus phenol reagent. Bars represent means and s.d. of three to four independent experiments. **B,** Effect of partially purified phenolics isolated from root exudates of white lupin on citrate excretion in cluster roots. Exudate phenolics were isolated from root washings after a collection period of 2 h by solid phase extraction with Sep-Pak RP-18 cartridges (Millipore, Milford, USA), and subsequently eluted with methanol. The concentrated methanolic solution was separated by preparative thin-layer chromatography (TLC) on Avicel cellulose plates using 30% (v/v) acetic acid as a solvent. Flavonoids were detected by spraying one part of the TLC plates with indicator reagents according to Linskens (1959), and colour reactions were examined under UV/Vis light. Separated bands were scraped off from the untreated parts of the TLC plates and extracted with 80% (v/v) methanol. Methanol was removed under vacuum and the aqueous phenolic solutions were applied to filter papers used for 1.5 h of exudate collection from single root clusters (Neumann et al, 1999). To account for the high variability of citrate exudation from different clusters, exudation rates were recorded relative to a preceeding 1.5 h control incubation of the same clusters using filter papers without phenolics. Means and s.d. of four independent replicates are presented.

or even directly affect the exudation process. Accordingly, root excretion of citrate in mature root clusters was rapidly stimulated within 1.5 h by exogenous application of certain fractions of partially purified root exudate phenolics (Fig. 2B). Effects of flavonoids and other phenolics on membrane transport of inorganic and organic compounds are well documented, and depending on concentration and type of phenolics, stimulatory as well as inhibitory effects have been observed (for review see Rao, 1990). Recently, Pinton *et al.* (1997) reported a stimulation of proton extrusion associated with increased activity of root plasmalemma H^+-ATPase by exogenous application of humic acids to intact oat seedling roots. It remains to be established whether the increased exudation of root phenolics (Fig. 2A) or citrate-mediated mobilization of humic acids (Gerke *et al.*, 1994) in the rhizosphere of cluster roots impacts on changes of root exudation or ion uptake during cluster root development.

Phosphorus mobilization from the organic soil P fraction in the proteoid rhizosphere seems to be related to the release of large amounts of a P deficiency-inducible isoform of acid phosphatase (APase) (Li *et al.*, 1997; Gilbert *et al.*, 1999; Neumann *et al.*, 1999), predominantly restricted to cluster root zones of P-deficient white lupin (Fig. 3A). Accordingly, AFLP analysis of gene expression in cluster roots revealed enhanced expression of a clone with 78% homology to a purple acid phosphatase precursor in mature root clusters (see Table 7), which may represent the secretory precursor form of the isoenzyme released into the rhizosphere. In many soils, however, the availability of organic P is limited mainly by the low solubility of recalcitrant organic P forms such as Ca/Mg-, and Fe/Al-phytates, which can comprise a major proportion of the soil-organic P (Adams and Pate, 1992), and by the limited mobility of the root-borne phosphohydrolases (APase, phytase), mainly associated with the root cell wall and with mucilage in apical root zones (Dinkelaker *et al.*, 1997). The concomitant release of carboxylates and phosphohydrolases in the proteoid rhizosphere may help to mobilize sparingly soluble organic P esters and thereby increase their availability for enzymatic hydrolysis by phosphohydrolases adsorbed on the root surface. Accordingly, in a P-deficient sandy soil, more Pi was liberated by simultaneous application of acid phosphatase combined with organic acids in a concentration range determined for the rhizosphere soil of cluster roots (Dinkelaker *et al.*, 1997; Li *et al.*, 1997), than by separate application of organic acids or acid phosphatases alone (Fig. 3B).

Citrate-mediated mobilization of P-adsorbing Fe/Al humic acid complexes in the rhizosphere of cluster roots has been reported by Gerke *et al.* (1994).

Fig. 3: A, Activity of root-secretory acid phosphatase in different root zones of P-deficient white lupin. Means and s.d. of three to five replicates are presented (Source: Neumann et al, 1999). **B,** Water extractable Pi in a P-deficient sandy soil from Niger (West Africa) after separate or simultaneous application (24 h) of acid phosphatase and of organic acids according to concentration levels detected in the rhizosphere soil of cluster roots. Organic acids: Malic 7.5 mM; Citric 2 mM; Fumaric 1 mM, t-Aconitic 0.6 mM Acid phosphatase: Wheat germ APase (0.7 U g^{-1} soil). Means and s.d. of four independent replicates. (Source: Neumann and Römheld, 2000).



Phosphorus complexed with Fe and Al in

this kind of organic P fraction is not susceptible to enzymatic hydrolysis by root-secretory or microbial phosphohydrolases due to the absence of P esters. It is not clear to what extent P-adsorbing humic acid complexes are plant available.

Another function of enhanced root secretion of phosphohydrolases under P-deficient conditions may be the rapid retrieval of P from organic P forms permanently released into the rhizosphere from damaged or sloughed-off root cells and microorganisms (Lefebvre *et al*., 1990).

Induction of cluster roots

Phosphorus deficiency is a major factor for induction of cluster roots in *Lupinus albus* but these structures may be stimulated to a lesser extent by low Fe- Mn- and Zn-supply. Low levels of N enhance P deficiency-induced formation of cluster roots, whereas high N supply has inhibitory effects (Dinkelaker *et al*., 1995). A limited number of root clusters are formed even at moderate or adequate P levels (Marschner *et al*., 1987; Johnson *et al*., 1994; Keertisinghe *et al*., 1998). Increased formation of cluster roots is the earliest visible symptom of P deficiency in seedlings of white lupin grown in a hydroponic culture system without P supply, appearing 12±14 d after seed imbibition (see Fig. 4B). Suppression of cluster root formation by foliar P application (Marschner *et al*., 1987) suggests that induction is determined by internal P concentration rather than by P levels of the substrate.

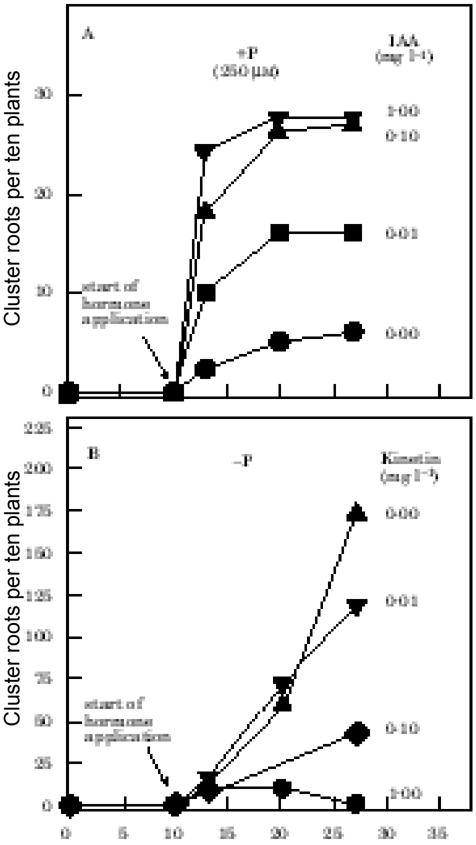


Fig. 4: A, Effects of exogenous application of indole-3-acetic acid (IAA) on cluster root formation in white lupin grown in a hydroponic culture system under P-sufficient conditions. IAA and nutrient solution supplied in 2 d intervals. **B,** Effects of exogenous application of kinetin on cluster root formation in white lupin grown in a hydroponic culture system under P-deficient conditions. Kinetin and nutrient solution supplied in 2 d intervals. Means of two experiments with each three replicates of ten plants.

There is increasing evidence that, similar to lateral root initiation in other plant species, cluster root formation might be under hormonal control. Exogenous application of indole-3-acetic acid (IAA) to the nutrient solution of hydroponically grown white lupin stimulated cluster root formation even in the presence of high P levels (Fig. 4A). In contrast, induction of cluster roots in P-deficient plants was suppressed by application of TIBA (2,3,5-triiodobenzoic acid) and NPA (N-(1-naphtyl)phtalamic acid), known to be potent inhibitors of the polar shoot-to-root transfer of auxins in higher plants (Gilbert et al., 1998).

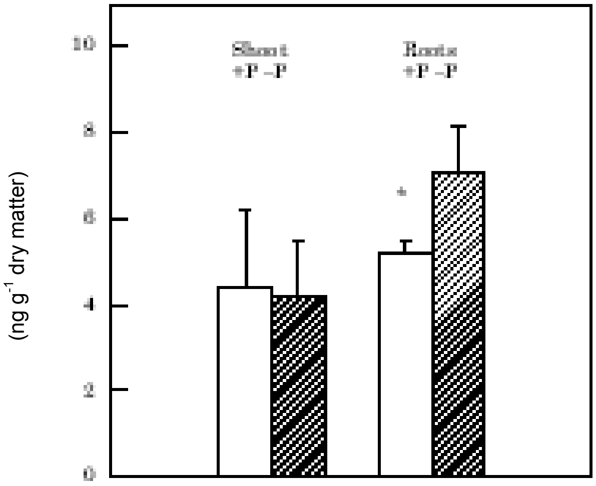


Fig. 5: Tissue concentrations of zeatin and zeatin riboside in shoot and roots of white lupin grown in a hydroponic culture system for 28 d with or without P supply. Cytokinins were determined by radioimmunoassay according to Bohner and Bangerth (1988). Means and s.d. of three independent replicates are presented. Significant differences are marked by an asterisk.

Similarly, a drastic inhibition of cluster root formation and of lateral rootlet elongation was observed when kinetin was applied to the growth medium of P-deficient white lupin at concentration levels 50.01 mg l^{-1} (Fig. 4B), suggesting a possible role of cytokinins as well. A similar auxin-cytokinin antagonism has been previously reported for lateral root formation in other plant species (Wightman *et al.*, 1980; Hinchee and Rost, 1986). Elevated concentrations of the zeatin/zeatin riboside cytokinin fraction were detected in the root tissue of 4-week-old P-deficient white lupin compared with P-sufficient control plants (Fig. 5). Based on these findings it may be speculated that auxin-stimulated emergence of juvenile cluster rootlets in P-deficient plants results in increased production of cytokinins due to the large number of root tips. Enhanced accumulation of cytokinins in the juvenile clusters may in turn contribute to inhibition of rootlet elongation during cluster root development.

Nutrient deficiency is also frequently associated with increased ethylene production (Lynch and Brown, 1997), and ethylene has been implicated in alterations of root morphology under P-deficient conditions (Borch *et al.*, 1999). However, an involvement of ethylene in cluster root induction remains to be established. In view of the highly differentiated morphology of cluster roots, hormonal interactions during cluster root development are likely to be quite complex.

Mechanism of carboxylate exudation

In contrast to cluster root induction, which is an early response to P starvation (12±14 d after seed imbibition, Fig. 4B), increased carboxylate accumulation in the root tissue and enhanced excretion of citrate and protons from root clusters is a symptom associated with later stages of P deficiency (21±28 d after seed imbibition, Fig. 6). These findings suggest that organic acid excretion is related to P deficiency-induced metabolic changes during cluster root development.

In white lupin grown in hydroponic culture without P supply over a period of 5±6 weeks, a distinct spatial distribution pattern with different stages of cluster root development could be distinguished along single first-order lateral roots (Neumann *et al*., 1999). Emergence of the youngest white-coloured juvenile cluster rootlets started approximately 2-3 cm from the lateral root tip. In the light-brown coloured, mature clusters, located 4-7 cm from the root tip, lateral rootlets had reached their final length of approx. 5 mm, and the colour turned to grey brown in senescent clusters close to the lateral root base.

Citric acid accumulated at high levels mainly in mature and senescent cluster roots, whereas malic acid was dominant in root tissue and in root exudates of non-proteoid roots and juvenile root clusters (Fig. 7). A peak of citrate exudation (up to 1 mmol h^{-1} g^{-1} f.wt) was induced when citrate accumulation in mature root clusters reached a threshold level of approx. 25±30 mmol g^{-1} f.wt, and was associated with a simultaneous acidification of the rhizosphere (Figs 1 and 7).

In senescent clusters, however, almost no citrate exudation was detectable despite high internal citric acid concentrations in the root tissue, indicating that a high rate of citrate release in mature root clusters is not simply leakage as a result of P deficiency-induced impaired plasma membrane integrity (Ratnayake *et al*., 1978). Citric acid applied exogenously during a 1.5 h period of localized root exudate collection (using the filter paper technique of Neumann *et al*., 1999) in senescent clusters, was 90% recoverable, suggesting that there was little effect of increased microbial degradation due to a higher microbial colonization rate at the surface of older root tissues (Foster, 1986). These findings suggest that the transient release of high amounts of citrate in mature root clusters of white lupin is mediated by a specific transport mechanism located at the plasma membrane. At the cytosolic pH of approx. 7.1-7.4 (Marschner, 1995), carboxylic acids usually exist as anions with low plasma membrane permeability. When the root systems of P-deficient white lupin were exposed for 1.5 h to the anion channel inhibitors ethacrynic acid and anthracene-9-carboxylic acid, the release of citrate declined by 50% (Table 2). This strongly suggests that citrate excretion from cluster roots is mediated by anion channels, with a concomitant release of protons in order to maintain charge balance, and accounting for rhizosphere acidification (Neumann *et al*., 1999). Similarly, inhibitory effects of anion-channel antagonists on root excretion of carboxylates have been reported for the Al-stimulated exudation of malate in root apices of wheat (Ryan *et al*., 1995), and the diurnal release of mugineic acid phytosiderophores in sub-apical root zones of Fe-deficient barley and maize (Sakaguchi *et al*., 1999; Neumann and Römheld, 2000). Future characterization of this transport mechanism will require studies of membrane physiology using patch clamp techniques and other tools.

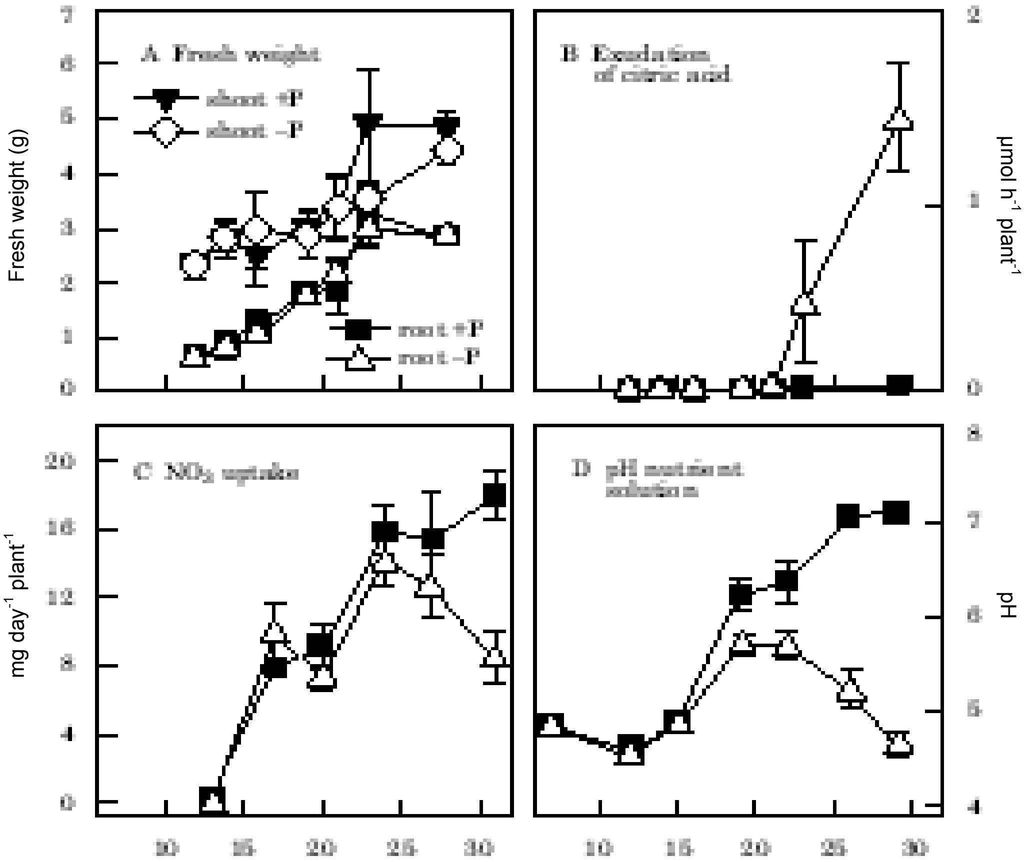


Fig. 6: Root and shoot biomass (A), root exudation of citrate (B), nitrate uptake (C) and pH (D) of the growth medium during seedling development of white lupin grown in hydroponic culture under P-sufficient or P-deficient conditions supplied with NO¡3 as nitrogen source. Means± s.d. of three independent replicates (Source: Neumann et al, 1999).

P deficiency-induced changes in carboxylate metabolism

Differential ^{14}CO2 pulse-chase labelling experiments with shoots and roots of white lupin revealed biosynthesis of carboxylates in the root tissue and particularly in cluster roots under P-deficient conditions (Johnson *et al*., 1996b). This is in good agreement with enhanced expression and *in vitro* activities of enzymes such as sucrose synthase, phosphoglucomutase, fructokinase (Table 3), and Phosphoenolpyruvate carboxylase (PEPC) (Table 4), involved in the catabolism of carbohydrates and in the biosynthesis of organic acids particularly in juvenile and mature root clusters (Johnson *et al*., 1996a; Keerthisinghe *et al*., 1998; Neumann *et al*., 1999; Watt and Evans, 1999). Compared with conventional glycolysis in P-sufficient plants, these enzymes may operate as an alternative pathway of carbohydrate catabolism under conditions of P starvation, which facilitates a more efficient P utilization by P recycling, reduced P consumption and utilization of alternative P pools such as Inorganic Phosphate (Ppi) (Theodoru and Plaxton, 1993; Plaxton, 1998). The ability to express those P-independent metabolic bypass reactions under P-deficient conditions determines a high sink strength of cluster roots for carbohydrates supplied by the shoot, which is a prerequisite for the biosynthesis of carboxylates in the root tissue.

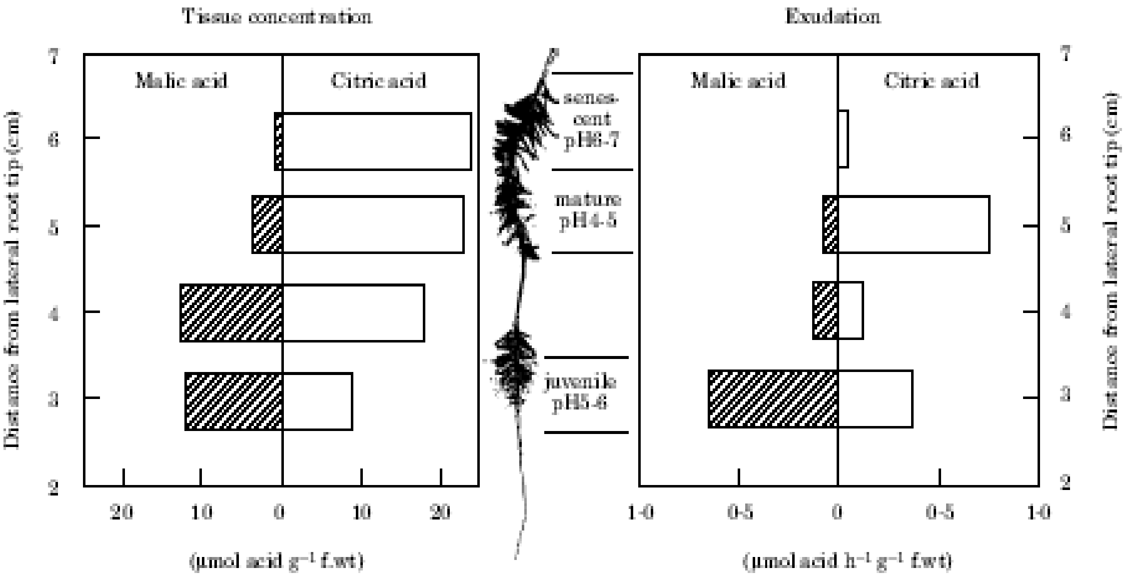


Fig. 7: Spatial variation of tissue concentrations and root exudation of major carboxylates (malate, citrate) and rhizoplane pH in different developmental stages of cluster roots in white lupin grown in hydroponic culture for 35 d without P supply. Means of three replicates (each including four root segments) are presented (adapted from Neumann *et al.*, 1999).

Table 2: Effect of anion-channel inhibitors on root exudation of citrate in P-deficient white lupin grown in a hydroponic culture system

Treatment	H_2O	Ethracrynic acid (50 μ M)	Anthracene-9-carboxylic acid (50 μ M)
Citrate exudation (% of control)	77.8^a ±15.1	41.6^b ±15.3	32.1^b ±19.7

Release of citrate was monitored during 1.5 h immersion of the whole root system into aerated inhibitor solutions or distilled H2O and compared to the exudation rates during a preceeding control incubation (1.5 h) of the same plants in distilled H2O. Due to large variations of citrate exudation between single plants (depending on activity status and number of root clusters per plant), changes in exudation rates were measured with the same plants before and after inhibitor application. Means+s.d. of three independent replicates. Significant differences are indicated by different superscripts (Source: Neumann *et al.*, 1999).

Additionally, non-photosynthetic CO_2 fixation via PEPC can contribute a substantial proportion of carbon (<30%) to carboxylate production in cluster roots (Johnson *et al.*, 1996a,b). Thus, PEPC-mediated CO_2 fixation was interpreted as an anapleurotic carbon supply to compensate for carbon losses associated with root exudation of carboxylates, which can comprise up to 25% of the photosynthetic CO_2 net fixation (Dinkelaker *et al.*, 1989; Johnson *et al.*,1996a,b).

Increased citrate accumulation in mature and senescent root clusters was associated not only with enhanced activity of PEPC but also with a reduced aconitase activity (Table 4, Neumann *et al.*, 1999). Aconitase is involved in the turnover of citrate within the TCA cycle and probably also in the cytosol (Brouquisse *et al.*, 1987). Thus, citrate accumulation is probably a consequence of both increased biosynthesis and reduced metabolism of citrate under P-deficient conditions. Induction of PEPC, reduced activity of aconitase, and increased accumulation of citric acid in the root tissue in response to P starvation has been similarly reported for other plant species such as tomato and chickpea (Neumann and Römheld, 1999).

Citrate accumulation during proteoid root development in white lupin was also associated with reduced respiration (Johnson *et al.*, 1994; Neumann *et al.*, 1999) and a concomitant decrease of soluble intracellular Pi (Table 5, Neumann *et al.*, 1999). Phosphorus limitation of the respiratory chain may induce feed-back inhibition of citrate turnover in the TCA cycle in order to prevent excessive production of reducing equivalents (Lance and Rustin, 1984).

Table 3: Representative distribution of specific in vitro activities of glycolytic enzymes in different root zones of P-sufficient and P-deficient white lupin grown in hydroponic culture

	Sucrose Synthase	Phospho-glucomutase	Fructokinase
Root type / Treatment	nmol substrate turnover h^{-1} mg^{-1} protein		
+P non-proteoid	103	47551	52
-P non-proteoid	139	140355	158
-P proteoid juvenile	307	295339	337
-P proteoid mature	396	230196	159
-P proteoid senescent	20	28602	33

Similar distribution patterns were obtained in three independent experiments. For enzyme extraction, roots were ground in liquid nitrogen and homogenized in 0.1 M Hepes (pH 7.5), 5 mM MgCl2, 2.5 mM DTT, 3 mM Na-DEDTC, 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF, 3% PVPP K30, in the ratio of 3 ml per g of fresh tissue. After two centrifugation steps (12000g, 15 min and 10 min, 4°C) the final supernatant was used to detect enzymatic activities. All enzymes were assayed spectro-photometrically by monitoring the changes of NADH concentrations at $A_{340\ nm}$.

Table 4: Specific in vitro activities of phosphoenolpyruvate carboxylase (PEPC), and aconitase in relation to tissue concentrations of citrate and cis-aconitate in roots of P-sufficient and P-deficient white lupin 23 d after sowing

	PEPC	Aconitase	Citrate	Cis-Aconitate
Root tissue	nmol product min^{-1} mg^{-1} protein		µmol g^{-1} FW	
+ P non-proteoid	$116^{a}\pm15$	$19^{a}\pm3$	$2.5^{a}\pm0.5$	$0.012^{a}\pm0.005$
-P non-proteoid	$212^{b}\pm35$	$14^{b}\pm1$	$8.8^{b}\pm2.0$	$0.016^{a}\pm0.004$
-P proteoid	$341^{c}\pm71$	$14^{a,b}\pm2$	$22.1^{c}\pm4.3$	$0.036^{b}\pm0.010$

Means ± s.d. of three independent replicates. In each column significant differences (one way ANOVA) are indicated by different superscripts. (Source: Neumann *et al.*, 1999)

Table 5: Relationship between P status (intracellular soluble Pi and RNA), respiration (O2 consumption), fermentation (specific activity of alcohol dehydrogenase), and citrate accumulation in different root zones of P-sufficient and P-deficient white lupin

	+P non-proteoid apical (2cm)	-P non-proteoid apical (2cm)	-P proteoid juvenile	-P proteoid mature	-P proteoid senescent
Soluble P_i (μ mol g^{-1} FW)	nd	1.29^{a}	1.86^{b}	$0.95^{a,c}$	0.51^{c}
RNA (mg g^{-1} FW)	0.25^{a}	0.36^{a}	0.57^{a}	0.06^{b}	0.02^{b}
Oxygen consumption (μ mol min^{-1} g^{-1} FW)	0.90^{a}	$0.88^{a,b}$	1.22^{a}	0.53^{b}	0.47^{b}
Alcohol dehydrogenase (μ mol NAD^{+} h^{-1} mg^{-1} protein)	25^{a}	18a	18^{a}	81^{b}	61^{b}
Citrate (μ mol g^{-1} FW)	nd	10.22^{a}	13.70^{b}	32.16^{c}	28.23^{c}

n.d = not determinated. Means of three independent replicates are presented. In each row, different superscripts indicate significant differences by one-way ANOVA (adapted from Neumann *et al.*, 1999)

Accordingly, in roots of *Phaseolus vulgaris* L., P deprivation decreased the capacity of the respiratory cytochrome pathway, but increased the ratio of NADH/NAD and cyanide-resistant respiration (Juszczuk and Rychter, 1997).

A P deficiency-induced inhibition of nitrate uptake in white lupin, both at the whole plant level (Fig. 6C) and based on root biomass (different rates of NO^{3-} uptake per plant but identical root fresh weight in +P and -P treatments, Fig. 6A,C), may further contribute to increased citrate accumulation in the root tissue by down-regulation of citrate conversion to 2-oxoglutarate, which is an important acceptor for amino N as a product of NO^{3-} reduction (Lancien et al., 1999). Inhibition of NO^{3-} uptake and assimilation has been previously reported as a common P-deficiency response in many other plant species (Le Bot *et al.*, 1990; Rufty *et al.*, 1990; Pilbeam *et al.*, 1993; Gniazdowska *et al.*, 1999), and was frequently associated with enhanced root accumulation of citric acid (Neumann and Römheld, 1999).

From these findings, it may be concluded that increased accumulation of citrate in the mature root clusters of white lupin is a P deficiency-induced metabolic disorder, and the release of high amounts of citrate and protons into the rhizosphere might serve as a detoxification mechanism to prevent cytoplasmic acidosis and over-accumulation of citrate exceeding the vacuolar storage capacity. A similar mechanism has been discussed for the detoxification of lactic acid, which accumulates in root tips of maize under hypoxic conditions (Xia and Roberts, 1994) with comparable intracellular carboxylate concentrations (20±30 μM) and similar exudation rates (2000±5700 nmol h^{-1} g^{-1} root f.wt) as observed in cluster roots of P-deficient white lupin (Xia and Roberts, 1994; Jones, 1998; Neumann *et al.*, 1999). However, accumulation of the tri-carboxylic citric acid in the cluster roots of white lupin may cause much stronger cytosolic acidification than similar concentrations of the mono-carboxylic lactic acid in the apical root zones of maize under hypoxia. Moreover, due to the chelating properties of citrate anions, cytosolic over-accumulation may interfere with the cytosolic Ca- and Mg-homeostasis in the root tissue. In both hypoxic maize root tips and mature root clusters of P-deficient white lupin, enhanced activity of alcohol dehydrogenase (AlDH) was detectable (Table 5). This enzyme is induced by energy limitation and low cytosolic pH (Pfister-Sieber and Brändle, 1994). In hypoxic plant tissues, AlDH induction is associated with enhanced production of ethanol, which is membrane permeable and easily released into the environment. This response is interpreted as an alternative pathway of carbohydrate fermentation to avoid excessive lactic acid accumulation (Pfister-Sieber and Brändle, 1994). Similarly, in P-deficient white lupin, AlDH induction may: (1) reflect severe energy shortage due to P limitation of root respiration; (2) indicate acidification of the cytosol by excessive accumulation of citric acid; or (3) counteract overproduction of NADH. Catabolism of sucrose, mediated by sucrose synthase instead of invertase, is another obvious similarity between metabolic alterations induced by limited oxygen supply (Guglielminetti et al., 1997) and P deficiency in cluster roots of white lupin.

Metabolic changes related to P deficiency are particularly expressed in mature and senescent cluster roots where concentrations of soluble Pi and RNA are extremely low. In contrast, Pi and RNA levels in juvenile clusters are comparatively high (Table 5). This may be attributed to re-mobilization and re-translocation of Pi from the older cluster regions, which exhibit no more growth activity (Watt and Evans, 1999), to the emerging juvenile clusters which include a high proportion of meristematic tissue with a high P demand. As a consequence, P limitation becomes more severe in mature and senescent clusters, associated with enhanced expression of P deficiency-induced metabolic alterations such as induction of

PEPC and P-independent glycolytic bypass reactions, inhibition of NO^{3-} uptake and respiration, and finally increased accumulation of citric acid in the root tissue.

P uptake mechanisms

^{33}Pi uptake per unit root fresh weight in P-deficient white lupin was approx. 2-fold higher in juvenile and mature root clusters compared to the apices of non-proteoid roots (Fig. 8, Neumann *et al*., 1999). Similar results were obtained for ^{33}Pi uptake based on root surface area of juvenile root clusters compared with non-proteoid root apices (Table 6). This implies that mature cluster roots are the sites of both P mobilization and of effective P uptake as well. Kinetic studies revealed a Km of 30.7 µM for P uptake of non-proteoid root apices in P-sufficient plants vs. Km values of 8.5±8.6 µM for non-proteoid and juvenile root clusters in white lupin exposed to P starvation (Table 6, Neumann *et al*., 1999), suggesting the induction of a high-affinity P uptake system under P-deficient conditions (Schachtman *et al*., 1998). A higher Vmax (6.5 nmol h^{-1} cm^{-2} root surface) for P uptake in juvenile root clusters compared with apical root zones of non-proteoid roots (2.0 nmol h^{-1} cm^{-2} root surface) may indicate a higher density of high-affinity P transporters in the plasma membrane of cluster roots (Table 6).

Conclusions

In P-deficient white lupin, adaptations towards enhanced spatial acquisition of available P (e.g. stimulation of root growth, enhanced formation of fine roots and root hairs, mycorrhizal associations) are poorly expressed (Marschner *et al*., 1987). In contrast, white lupin exhibits all the adaptations which have been shown to play a role in root-induced chemical mobilization of sparingly available P sources in soils. These include rhizosphere acidification, release of chelating root exudates, root-excretion of phosphohydrolases and induction of high-affinity P uptake systems. This system offers the opportunity to study both chemical modifications in the rhizosphere as well as physiological changes induced by limited P supply, in comparison with other plant species.

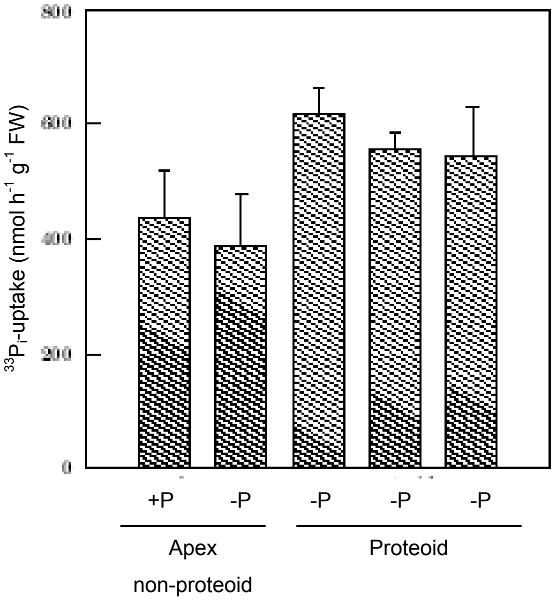


Fig. 8: Uptake of 33Pi in different root zones of P-sufficient and P- deficient white lupin after a culture period of 4 weeks in a hydroponic culture system. Means and s.d. of three to five replicates. (Source: Neumann *et al*, 1999).

Moreover, white lupin may be a good model system to investigate the molecular biology of plant adaptations to P starvation, which could help to define new targets for plant breeding or genetic engineering.

Table 6: Uptake characteristics of $^{33}P_i$ in different root zones of P-sufficient and P-deficient white lupin based on root surface area

Root zone / Treatment	Surface area (cm^2 g^{-1} root FW)	$^{33}P_i$ uptake (nmol h^{-1} cm^{-2} root surface)	Km (μ M)	Vmax (nmol h^{-1} cm^{-2} root surface)
Non-proteoid apical (2 cm) +P	137.5	3.2±0.6	30.7	4.8
Non-proteoid apical (2 cm) –P	137.5	2.8±0.7	8.6	2.0
Proteoid juvenile -P	75.1	8.3±0.3	8.5	6.5

Only 2 cm-apical root zones and juvenile root clusters are compared since reliable surface area determination of mature and senescent clusters was not possible due to strong proliferation of root hairs in these root zones. Calculations are based on average surface area determinations of ten apical root segments and ten juvenile clusters respectively. Pi uptake data represent means+s.d. of three to five independent replicates.

Table 7: Homology analysis and putative functions of selected AFLP cDNA clones predominantly expressed in different stages of cluster root development

Clones identified	Putative function
Proteoid juvenile	
Sucrose synthase (EC 2.4.1.13) (86% for 666 bp) Fructokinase (EC 2.7.1.4) (77% for 361 bp) Phosphoglucomutase (EC 5.4.2.2) (56% for 277 bp)	(P_i independent glycolytic bypass reactions) Increased carbohydrate catabolism
Proteoid mature	
Purple acid phosphatase precursor (EC 3.1.3.2) (78% for 135 bp)	Precursor for root-secretory Apase
Chitinase (77% for 88 bp)	Anti-fungal activity
Proteoid senescent	
Chorismate synthase precursor (80% for 296 bp)	Increased activity of shikimate pathway, synthesis of phenolics, P recycling

The analysis of root zone-specific gene expression in different stages of cluster root development using the AFLP approach revealed more than 80 clones with homologies to genes encoding for protein kinases and phosphatases, transcription factors, membrane transport proteins, pathogen-related proteins, cell wall-modifying enzymes, and enzymes of carbohydrate and phenolic metabolism. Table 7 represents a selection of those clones, most closely related to P deficiency-induced metabolic changes associated with the production and release of P mobilizing root exudates. A detailed understanding of these processes will be required to develop strategies for genetic manipulation to result in improved acquisition efficiency for P by crop plants. These investigations also have a bearing on the acquisition of iron and other micronutrients, aluminium tolerance, phytomining and phytoremediation strategies.

References

Adams MA and Pate JS 1992 Availability of organic and inorganic forms of phosphorus to lupins (Lupinus spp.). *Plant and Soil* **145**: 107-113.

Bohner J and Bangerth F 1988 Cell number, cell size and hormone levels in semi-isogenic mutants of Lycopersicon pimpinellifolium differing in fruit size. *Physiologia Plantarum* **72**: 316-320.

Borch K, Bouma TJ, Lynch JP and Brown KM 1999 Etylene: a regulator of root architectural responses to soil phosphorus availability. *Plant, Cell and Environment* **22**: 425-431.

Brouquisse R, Nishimura M, Gailard J and Douce R 1987 Characterization of a cytosolic aconitase in higher plant cells. *Plant Physiology* **84**: 1402-1407.

Dinkelaker B, Hahn G and Marschner H 1993a Non-destructive methods for demonstrating chemical changes in the rhizosphere. II. Application of methods. *Plant Soil* **155/156**: 71-74.

Dinkelaker B, Hengeler C and Marschner H 1995 Distribution and function of proteoid roots and other root clusters. *Botanica Acta* **108**: 183-200.

Dinkelaker B, Rö mheld V and Marschner H 1989 Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (Lupinus albus L.). *Plant, Cell and Environment* **12**: 285-292.

Dinkelaker B, Hahn G, Rö mheld V, Wolf GA and Marschner H 1993b Non-destructive methods for demonstrating chemical changes in the rhizosphere. I. Description of methods. *Plant and Soil* **155/ 156**: 67-70.

Dinkelaker B, Hengeler C, Neumann G, Eltrop L and Marschner H 1997 Root exudates and mobilization of nutrients. In: Rennenberg H, Eschrich W, Ziegler H, eds. Trees Contributions to modern tree physiology. Leiden, The Netherlands: Backhuys, 441-452.

Foster RC 1986 The ultrastructure of the rhizoplane and the rhizosphere. *Annual Review of Phytopathology* **24**: 211-234.

Gardner WK, Barber DA and Parbery DG 1983 The acquisition of phosphorus by Lupinus albus L. III. The probable mechanism, by which phosphorus movement in the soil-root interface is enhanced. *Plant Soil* **70**: 107-114.

Gerke J, Röhmer W and Jungk A 1994 The excretion of citric and malic acid by proteoid roots of Lupinus albus L.: effects on soil solution concentrations of phosphate, iron, and aluminium in the proteoid rhizosphere samples of an oxisol and a luvisol. *Zeitschrift für Pflanzenernährung und Bodenkunde* **157**: 289-294.

Gilbert GA, Vance CP and Allan DL 1998 Regulation of white lupin root metabolism by phosphorus availability. In: Lynch JP, Deikman J, eds. Phosphorus in plant biology: regulatory roles in molecular, cellular, organismic, and ecosystem processes. *American Society of Plant Physiologists*, 157-167.

Gilbert GA, Knight JD, Vance CP and Allan DL 1999 Acid phosphatases in phosphorus-deficient white lupin roots. *Plant, Cell and Environment* **22**: 801-810.

Gniazdowska A, Krawczak A, Mikulska M and Rychter AM 1999 Low phosphate nutrition alters bean plants ability to assimilate and translocate nitrate. *Journal of Plant Nutrition* **22**: 551-563.

Guglielminetti L, Wu Y, Boschi E, Yamaguchi J, Favati A, Vergara M, Perata P and Alpi A 1997 Effects of anoxia on sucrose degrading enzymes in cereal seeds. *Journal of Plant Physiology* **150**: 251-258.

Hinchee MA and Rost TL 1986 The control of lateral root development in cultured pea seedlings. I. The role of seedling organs and plant growth regulators. *Botanical Gazette* **142**: 137-147.

Johnson JF, Allan DL and Vance CP 1994 Phosphorus stress-induced proteoid roots show altered metabolism in Lupinus albus L. *Plant Physiology* **104**: 657-665.

Johnson JF, Vance CP and Allan DL 1996a Phosphorus deficiency in Lupinus albus: altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant Physiology* **112**: 31-41.

Johnson JF, Allan DL, Vance CP and Weiblen G 1996b Root carbon dioxide fixation by phosphorus deficient Lupinus albus. Contri- bution to organic acid exudation by proteoid roots. *Plant Physiology* **112**: 19-30.

Jones DL 1998. Organic acids in the rhizosphere-a critical review. *Plant Soil* **205**: 25-44.

Juszczuk IM and Rychter AM 1997 Changes in pyridine nucleotide levels in leaves and roots of bean plants (*Phaseolus vulgaris* L.) during phosphate deficiency. *Journal of Plant Physiology* **151**: 399-404.

Keerthisinghe G, Hooking PJ, Ryan PR and Delhaize E 1998 Effect of phosphorus supply on the formation and function of proteoid roots of white lupin (Lupinus albus L.). *Plant, Cell and Environment* **21**: 467-478.

Lance C and Rustin P 1984 The central role of malate in plant metabolism. *Physiologie Vegetal* **22**: 625-641.

Lancien M, Ferrario-Méry S, Boux Y, Bismuth E, Masclaux C, Hirel B, Gadal P and Hodges M 1999 Simultaneous expression of NAD-dependent isocitrate dehydrogenase and other Krebs cycle genes after nitrate resupply to short-term nitrogen starved tobacco. *Plant Physiology* **120**: 717-726.

Le Bot J, Alloush GA, Kirkby EA, Sanders FE 1990 Mineral nutrition of chickpea plants supplied with NO3 or NH4-N. II. Ionic balance in relation to phosphorus stress. *Journal of Plant Nutrition* **13**: 1591-1601.

Lefebvre DD, Duff SMG, Fife CA, Julien-Inalsingh C and Plaxton WC 1990 Response to phosphate deprivation in Brassica nigra suspension cells. *Plant Physiology* **93**: 504-511.

Li M, Shinano T and Tadano T 1997 Distribution of exudates of lupin roots in the rhizosphere under phosphorus deficient conditions. *Soil Science and Plant Nutrition* **43**: 237-245.

Linskens HF 1959 Papierchromatographie in der Botanik. 2. Aufl. Berlin: Springer.

Lynch L and Brown KM 1997 Ethylene and plant responses to nutritional stress. *Physiologia Plantarum* **100**: 613-619.

Marschner H 1995 Mineral nutrition of higher plants, 2nd edn. London: Academic Press.

Marschner H, Römheld V and Cakmak I 1987 Root-induced changes of nutrient availability in the rhizosphere. *Journal of Plant Nutrition* **10**: 1175-1184.

Neumann G and Römheld V 1999 Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant and Soil* **211**: 121-130.

Neumann G, Römheld V 2000 The release of root exudates as affected by the plant physiological status. In: Pinton R, Varanini Z, Nannipieri Z, eds. The rhizosphere: biochemistry and organic substances at the soil-plant interface. Basel, Switzerland: Marcel Dekker (in press).

Neumann G, Massonneau A, Martinoia E and Römheld V 1999 Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* **208**: 373-382.

Pfister-Sieber M and Brändle R 1994 Aspects of plant behaviour under anoxia and post-anoxia. *Proceedings of the Royal Society of Edinburgh* **102B**: 313-324.

Pilbeam DJ, Cakmak I, Marschner H and Kirkby EA 1993 Effect of withdrawal of phosphorus on nitrate assimilation and PEP carboxylase activity in tomato. *Plant and Soil* **154**: 111-117.

Pinton R, Cesco S, Santi S and Varanini Z 1997 Soil humic substances stimulate proton release by intact oat seedling roots. *Journal of Plant Nutrition* **20**: 857-869.

Plaxton WC 1998 Metabolic aspects of phosphate starvation in plants. In: Lynch JP, Deikman J, eds. Phosphorus in plant biology: regulatory roles in molecular, cellular, organismic, and ecosystem processes. *American Society of Plant Physiologists*, 229-240.

Rao AS 1990 Root flavonoids. Botanical Review 56: 1±84.
Ratnayake M, Leonard RT, Menge A. 1978. Root exudation in relation to supply of phosphorus and its possible relevance to mycorrhizal infection. *New Phytologist* **81**: 543-552.

Rufty TW and MacKnown CT, Israel DW 1990
Phosphorus stress effects on assimilation of nitrate. *Plant Physiology* **94**: 328-333.

Ryan PR, Delhaize E and Randall PJ 1995 Characterization of Al-stimulated e.ux of malate from the apices of Al-tolerant wheat roots. *Planta* **196**: 103-110.

Sakaguchi T, Nishizawa NK, Nakanishi H, Yoshimura E and Mori S 1999 The role of potassium in the secretion of mugineic acids family phytosiderophores from iron-deficient barley roots. *Plant and Soil* **215**: 221-227.

Schachtman DP, Reid RJ and Ayling SM 1998 Phosphorus uptake by plants: from soil to cell. *Plant Physiology* **116**: 447-453.

Theodoru ME and Plaxton WC 1993 Metabolic adaptations of plant respiration to nutritional phosphate deprivation. *Plant Physiology* **101**: 339-344.

Watt M and Evans J 1999 Linking development and determinacy with organic acid e.ux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO2 concentration. *Plant Physiology* **120**: 705-716.

Wightman F, Schneider EA and Thimann KV 1980 Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in pea roots. *Physiologia Plantarum* **49**: 304-314.

Wojtaszek P, Stobiecki M and Gulewicz K 1993 Role of nitrogen and plant growth regulators in the exudation and accumulation of isoflavonoids by roots of intact white lupin (Lupinus albus L.) plants. *Journal of Plant Physiology* **142**: 689-694.

Xia JH and Roberts JKM 1994 Improved cytoplasmic pH regulation, increased lactate e.ux, and reduced cytoplasmic lactate levels are biochemical traits expressed in root tips of whole maize seedlings acclimated to a low-oxygen environment. *Plant Physiology* **105**: 651-657.

CHAPTER II

METABOLIC CHANGES ASSOCIATED WITH CLUSTER

ROOT DEVELOPMENT IN WHITE LUPIN (*LUPINUS ALBUS* L.): RELATIONSHIP BETWEEN ORGANIC ACID EXCRETION, SUCROSE METABOLISM AND ENERGY STATUS

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Summary

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

Résumé

En situation de carence en phosphore le lupin blanc (*Lupinus albus*, L.), développe des racines protéoïdes, appelées ainsi du fait de leur présence préférentielle chez les Protéacées. Dans leur stade mature ces racines acidifient le sol et excrètent de grandes quantités d'acides organiques (jusqu'à 1,5 et 7 µmol $.h^{-1}$ $.g^{-1}$ MF de malate et citrate, respectivement) permettant aux lupins d'utiliser des sources très faiblement accessibles de phosphate. A l'aide de la technique AFLP, nous avons identifié des gènes exprimés préférentiellement dans les racines protéoïdes juvéniles et matures. Les transcrits de deux enzymes impliquées dans la glycolyse, la fructokinase et la phosphoglucomutase, ont été identifiés dans les racines protéoïdes juvéniles et une, la sucrose synthase, dans les racines protéoïdes matures. Afin de vérifier ces observations nous avons réalisé des RT-PCR quantitatives et nous avons pu confirmer l'augmentation du taux de transcrit. La mesure des activités enzymatiques a montré que l'activité fructokinase et phosphoglucomutase augmente dans les racines protéoïdes juvéniles, alors que l'activité sucrose synthase est maximale daans les racines protéoïdes matures. Ces résultats indiquent que la formation de racines protéoïdes et l'excrétion de citrate augmentent localement la force de puits. La production de citrate et l'inhibition de la respiration sont susceptibles de résulter en une augmentation du ratio $NADH/NAD^+$, ce qui peut être toxique pour la plante. La voie de la fermentation permettrait l'oxydation du NADH par décarboxylation du pyruvate et la subséquente réduction de l'acétaldéhyde produit. La détermination de l'activité alcool déshydrogénase montre que cette enzyme est fortement induite dans les racines protéoïdes matures. Toutefois, la production d'éthanol n'est pas augmentée, indiquant que le pyruvate est shunté vers la synthèse de citrate et non vers la production d'éthanol.

Introduction

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

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

Most of the soil phosphorus is present as organic phosphate or insoluble mineral phosphate, which both cannot be absorbed by plants. Organically bound phosphate, which may account for up to 80 % of the total phosphorus in some cultivated soils (Li *et al*. 1997a; Gilbert *et al*. 1998), has to be mineralised to inorganic phosphate before plants can take it up. Plants and soil microorganisms have developed the capacity to release phosphohydrolases into the rhizosphere, which convert organic phosphate to inorganic phosphate. Among these phosphohydrolases, acid phosphatase (APase) is the most documented and widespread enzyme secreted in response to phosphorus deficiency (Lefebvre *et al*. 1990; Duff *et al*. 1994), but also other enzymes, such as ribonucleases and phytases have been found in rhizodeposition and cell culture media (Dodds *et al*. 1996; Li *et al*. 1997a,b; Köck *et al*. 1998).

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

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

The present study provides a detailed comparison of relations between organic acid metabolism, catabolism of carbohydrates and energy status in different stages of cluster root development.

Material and methods

Plant material.

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

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

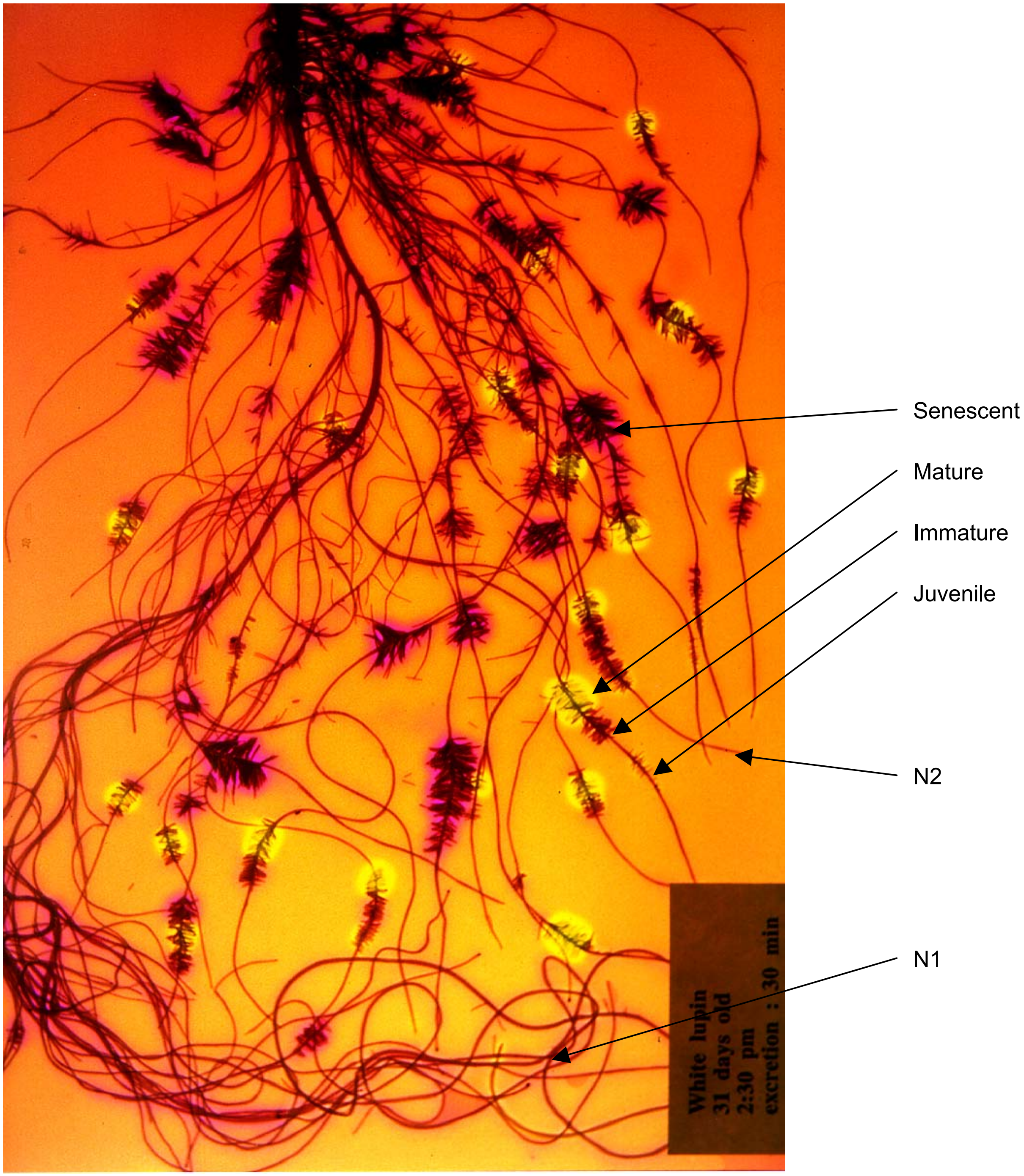
Bromocresol purple was used as a pH indicator to visualise pH changes along the roots. The root system was spread between two glass plates covered by a 2 mm layer of 1 % agar containing 0.04 % (w/v) of bromocresol purple. The pH of the gel was adjusted to 6 – 6.5 in order to get a colour (Fig. 1) which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

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

Acid extraction and determination of Pi and ATP.

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

Fig. 1: Root system of 31 days old P-deficient white lupin. The roots were placed between 2 plates of agar containing 0.04% (w/v) bromocresol purple as a pH indicator. The dye turns to yellow (pH<5) when the roots are acidifying their surrounding and become purple if alkalinisation (pH>7) occurs. The different developmental stages of the cluster roots are indicated. N1 apex (1 cm) of the secondary roots which do not contain cluster roots, N2 apex (1 cm) of the secondary roots containing cluster roots.



Exudate collection and analysis.

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

Extraction of total RNAs.

Frozen tissues were homogenised in liquid nitrogen using pre-cooled mortars and pestles, extracted with 8 volumes of extraction buffer (20% (v/v) phenol (in 10 mM Tris HCl pH 9.0), 20% (v/v) chloroform, 60 mM NaCl, 6 mM Tris HCl (pH 7.5), 0.6 mM EDTA, 0.6% SDS, 1.2 mM ß-mercaptoethanol, 9 mM Na-ascorbate) and incubated during 30 min at 4°C under constant stirring (all subsequent steps were carried out at 4°C). After centrifugation for 20 min at 9000 g, nucleic acids were recovered from the upper phase and precipitated overnight at 4°C with 1/10 volume of 3 M Na-acetate and 2 volumes of ethanol. Nucleic acids were subsequently pelleted during 20 min at 9000 g, washed with 70% (v/v) ethanol and resuspended in water. RNAs were specifically precipitated with 2 M Li-acetate during 3h at 4°C, pelleted and washed as mentioned above. The RNA pellet was resupended in water and precipitated by adding 1/10 vol of 3 M Na-acetate and 2 vol. of ethanol. After a 20 min centrifugation at 9000 g, the RNAs were washed with ethanol and resuspended in water.

cDNA-AFLP and clones identification.

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

RT-PCR.

Total RNA was treated with RQ1 DNase (Promega) following the manufacturer's instructions and then loaded on a gel to check concentrations. About 2.5µg of total RNA of each root type, supplemented with 10^6 copies of pAW109 RNA (provided by Perkin-Elmer Biosystems, Roche Molecular Systems, Branchburg NJ, USA, #N808-0037), have been reverse transcribed using Reverse Transcription System provided by Promega with oligo-dT and following manufacturer's instructions. 1/100 of the RT product was used for each PCR reaction.

PCRs have been performed with Promega's Taq DNA Polymerase in the buffer supplied containing 2.5mM $MgCl_2$. Final concentrations nucleotides was 0.1 mM for dATP and 0.2 mM for the other dNTPs. The PCR reaction mix was supplemented with 0.5MBq of ^{33}P labelled dATP. The sucrose synthase (SuSy), phosphoglucomutase (PGM) and fructokinase (FK) primers were designed based on the clones obtained with the cDNA-AFLP. A pAW109 specific set of primers was introduced in the same tube to verify the RT-PCR processing. Primers for SuSy were $^{5'}$AGTGATGGTCCCTTTGGTGA$^{3'}$ and $^{5'}$ACACGCTCAACCTTGTCTCC$^{3'}$ amplifying a 554 bp fragment, control RNA was amplified using AW112 $^{5'}$CAGAGGGAAGAGTTCCCCAG$^{3'}$ and AW113 $^{5'}$CCTTGGTCTGGTAGGAGACG$^{3'}$ giving a 301bp fragment. For PGM: $^{5'}$GAGCTCTGAAGGAGAATCATTG$^{3'}$ and $^{5'}$AGGATCATGGAGTGACAGTC$^{3'}$ amplifying a 357 bp fragment and AW125 $^{5'}$CAATGTCTCACCAAGCTCTG$^{3'}$ and AW159 $^{5'}$GAGGAGGTGTTGACTTCATTC$^{3'}$ amplifying an approximate 450 bp fragment. For FK: $^{5'}$TCCTGAAGAAGCTCGTCAAC$^{3'}$ and $^{5'}$GAGTACCTCTCTTAACCTTGG$^{3'}$ amplifying a 336 bp fragment and AW125 and AW 159 were used. The PCR reactions were 45s at 95°C, 1 min at 60°C (SuSy) or 56°C (PGM and FK) and 1 min at 72°C for 24 cycles. The number of cycle has been checked to be in the linear range of the PCR reactions under this conditions.

RT-PCR products were separated by 2.5% (w/v) agarose gel electrophoresis. The gel were treated 10 min with 0.25M HCl and then transferred using a vacuoblot system with 2XSSC on a polarised nylon membrane (Porablot NY plus, Düren, Germany) during 2h. Blots were rinsed and exposed to Biomax MR film (Eastman Kodak, Rochester, NY,USA) during a few hours.

Extraction of soluble proteins and enzyme assays.

Roots were ground in liquid nitrogen and homogenised with 3 volumes of 0.1 M Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 2.5 mM DTT, 3 mM Na-DEDTC, 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF, 3 % PVPP K30. After a 25 min centrifugation at 12000 g (4°C), the supernatant was used to determine the extractable enzymatic activities and protein concentrations (DC Protein Assay kit, Bio-Rad, Hercules, CA, USA).

All enzymes were assayed spectrophotometrically by monitoring the appearance or the disappearance of NADH at 340 nm. All reactions were initiated by addition of the root extract.

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

Respiration measurements.

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

Results

Description of the root morphology.

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

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

In order to make general comparison between the different root types we measured some further parameters related to the phosphate status (Table 1). Comparison of the protein content shows that with the exception of N1 and the senescent cluster roots the protein contents was quite similar (Table 1). In contrast the total RNA contents decrease strongly from the immature to the mature and senescent cluster roots. This fact is important, since the phosphate liberated and reallocated by the degradation of RNA is likely to play an important role for the growth of young root tissues (see discussion). Phosphate and ATP contents (Table1) are highest in juvenile and lowest in senescent cluster roots. The other root types have a very similar phosphate content but immature and mature cluster roots contain about twofold as much ATP compared to the root apices. Respiration is about 1.5 fold higher in juvenile cluster roots compared to most of the other root types. However, a decrease is observed in mature cluster roots and senescent cluster roots which exhibit only about 20% of the respiratory activity observed in juvenile cluster roots.

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

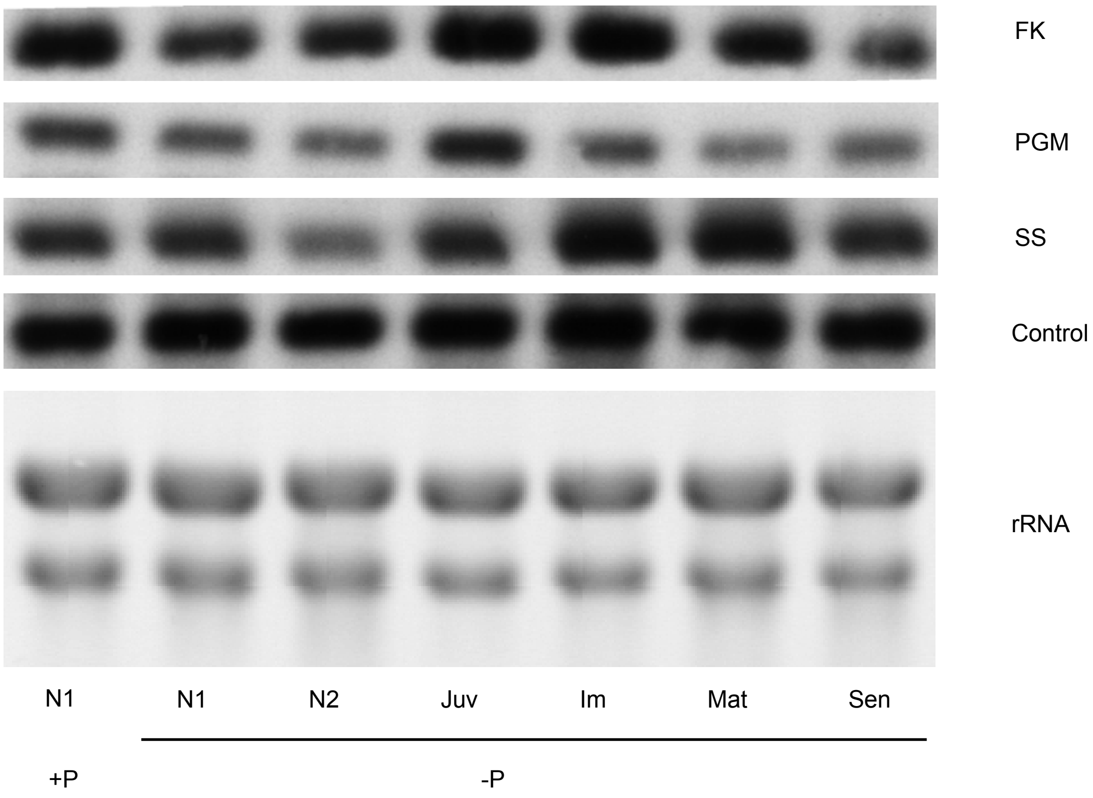
Root zone		Excretion		Root content				Respiration
		Citrate (nmol .h^{-1})	Malate (nmol h^{-1})	Proteins (μ g)	RNA (μ g)	Phosphate (μ mol)	ATP (nmol)	(μ mol O_2 min^{-1})
+P	N1	330±92	646±126	660±50	247±68	nd	nd	nd
-P	N1	471±142	369±126	520±60	68±49	0.15±0.03	24.3±4.5	2.0±0.2
	N2	432±42	712±140	910±90	655±3	0.18±0.09	23.0±10.1	2.9±0.4
	Juvenile	756±150	1654±249	1180±20	573±73	0.38±0.10	90.9±8.7	3.3±0.4
	Immature	2464±330	1696±489	820±10	247±88	0.21±0.02	47.4±6.8	2.5±0.3
	Mature	7051±2066	1539±327	900±90	62±29	0.18±0.05	42.6±8.9	1.9±0.4
	Senescent	2297±788	90±788	470±10	16±2	0.08±0.04	12.1±3.9	0.6±0.1

Sucrose synthase, fructokinase, phosphoglucomutase expression pattern and activities.

In order to identify stage-specific expression of mRNA we performed a cDNA-AFLP analysis (Bachem et al. 1996) with juvenile, mature and senescent cluster roots. Cloning and sequencing of a range of differentially expressed bands showed that several enzymes implicated in glycolysis, namely fructokinase, sucrose synthase, phosphoglucomutase where upregulated in juvenile and mature cluster roots (Table 2). The very high similarity observed over stretches of 120 and 227 bases for fructokinase and sucrose synthase makes it very likely that the clones indeed correspond to the genes coding for the enzymes identified in the data base. For phosphoglucomutase the similarity was less pronounced.

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

Fig. 2: Transcript levels of fructokinase (FK), phosphoglucomutase (PGM) and sucrose synthase (SuSy) in different parts of lupin roots. Quantitative RT-PCR was performed as described in materials and methods including a synthetic RNA as control (control shown for PGM). As a further control a RNA agarose gel was stained with ethidium bromide.



Since mRNA levels may not reflect the enzyme activity in a tissue, and transition of one cluster root stage to another is a rapid process, we measured the *in vitro* activities of the three enzymes (Fig. 3). Based on fresh weight, the activities of fructokinase and phosphoglucomutase, were increased in response to phosphorus starvation. In -P plants, the juvenile and mature cluster roots had the highest activities, whereas senescent clusters and normal N1, exhibited a lower activity. Due to the variation in the protein content between the different -P root types, the specific activity of fructokinase showed similar but less pronounced fluctuations. However, specific activities of fructokinase and phosphoglucomutase were much higher in every root part of -P compared to +P plants.

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

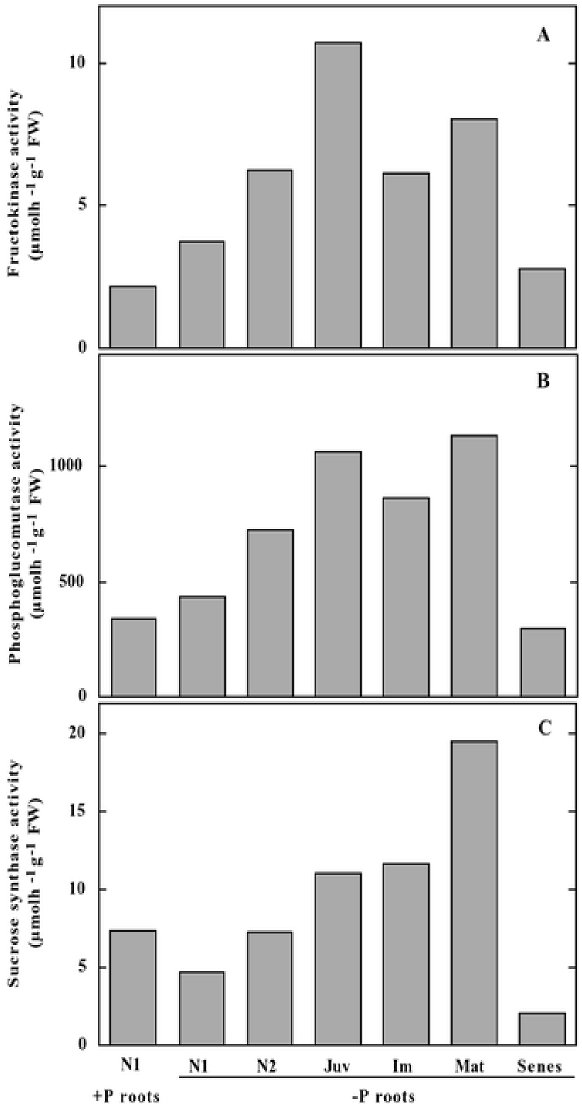


Fig 3: In vitro activities of fructokinase (A), phosphoglucomutase (B) and sucrose synthase (C), measured in –P and +P roots of white lupin. The experiments were repeated at least four times, and the distribution pattern of enzymatic activities obtained in different root zones was reproducible. Due to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

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

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

Alcohol dehydrogenase and pyruvate decarboxylase activity.

Low respiration and production of large amounts of organic acids suggests that the plant accumulate reducing equivalents. A pathway to cope with overproduction of NADH consists in the reduction of acetaldehyde originating from pyruvate decarboxylation and formation of ethanol. Indeed, alcohol dehydrogenase activity is strongly increased in cluster roots excreting citric acid (Fig. 4).

While an increase can already be observed in immature cluster roots compared to N2 roots, a more than threefold increase can be observed in mature cluster roots. Based on fresh weight, a slight increase is already observed in juvenile cluster roots. In contrast, induction of pyruvate decarboxylase activity is far lower and no differences could be observed between N2 and juvenile, immature and mature cluster roots. In order to investigate whether the increase in alcohol dehydrogenase is linked to ethanol production and excretion we determined ethanol excreted by the different root types. Only a very low ethanol tissue concentration was detected within the different root parts and no difference could be observed (not shown). Surprisingly, N2 roots and juvenile cluster roots excreted more ethanol than immature or mature cluster roots.

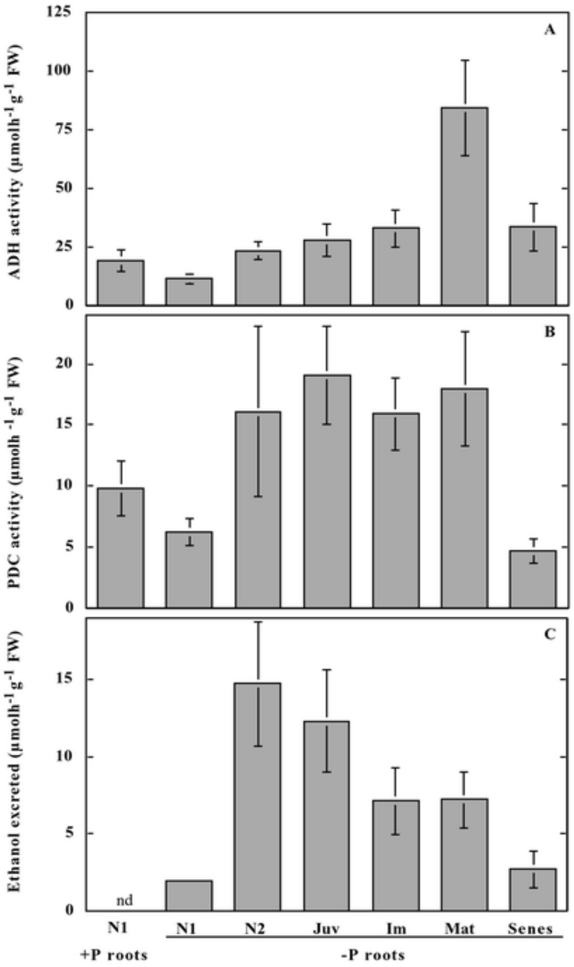


Fig. 4. *In vitro* activities of alcohol dehydrogenase **(A)**, pyruvate decarboxylase **(B)** and ethanol excretion **(C)** in different root zones of white lupin. Data represent the mean ± SD of 4 independent experiments, each with three replicates.

Discussion

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

Using the cDNA-AFLP we identified three clones differentially expressed in different root types exhibiting a high homology to genes coding for enzymes involved in the transformation of sucrose to fructose-6-P: sucrose synthase, fructokinase and phosphoglucomutase. Since these enzymes are the starting points of glycolysis we were interested to see, whether the transcript levels for these genes are indeed induced. RT-PCR confirmed the results obtained with cDNA-AFLP. The observation that sucrose synthase was originally found in juvenile cluster roots may be due to the fact that at the beginning of our studies we were not able to differentiate between immature and mature cluster roots and that immature cluster roots were, at least partially, collected together with juvenile cluster roots since they exhibit a negligible acidification of the medium. Sucrose unloaded from the phloem sap serves as a carbon source for the roots, either for growth or organic acid excretion and is either processed by invertases or sucrose synthase (Ciereszko et al. 1998). $^{14}CO_2$ shoot-labelling experiments with P-deficient white lupin demonstrated carbon translocation from shoots to roots with subsequent conversion of sugars to organic acids in the root tissue (Johnson *et al*. 1996a). Organic acid excretion (malate and citrate) increased from about 1000 nmol $.h^{-1.}.g^{-1}$ FW in -P or +P normal roots to more than 7000 nmol$.h^{-1}.g^{-1}$ FW in the mature cluster roots (Table 1). $^{14}CO_2$ labelling studies showed that organic acids exuded by the roots of P-deficient white lupin are also linked to PEP carboxylase-mediated non-photosynthetic CO_2 fixation in the root tissue (Johnson et al. 1996a,b), which can account for one third of the exuded carbon. In the case of the mature cluster roots, this implies that root CO_2 fixation is responsible for an exudation of about 2860 nmol$.h^{-1}.g^{-1}$ FW. Compared with the total organic acids exuded from mature cluster roots (7051 nmol$.h^{-1}.g^{-1}$ FW), 5730 nmol$\cdot h^{-1}\cdot g^{-1}$ FW has to be sustained by C supplied from the shoots as sugars. Our enzymatic studies showed that fructokinase, phosphoglucomutase and sucrose synthase (Fig. 3) exhibited increased activity in juvenile, immature and mature cluster roots compared with normal roots. We also measured hexokinase activity in -P roots (results not shown) which was found to be very low compared to sucrose synthase and fructokinase. For fructokinase as well as for phosphoglucomutase the highest transcript levels as well as enzymatic activities could be detected in the juvenile cluster roots. However, our RT-PCR data indicate that the decrease in transcript level in immature and mature cluster roots is faster than the decrease in enzymatic activity. It is therefore likely that the protein turnover is less rapid than mRNA turnover. The transition of juvenile to immature cluster roots takes approximately two days, and about one day from the immature to the mature stage. Therefore, enzymes which are synthesised in an earlier stage and which do not exhibit a rapid turnover will remain still active in a later stage of development. In contrast to phosphoglucomutase and fructokinase maximal transcript levels of sucrose synthase can be detected in the immature stage. Maximal enzymatic activity of sucrose synthase is found in the mature state and it must again be postulated that turnover of the enzyme is slower than that of the corresponding mRNA. A decreased protein turnover under phosphate starvation could save ATP and hence help to survive phosphate stress.

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

The use of sucrose synthase by cluster roots is also of energetic importance for the cells. During phosphorus deficiency, pools of Pi and NTPs are significantly reduced, whereas the PPi pool is maintained. A metabolic "bypass" using PPi-dependent enzymes instead of ATP-dependent enzymes has been proposed in P-deficient plants (for review see Theodorou and Plaxton, 1993; Plaxton 1996 and 1998). The sucrose synthase pathway allows sucrose conversion to hexose-Ps via a PPi-dependent pathway, and compared with the invertase pathway requires 2-fold less ATP.

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

References

Bachem CW, Van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M and Visser RG 1996 Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant J* **9**: 745-753

Chan K-M, Delfert D, Junger KD 1986 A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. *Anal Biochem* **157**: 375 – 380

Ciereszko I, Zambrycka A, Rychter A 1998 Sucrose hydrolysis in bean roots (*Phaseolus vulgaris* L.) under phosphate deficiency. *Plant Sci* **133**: 139-144

Dinkelaker B, Hengeler C, Neumann G, Eltrop L, Marschner H 1997 Root exudates and mobilization of nutrients. In Trees. Contributions to modern tree physiology. Rennenberg, H. Eschrich, W., Ziegler H., eds., Backhuys Publishers, Leiden, The Netherlands, pp 441-452

Dodds PN, Clarke AE, Newbigin ED 1996 Molecular characterisation of an S-like RNase of *Nicotiana alata* that is induced by phosphate starvation. *Plant Mol Biol* **31**: 227-238

Duff SMG, Sarath G, Plaxton WC 1994 The role of acid phosphatases in plant phosphorus metabolism. *Physiol Plant* **90**: 791-800

Gardner WK, Parbery DG, Barber DA 1981 Proteoid root morphology and function in *Lupinus albus*. *Plant Soil* **60**: 143-147

Gilbert GA, Allan DL, Vance CP 1997 Phosphorus deficiency in white lupin alters root development and metabolism. In Flores HE, Lynch JP, Eissenstat D, eds, Radical biology: advances and perspectives on the function of plant roots, Rockville, pp 92-103

Gilbert GA, Vance CP, Allan DL 1998 Regulation of white lupin root metabolism by phosphorus availability. In JP Lynch, J Deikman, eds, Phosphorus in plant biology: regulatory roles in molecular, cellular, organismic and ecosystem processes. Rockville, pp 157-167.

Gniazdowska A, Krawczak A, Mikulska M, Rychter AM 1999 Low phosphate nutrition alters bean plants ability to assimilate and translocate nitrate. *J Plant Nutr* **22**: 551-563.

Hoffland E, Findenegg GR, Nelemans A 1989 Solubilization of rock phosphate by rape. II. Local root exudation of organic acids as a response to P-starvation. *Plant Soil* **113**: 161-165.

Johnson JF, Allan DL, Vance CP 1994 Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. *Plant Physiol* **104**: 657-665

Johnson JF, Allan DL, Vance CP, Weiblen G 1996a Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus*. Contribution to organic acid exudation by proteoid roots. *Plant Physiol* **112**: 19-30

Johnson JF, Vance CP, Allan DL 1996b Phosphorus deficiency in *Lupinus albus*. Altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant Physiol* **112**: 31-41

Juszczuk IM, Rychter AM 1997 Changes in pyridine nucleotide levels in leaves and roots of bean plants (*Phaseolus vulgaris* L during phosphate deficiency. *J Plant Physiol* **151**: 399-404

Kakie T 1969 Phosphorous fractions in tobacco plants as affected by phosphate application. *Soil Sci Plant Nutr* **15**: 8-85

Köck M, Theierl K, Stenzel I, Glund K 1998 Extracellular administration of phosphate-sequestering metabolites induces ribonucleases in cultured tomato cells. *Planta* **204**: 404-407

Lamont B 1972 The morphology and anatomy of proteoid roots in the genus *Hakea*. *Aust J Bot* **20:** 155-174

Lance C, Rustin P 1984 The central role of malate in plant metabolism. *Physiol Vég* **22**: 625-641

Lefebvre DD, Duff SMG, Fife CA, Julien-Inalsingh C, Plaxton WC 1990 Response to phosphate deprivation in *Brassica nigra* suspension cells. *Plant Physiol* **93**: 504-511

Li M, Osaki M, Rao IM, Tadano T 1997a Secretion of phytase from the roots of several plant species under phosphorus-deficient conditions. *Plant Soil* **195**: 161-169

Li M, Shinano T, Tadano T 1997b Distribution of exudates of lupin roots in the rhizosphere under phosphorus deficient conditions. *Soil Sci Plant Nutr* **43**: 237-245

Marschner H, Romheld V, Cakmak I 1987 Root-induced changes of nutrient availability in the rhizosphere. *J Plant Nutr* **10**: 1175-1184

Marschner H 1995 Rhizosphere pH effects on phosphorus nutrition. In: Genetic manipulation of crop plants to enhance integrated nutrient management in cropping systems -1- Phosphorus: proceedings of an FAO/ICRISAT expert consultancy workshop. Lee JC, Sharma KK, Subbaro GV, Kueneman EA, eds., ICRISAT Asia Center India, pp 107-115

Neumann G, Massonneau A, Martinoia E, Römheld V 1999 Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* **208**: 373-382

Neumann G, Römheld V 2000 Phosphorus-deficiency induced root excretion of carboxylic acids and protons in different plant species. *Plant Soil* (in press)

Plaxton WC 1996 The organization and regulation of plant glycolysis. *Ann Rev Plant Physiol Plant Mol Biol* **47**: 185-214

Plaxton WC 1998 Metabolic aspects of phosphate starvation in plants. In JP Lynch, J Deikman, eds, Phosphorus in plant biology: regulatory roles in molecular, cellular, organismic and ecosystem processes. Rockville, pp 229-241

Raghothama KG 1999 Phosphate acquisition. *Ann Rev Plant Physiol Plant Mol Biol* **50**: 66-693

Rufty TW, MacKown CT, Israel DW 1990 Phosphorus stress effects on assimilation of nitrate. *Plant Physiol* **94**: 328-333

Ruiz JM, Romero L 1998 Calcium impact on phosphorus and its main bioindicators: response in the roots and leaves of tobacco. *J Plant Nutr* **21**: 2273-2285

Sambrook, Fritsch EF, Maniatis T 1989 Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Tadege M, Braendle R, Kuhlemeier C 1998 Anoxia tolerance in tobacco roots: effect of overexpression of pyruvate decarboxylase. *Plant J* **14**: 327-335

Theodorou ME, Plaxton WC 1993 Metabolic adaptations of plant respiration to nutritional phosphate deprivation. *Plant Physiol* **101**: 339-344

Watt M, Evans JR 1999 Linking development and determinacy with organic acid efflux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO_2 concentration. *Plant Physiol* **120**: 705-716

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U 1995 Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *Plant J* **7**: 97-107

CHAPTER III

ATP CITRATE LYASE: CLONING, HETEROLOGOUS EXPRESSION AND ROLE IN ROOT EXUDATION OF ORGANIC ACIDS

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Summary

In order to cope with phosphate deficiency, white lupin produces cluster roots, which excrete large amounts of malate and citrate. Young, developing cluster roots mainly excrete malate, mature cluster roots mainly citrate. Mature proteoid roots excrete four to six times more carboxylates compared to juvenile proteoid roots. Using a cDNA-AFLP approach we identified a gene coding for a putative ATP-citrate lyase (ACL) upregulated in young cluster roots. Cloning of the ACL genes revealed that in contrast to animals, plant ACL is constituted by two polypeptides. The function of the gene product as ACL could be demonstrated by heterologous expression in yeast. Both subunits are required for ACL activity. ACL activity was positively correlated with malate exudation in cluster roots of lupin and in primary roots of lupin and maize. A negative correlation was observed with citrate exudation in cluster roots. These results show that ACL is implicated in root exudation of organic acids and hence plays a novel role in addition to lipid synthesis.

Résumé

Pour venir à bout de la carence en phosphate, le lupin blanc produit des racines protéoïdes, qui excrètent de grandes quantités de malate et citrate. Les racines protéoïdes jeunes, en développement, excrètent principalement du malate, alors que les racines protéoïdes matures principalement du citrate. Les racines protéoïdes matures excrètent quatre à six fois plus de carboxylates comparées aux racines protéoïdes juvéniles. A l'aide d'une approche par cDNA-AFLP, nous avons identifié un gène codant une possible ATP-citrate lyase (ACL) induite dans les racines protéoïdes jeunes. Le clonage des gènes de l'ACL révèle qu'au contraire des animaux, l'ACL végétale est constituée de deux polypeptides. La fonction des produits des gènes comme ACL a pu être démontrée par leur expression hétérologue dans la levure. Les deux sous-unités sont requises pour l'activité ACL. L'activité ACL est positivement corrélée à l'exudation de malate dans les racines protéoïdes de lupin et dans les racines primaires de lupin et maïs. Une corrélation négative est observée avec l'exudation de citrate dans les racines protéoïdes. Ces résultats montrent que l'ACL est impliquée dans l'exudation racinaire des acides organiques et donc joue un nouveau rôle en plus de la synthèse lipidique.

Introduction

Phosphorus (P), is one of the most limiting nutrients in natural and agricultural ecosystems, due to the low concentrations of free inorganic phosphate (P_i) in the soil solution, which is the sole form of phosphorus absorbed by plant roots (Marschner, 1995). The strategies of most plants to overcome phosphate starvation are the synthesis of high affinity transporters which can absorb P_i even if the concentration in the soil solution is very low; modifications in root morphology and the association with mycorrhiza, mainly improving spatial acquisition of available phosphate. Further mechanisms for chemical P mobilization are i) the secretion of phosphohydrolases which liberate Pi by hydrolysis of organic P forms, ii) the acidification of the rhizosphere by enhanced net extrusion of protons for mobilization of acid-soluble P fractions in calcareous soils and iii) the exudation of carboxylates as organic chelators, mobilizing sparingly soluble Fe, Al and Ca phosphates by complexation of the metal cations. Rhizosphere acidification is a widespread response to phosphorus deficiency, particularly in dicotyledonous plants. In contrast, exudation of considerable amounts of carboxylates effective in phosphate solubilization seems to be restricted to a limited number of plant species (Marschner 1995; Neumann and Römheld 1999). White lupin and members of the Proteaceae are able to excrete far larger amounts of carboxylates than any other plant species investigated so far (Dinkelaker *et al.* 1997). Compared with rhizosphere acidification, this strategy has the advantage that phosphate is not only liberated from acid soluble P fractions, but also by anion exchange from Fe-Al-P complexes found in many acidic soils.

In these plant species, exudation of organic acid anions occurs at well-defined parts of roots, where short clustered lateral roots (proteoid roots), are formed. White lupin is the best-described system forming proteoid roots. Formation of proteoid roots follows a well-defined developmental pattern. Young, growing proteoid roots release mainly malate and only low amounts of citrate. Immature proteoid roots excrete similar amounts of citrate and malate. Mature proteoid roots excrete far higher amounts of carboxylates, mainly citrate and strongly acidify the rhizosphere. This is associated with a shift from malate to citrate accumulation in the root tissue with increasing age of the clusters (Neumann *et al.*, 1999; 2000). The high demand for carbon used for synthesis of carboxylates is sustained by the upregulation of several enzymes, i.e. PEPcarboxylase (Johnson *et al.*, 1996 a and b), sucrose synthase, phosphoglucomutase and fructokinase which are involved in the glycolytic pathway (Massonneau *et al.*, 2001).

While upregulation of these enzymatic activities can explain increased exudation of carboxylates from cluster roots in general, the reasons for the differential exudation of malate or citrate in different root zones (juvenile and mature clusters) still remains an open question. Using the AFLP technique we identified a large number of genes with putative differential expression in young and mature proteoid roots. Among these genes, we also identified a putative ATP-dependent citrate lyase (ACL). ACL (EC 4.1.3.8) catalyses the formation of acetyl-CoA and oxaloacetate from citrate and CoA with a concomitant hydrolysis of ATP. Due to its instability the study and cloning of the ACL has been delayed and it was only in 1990 that the first animal ACL has been cloned (Elshourbagy *et al.*, 1990). Several reports on the plant ACL have been published and its activity has been found to reside mainly in young growing parts and during seed maturation (Kaethner and Rees, 1985, Ratledge *et al.*, 1997). In developing seeds of *Brassica napus*, Ratledge *et al.* (1997) found that the most of the ACL activity was localized in chloroplasts and established a correlation between ACL activity and the rate of lipid synthesis. A role for ACL in lipid synthesis was therefore deduced. In other plants, ACL was exclusively localized in the cytosol and a role in terpenoid synthesis was postulated (Kaethner and Rees, 1985).

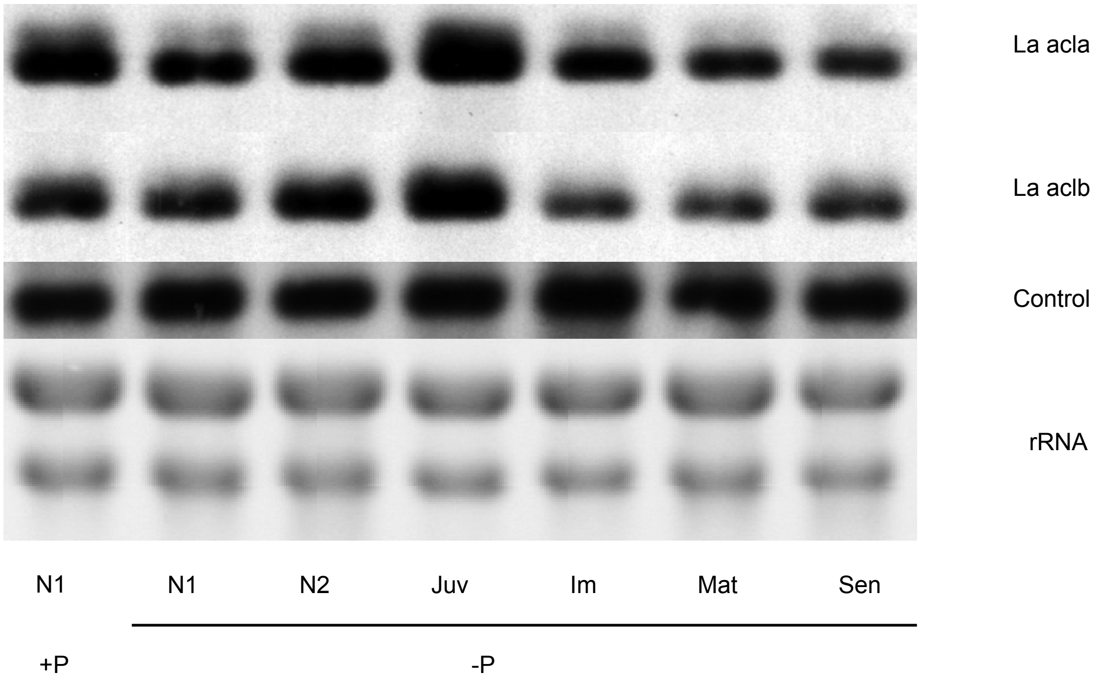
Despite the importance of the ACL, to our knowledge plant ACL has yet not been cloned. Molecular identification of ACL will allow to learn more on the role of the corresponding gene product in plant metabolism. Indeed, as shown in this report, cloning of lupin ACL revealed that in contrast to animals plant ACL is constituted by two different subunits and that in addition to its well-established role in lipid synthesis it may play a role in malate exudation in roots.

Results

One of our interests is to understand the mechanisms leading to the exudation of the huge amounts of carboxylates by mature proteoid roots and how the cellular metabolism changes during the conversion of young, growing proteoid roots to mature proteoid roots. In order to identify stage-specific expression of mRNA we performed a cDNA-AFLP analysis (Bachem *et al.* 1996) with juvenile, mature and senescent root clusters. In a former contribution we showed that several enzymes implicated in glycolysis, namely fructokinase, sucrose synthase and phosphoglucomutase were upregulated in juvenile and mature cluster roots (Massonneau *et al.* 2001). Among the genes identified to be differentially expressed, we also identified a putative ATP-dependent citrate lyase. ACL catalyses the reaction which forms acetyl-CoA and oxaloacetate from citrate, CoA and ATP.

The length of the putative ACL clone was 278 bp and exhibited 71% similarity with the rat ACL over a stretch of 92 amino acids. Transcript analysis using semi-quantitative RT-PCR confirmed that the sequence identified was indeed highly expressed in juvenile proteoid roots and downregulated in mature proteoid roots (see Figure 3).

Fig. 3: Transcript levels of the two subunits of ATP citrate lyase in different parts of lupin roots. Semi-quantitative RT-PCR was performed as described in materials and methods including a synthetic RNA as control. As a further control a RNA agarose gel was stained with ethidium bromide.



Since to our knowledge no plant ACL was cloned and functionally identified, we were interested to verify whether the AFLP fragment corresponds to the ACL gene. PCR based screening using primers deduced from the putative ACL of a lupin library made from juvenile proteoid roots, resulted in six independent clones, the longest being 2222 bp. Since this clone was nearly 2000 bases shorter than the full-length cDNA reported for rat, we performed a Nothern blot. The transcripts hybridizing with our probe showed a similar size for the mRNA as observed for the lupin cDNA (not shown). A comparison of the rat and lupin cDNAs with the *Arabidopsis* genome revealed that *Arabidopsis* contains three genes (Accession or gene numbers: Q9C522, At5g49460 and At3g06650) highly similar to our lupin cDNA exhibiting the same size. In addition our search revealed three putative *Arabidopsis* gene similar to the N-terminal part of the rat ACL (Accession or gene numbers: Q9SGY2, O22718 and O80526). ESTs from other plants corresponding to the N-terminal part of ACL are also present in the database. Using degenerated oligonucleotides corresponding to conserved parts of this putative subunit of ACL we screened the lupin library and identified several clones of approximately 1600 bp. An alignment of the lupin cDNA with the corresponding *Arabidopsis* genes shows that the deduced gene products are highly similar (89% identity for subunit B, corresponding to the N-terminal region of the animal protein, and 97 for subunits A which corresponds to the C-terminal region). The lupin gene exhibits also a high similarity to the rat ACL (45 and 54 % identity and 61 and 71% similarity, for subunit B and A, respectively). As in their animal counterparts we have identified within the subunit A the putative CoA binding domain and a conserved histidine, which is phosphorylated during the enzymatic reaction, two ATP-dependent citrate/succinate-CoA ligase signatures in this subunit and one in subunit B. Interestingly, both in fungi and bacteria a similar organization pattern as in plants can be observed (Kanao *et al.*, 2001, Nowrousian *et al.*, 2000). Compared to the animal ACL *Chlorobium limicola* and *Lupinus albus* miss a region of about 60 aminoacids located between subunit B and A in animal ACLs. In contrast, *Sordaria macrospora* has an N-terminal extension of subunit B which corresponds to the size of the linker region in animals. However, no homology between the animal sequence and that of *S. macrospora* can be observed.

Fig 1: Alignment of different ATP-citrate lyases. This graph is a manual combination of the alignment for each lupin subunit obtained with the PILE UP program (GCG Center) and edited using Genedoc (Version 2.6.001, Karl Nicholas). *Rattus norvegicus* ACLY (P16638), *Caenorhabditis elegans* ACLY (Z81028), *Lupinus albus* ACLB (AJ344108), *Sordaria macrospora* ACL2 (AJ243817), *Chlorobium limicola* ACLB (AB054669), *Lupinus albus* ACLA (AJ344107), *Sordaria macrospora* ACL1 (AJ224922) and *Chlorobium. limicola* ACLA (AB054670). i, ii and iii are the ATP-dependent citrate/succinate-CoA ligase signatures, * indicates the phosphorylation sites observed in animal ACLs, the squared histidine is the active site and Co-A BD the putative Coenzyme A binding site.

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R. norvegicus ACLY   : M AKAISEQTGKELLYKYICTT-----SAIQNR-FK--------YARVTPDTDWAHLLQD----HPWLLSQ-SL-VVKPD :   60
C. elegans ACLY     : MSAK VSELSGKEVLYKYFEPS-----GLLSAP-HA--------F-HVKAGENFDEIANK----YEWLARD- K VIKPD :   60
L. albus ACLB       : MARKKIREYDSKRLLKEHYKRL-----SGNDLP-IK--------SAQVTETTDFTELQEK----EPWL-S-SSKLVVKPD :   60
S. macrospora ACL2  : MSAKSILEADGKAILNYHLTRAPVIKPSTLPNP-TKHNPPPRLASLHFAEDADVNGVLSQAEVTYPWLLQDGARFVAKPD :   79
C. limicola ACLB    : M-AK-ILE--G--------------------PAMKLFNKWGIPVPNYVVIMDPDRLAQLGEA-NKWLRE--SKLVVKAH :   52
L. albus ACLA       :                                                                                  :    -
S. macrospora ACL1  :                                                                                  :    -
C. limicola ACLA    :                                                                                  :    -


R. norvegicus ACLY   : QLIKRRG LGL GVNLSLDGVKSWLKPRLGHEATVGKAKGFLKNFLIEPFVP SQAEEFYVCIYATREGDYVLFHHEGGV :  140
C. elegans ACLY     : QLIKRRGKLGLVKIGTPQE-LKAWFEKTGDSYVRVGQTEGRLHTFIVEPFCAHTEKDEMYIAIYSERFRDVIMFYEQGGV :  139
L. albus ACLB       : MLFGKRGKSGLVALNLDLAQVASFVKERLGNEVEMSGCKGPITTFIVEPFIPHN--EEFYLNIVSDRLGNSISFSECGGI :  138
S. macrospora ACL2  : QLIKRRG SGLLALNKTWPEAKAWIAERAGKAQKVEHTEGVLRQFLVEPFVP PQETEYYIN------GDWILFYHEGGV :  153
C. limicola ACLB    : EAIGGRFKLGLVKIGLNLEQAIEAAKEMLG--AKVGTAE--IGQVIIAEMLEHDA--EFYVSIIGNKDGAELLISKHGGV :  126
L. albus ACLA       :                                                                                  :    -
S. macrospora ACL1  :                                                                                  :    -
C. limicola ACLA    :                                                                                  :    -


R. norvegicus ACLY   : DVGDVDTKAQKLLVGVDEKLNA---EDIKRHLLVHAPEDKKEILASFISGLFNFYEDLYFTYLEINPLVVTKD------G :  211
C. elegans ACLY     : DIGDVEEKARTVSVPVQLNENAMTPSDEELTTLL-GPLKDSDIVRRFVVELYKAYKDLHFTYLEINPFVLLNN------Q :  212
L. albus ACLB       : EIEENWDKVKTVFVPTGVSLT----SEIIAPLVATLPLEIKGEIEEFLKVIFNLFQDLDFTFLEMNPFTLV------DGK :  208
S. macrospora ACL2  : DVGDVDAKAEKILIPVDLS-QYPSNEELASTLLKHVPKGIHNVLVDFIARLYAVYVDCQFTYLEINPLVVIPNEDATSAE :  232
C. limicola ACLB    : DIEDNWDSVRRIQIELDENPTIEQLTELAKD--AGFEGEIAERVGKICSRLILCFDNEDAQSIEINPLVIRKSDMRFAA- :  203
L. albus ACLA       :                                                                                  :    -
S. macrospora ACL1  :                                                                                  :    -
C. limicola ACLA    :                                                                                  :    -


R. norvegicus ACLY   : VYILDLAAKVDATADYICKVKW-------------------------GDIEFPPPFGREAYPEEAYIADLDAKSGASLKL :  266
C. elegans ACLY     : IHVLDLAARLDETANFLCADKWKSRLTPYG---------------GPNHVEFPAPFGRDLTSEEQYISEMDAKTGASLKL :  277
L. albus ACLB       : PYPLDMRGELDDTAAFKNFKKW-------------------------GNIEFPLPFGRVMSPTESFIHGLDEKTSASLKF :  263
S. macrospora ACL2  : VHFLDLAAKLDQTADFECGNKWAIARSPAALGIVAQSSNGGVNIDAGPPIEFPAPFGRELTKEEAYIAELDAKTGASLKL :  312
C. limicola ACLB    : ---LDAVMNVDYDARFRHAD-WDFKPV---------------------SE----IGRPFTEAEQQIMEIDSRIKGSVKF :  253
L. albus ACLA       :                                                                                  :    -
S. macrospora ACL1  :                                                                                  :    -
C. limicola ACLA    :                                                                                  :    -

                                  iii
                          _________________
R. norvegicus ACLY   : TLLNPKGRIWTMVAGGGASVVYSDTICDLGGVNELANYGEYSGAPSEQQTYDYAKTILSLMTR-EKHPDG--KILIIGGS :  343
C. elegans ACLY     : TILNRKGRVWTMVAGGGASVVFTDTVCDLGGASELANYGEYSGDPSESQTYEYAKTLLSVMTEGTPRPDG--KVLIIGGS :  355
L. albus ACLB       : TVLNPKGRIWTMVAGGGASVIYADTVGDLGFANELGNYAEYSGAPKEDEVLQYARVVIDCAT---ANPDGQKRALVVGGG :  340
S. macrospora ACL2  : TVLNPNGRIWTLVAGGGASVVYADAIASAGFADELA YGEYSGAPTESQTYHYARTVLDLMLRAPVSEQG--KVLFIGGG :  390
C. limicola ACLB    : VEV-PGGEIALLTAGGGASVFYADAVVARGGT--IANYAEYSGDPADWAVEALTETICRLP---------NIKHIIVGGA :  321
L. albus ACLA       :                                                                                  :    -
S. macrospora ACL1  :                                                                                  :    -
C. limicola ACLA    :                                                                                  :    -


R. norvegicus ACLY   : IANFTNVAATFKGIVRAIRDYQGSLKEHEVTIFVRRGGPNYQEGLRVMGEVGKTTGIPIHVFGTETHMTAIVGMAWA--- :  420
C. elegans ACLY     : IANFTNVAKTFGGIVRAFETFVSKLKEHKVTIFVRRGGPNYQEGLRRIKDAATKLELPIHVFGPETHMTAIVGAALGVKP :  435
L. albus ACLB       : IANFTDVAATFSGIIRALKEKESKLKAARMHIYVRRGGPNYQKGLAKMRELGEEIGIPIEVYGPEATMTGICKEAIQCIT :  420
S. macrospora ACL2  : IANFTNVASTFKGVIKALREYGKALIEHNTQIWVRRAGPNYQEGLKNLKAATQELGLNAKIFGPEMHVSGIVPLALVPGK :  470
C. limicola ACLB    : IANFTDVKATFSGIINGFRESKSKGYLEGVKIWVRRGGPNEAQGLAAIKQLQEE-GFDIHVYDRSMPMTDIVDLAMKS   :  398
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L. albus ACLA        :                                                                                  :    -
S. macrospora ACL1   :                                                                                  :    -
C. limicola ACLA     :                                                                                  :    -


R. norvegicus ACLY   : -PAIPNQPPTAAHT--ANFLLNASGSTSTPAPSRTASFSESRADEVAPAKKAKPAMPQD-SVPSPRSLQGKS-ATLFSRH :  495
C. elegans ACLY      : MPTVPTAPQT---TGQFLL-----------SPER-----NTGGTERAPPSPAANATPTEHPLTTAQQNKLKSFRGLFEDD :  496
L. albus ACLB        : ASA                                                                              :  423
S. macrospora ACL2   : WEESGAVEFQA                                                                      :  481
C. limicola ACLB     :                                                                                  :    -
L. albus ACLA        :                                                             -------   MATGQLFSRT :   10
S. macrospora ACL1   :        MPSATSTNGANGNGNGNGASASPAPGNLSANDNIR-------RFAAPSRPLS-PLP--------A-HALFNEK :   56
C. limicola ACLA     :                                                                         MSILANKD :    8


R. norvegicus ACLY   : TKAIVWG--MQTRAVQGMLDFDYVCSRDEPSVAAMVYPFTGDHKQKFYWGHKEILIPVFKNMADAMKKHPEVDVLINFAS :  573
C. elegans ACLY      : TKAIIWG--QQAKAIQGMLDFDYVCRRSSPSVVASTYPFTGDNKQKYYFGQKEILIPAYKSMAKAFATHPDASIMVTFAS :  574
L. albus ACLB        :                                                                                  :    -
S. macrospora ACL2   :                                                                                  :    -
C. limicola ACLB     :                                                                                  :    -
L. albus ACLA        : TQALFYN--YKQLPVQRMLDFDFLCGRETPSVAGIINP-GSEGFQKLFFGQEEIAIPVHATIEAACARHPTADVFINFAS :   87
S. macrospora ACL1   : TRCFVYG--LQPRAVQGMLDFDFICKRSTPSVAGIIYTFGGQFVSKMYWGTSETLLPVYQEVQKAIAKHPDVDVVVNFAS :  134
C. limicola ACLA     : TRAVIIGGVAGVNAAKRMAQFDFLVNRPL-TVQAFVYPPEAGQQKEIFRGGELKNVTVYDSLAPALEEHPDINTALI L  :   87


R. norvegicus ACLY   : LRSAYDSTMETMNYAQIRTIAIIAEGIPEALTRKLIKKADQKGVTIIGPATVGGIKPGCFKIGNTGGMLDNILASKLYRP :  653
C. elegans ACLY      : MRSVFETVLEALEFPQIKVIAIIAEGVPENQTRKLLKIAHDRGVTLVGPATVGGIKPGCFKIGNTGGMMDNILASKLYRP :  654
L. albus ACLB        :                                                                                  :    -
S. macrospora ACL2   :                                                                                  :    -
C. limicola ACLB     :                                                                                  :    -
L. albus ACLA        : FRSAAASSMAALKQSTIRVVAIIAEGVPESDTKQLIAY RSNNKVVIGPATVGGIQAGAFKIGDTAGTIDNIIQCKLYRP :  167
S. macrospora ACL1   : SRSVYSSTMELMEHPQIKTIAIIAEGVPERRAREIAYVAKKKGITIIGPATVGGIKPGCFKIGNTG MMDNIVASKLYRK :  214
C. limicola ACLA     : ASRAAQAAKEALESPNIQLVSMITEGVPEKDAKRLKKLAQKLGKMLNGPSSIGIMSAGECRLGVIGGEFKNLKLCNLYRQ :  167

                                            i
                                                                                *
R. norvegicus ACLY   : GSVAYVSRSGGMSNELNNIISRTTDGVYEGVAIGGDRYPGSTFMDH LR QDTPGVKMIVVLGEIGGTEEYKICRGIKEG :  733
C. elegans ACLY      : GSVAYVSRSGGMSNELNNIISQNTNGVYEGIAIGGDRYPGSTYTDHVIRYQNDDRVKMIVLLGEVGGVEEYKIVDLLKQK :  734
L. albus ACLB        :                                                                                  :    -
S. macrospora ACL2   :                                                                                  :    -

L. albus ACLA
S. macrospora ACL1   : GSVGYVSKSGGMSNELNNIISQTTDGVYEGVAIGGDRYPGTTFIDHLLRYQADPACKILVLLGEVGGVEEYKVIEAVKQG :  294
C. limicola ACLA     : GSFGVLTKSGGLSNEAMWLCAQNGDGITSAVAIGGDAYPGTDFVTYLEMFEKDPATKAVVMIGEVGGNLEEEAAEWLAAE :  247

                                         ii                         CoA BD
R. norvegicus ACLY
C. elegans ACLY
L. albus ACLB        :                                                                                  :    -
S. macrospora ACL2   :                                                                                  :    -
C. limicola ACLB
L. albus ACLA
S. macrospora ACL1   : IITKPIVAWAIGTCASMFKTEVQFGHAGAFANSQLE-TAATKNKSMREAGFYVPDTFEDMPALLKQVYDKLVADGTI-VP :  372
C. limicola ACLA     : PRRIKLIAAIGGTCQEVLPQGMKFGHAGAKEGKKGAGSARSKMNALRDAGAYVPDTFGGLSKEIKKVYEELIAAGEISTE :  327

R. norvegicus ACLY   : AQEVPPPTVPMDYSWARELGLIRKPASFMTSICDERGQELIYAGMPITEVFKEEMGIGGVLGLLWFQRRLPKYSCQFIEM :  891
C. elegans ACLY      : QPEVPPPAVPMDYAWARELGLIRKPASFMTSICDERGEELNYAGVPITKVLESDMGIGGVLGLLWFQKRLPPHANKFIEI :  892
L. albus ACLB        :                                                                                  :    -
S. macrospora ACL2   :                                                                                  :    -
C. limicola ACLB     :                                                                                  :    -
L. albus ACLA        : VKEFTPPPIPEDLNTAIKSGKVRAPTHIISTISDDRGEEPCYAGVPM SIIEKGFGVGDVISLLWFKRSLPRYCTQFIEI :  405
S. macrospora ACL1   : APEPVVPKIPIDYSWAQELGLIRKPAAFISTISDDRGQELLYAGMPISDVFREEIGIGGVMSLLWFRRRLPDYAAKFLEM :  452
C. limicola ACLA     : IDEAVLPELPPRVQEVMKQGEVIVEPLIRTTISDDRGEEPRYAGYAASELCSKGYGIEDVIGLLWNKKLPTREESEIIKR :  407


R. norvegicus ACLY
C. elegans ACLY      : CLMVTADHGPAVSGAHNTIICARAGKDLVSSLTSGLLTIGDRFGGALDAAAKMFSKAF-DSGIIPMEFVNKMKKEGKLIM :  970
L. albus ACLB        : CLMLTADHGPAVSGAHNTIVCARAGKDLISSLTSGLLTIGDRFGGALDGAARQFSEAF-DQGWSANQFVSEMRKKGKHIM :  971
S. macrospora ACL2   :                                                                                  :    -
C. limicola ACLB     :                                                                                  :    -
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L. albus ACLA       :                                                                                  :    -
S. macrospora ACL1  : CIMLCADHGPCVSGAHNTIVTARAGKDLVSSLVSGLLTIGPRFGGAIDDAARYFKDAR-DRSLTPYEFVESMKKKGIRVP :  484
C. limicola ACLA    : VLMLTADHGPAVSGAMNTIITTRAGKDLISSLVAGLLTIGSRFGGALDGAAEEFTKAF-DKGLSPREFVDTMRKQNKLIP :  531
                    : IVMISADHGPAVSGAFGSILAACAGIDMPQAVSAGMTMIGPRFGGAVTNAGKYFKMAVEDYPNDIPGFLSWMKKNVGPVP :  487


R. norvegicus ACLY  : GIGHRVKSINNPDMRVQILKDFV--KQHF-PATPLLDYALEVEKITTSKKPNLILNVDGFIGVAFVDMLRNCGSFTREEA : 1047
C. elegans ACLY     : GIGHRVKSINNPDKRVEILKRFAMDKKEFAQETPLFEYALEVEKITTAKKPNLILNVDGAIAILFVDILRHSGMFTKQEA : 1051
L. albus ACLB       :                                                                                  :    -
S. macrospora ACL2  :                                                                                  :    -
C. limicola ACLB    :                                                                                  :    -
L. albus ACLA       : GIGHRIKNRDNKDKRVELLQKFA--RTHF-PSVKYMEYAVEVETYTLTKANNLVLNVDGAIGSLFLDLLAGSGMFTKQEI :  561
S. macrospora ACL1  : GIGHRVKSRNNPDLRVELVKEYV--KAKF-PSSKLLDYALAVETVTTSKKDNLILNVDGCIAVCFVDLLRNCGAFSTEEA :  608
C. limicola ACLA    : GIGHRVKSVKNPDQRVKYLVSYIKNETSLH--TPCLDYALEVEKVTTAKKGNLILNVDGTIGCILMDL------------ :  553


R. norvegicus ACLY  : DEYVDIGALNGVFVLGRSMGFIGHYLDQKRLKQGLYRHPWDDISY-VLPEHMSM                           : 1100
C. elegans ACLY     : EETIEIGSLNGLFVLGRSIGFIGHYLDQSRLKQGLYRHPWDDISY-IMPESNLVKF                         : 1106
L. albus ACLB       :                                                                                  :    -
S. macrospora ACL2  :                                                                                  :    -
C. limicola ACLB    :                                                                                  :    -
L. albus ACLA       : DEIVEIGYLNGLFVLARSIGLIGHTFDQKRLKQPLYRHPWEDVLY-TK                                 :  608
S. macrospora ACL1  : EDYLSMGVLNGLFVLGRSIGLIAHYLDQKRLRTGLYRHPWDDITY-LLPSLQQPGPPGTEGRVEVQI              :  674
C. limicola ACLA    : --DFPVHSLNGFFVLARTIGMIGHWIDQNNQNSRLIRLYDYLINYAVKPEQEVPEKK                        :  608
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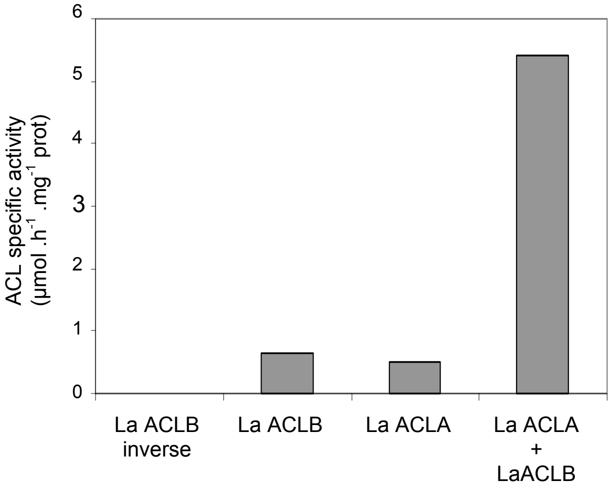
In order to prove that the two genes identified indeed code for the plant ACL, we performed expression studies. In a first attempt we expressed the two subunits in *E. coli*. On a SDS-PAGE gel we observed high levels of the corresponding proteins, however, no activity could be detected, most probably due to the fact that the ACL subunits were only present in inclusion bodies and hence insoluble. Therefore we decided to use yeast as expression system. The genome of *Saccharomyces cerevisae* does not contain genes corresponding to the ACL and in fact no ACL activity could be detected in wild type yeasts (Fig. 2). In contrast, when the two ACL subunits were expressed in yeast under the control of a constitutive promoter, ACL activity could be detected. Expression of the N- or C-terminal subunit alone resulted in negligible ACL activities, suggesting that both subunits are required for ACL activity.

Fig. 2: ATP citrate lyase activities measured in yeasts expressing the different lupin ACL subunits. The experiment has been repeated four times, giving always the same pattern. A typical result is presented. Since unspecific NADH oxidation occurred and lead to negative values when both subunits were not expressed together, results are corrected taking the values obtained for yeasts containing the *La aclb* antisense construct as reference.



After the demonstration that the heterologously expressed clones code for the ACL, we were interested to correlate the expression of ACL with the enzymatic activity of its gene product and carboxylate exudation in the different root types. Both subunits showed a very similar expression pattern, exhibiting high transcript levels in young, growing parts of roots (Figure 3). The ACL activity paralleled the expression pattern of both subunits in the different root types (Figure 4 A). ACL activity was highest in juvenile proteoid roots and decreased in immature, mature and senescent proteoid roots. Extracts of N1 and N2 roots exhibited ACL activities comparable to those of immature proteoid roots. Based on protein levels, young leaves showed a similar activity as N1 and N2 roots, while the ACL activity of adult leaves was in the same order of magnitude as senescent protein roots. Comparing the different stages of proteoid roots we observed that similar amounts of malate were excreted in juvenile, immature and mature proteoid roots (Figure 4B). However, the ratio of excreted malate to citrate was by far the highest in juvenile roots which exhibit the highest ACL activity (Figure 4C). This suggests a positive correlation between ACL activity and malate exudation and a negative between ACL activity and citrate exudation.

Malate exudation by roots has been reported for a large number of plants. In order to analyse whether a correlation between ACL activity and malate exudation also exists in non-proteoid roots, we compared these parameters in primary roots of lupin and maize. The first 30 mm of the roots were cut in four sections, 2 apical sections of 5 mm and the 2 next sections of 10 mm. In lupin as well as in maize the largest amounts of malate are excreted in the 5 to 10 mm section. This corresponds also to the part of the root exhibiting the highest ACL activity and has been shown to be an actively growing zone under similar culture condition in maize (Peters and Felle, 1999). Root sections behind the 5 to 10 mm section excrete reduced amounts of malate and exhibit lower ACL activities. This result confirms the correlation observed between ACL activity and malate exudation in the different parts of lupin proteoid roots.

Fig. 4: A: ATP citrate lyase in vitro activities measured in leaves and in -P and +P roots of white lupin. The experiments were repeated at least 4 times, and the distribution pattern of enzymatic activities obtained in different root zones was highly reproducible. Due to the variations of the absolute enzyme activities, one single, typical experiment is shown. Measurements were performed in triplicate, SD are not given since too small to be visualized.
B and C: Malate and citrate exudation results from a previous work (Massonneau *et al.*, 2001) are shown to visualize the correlation.

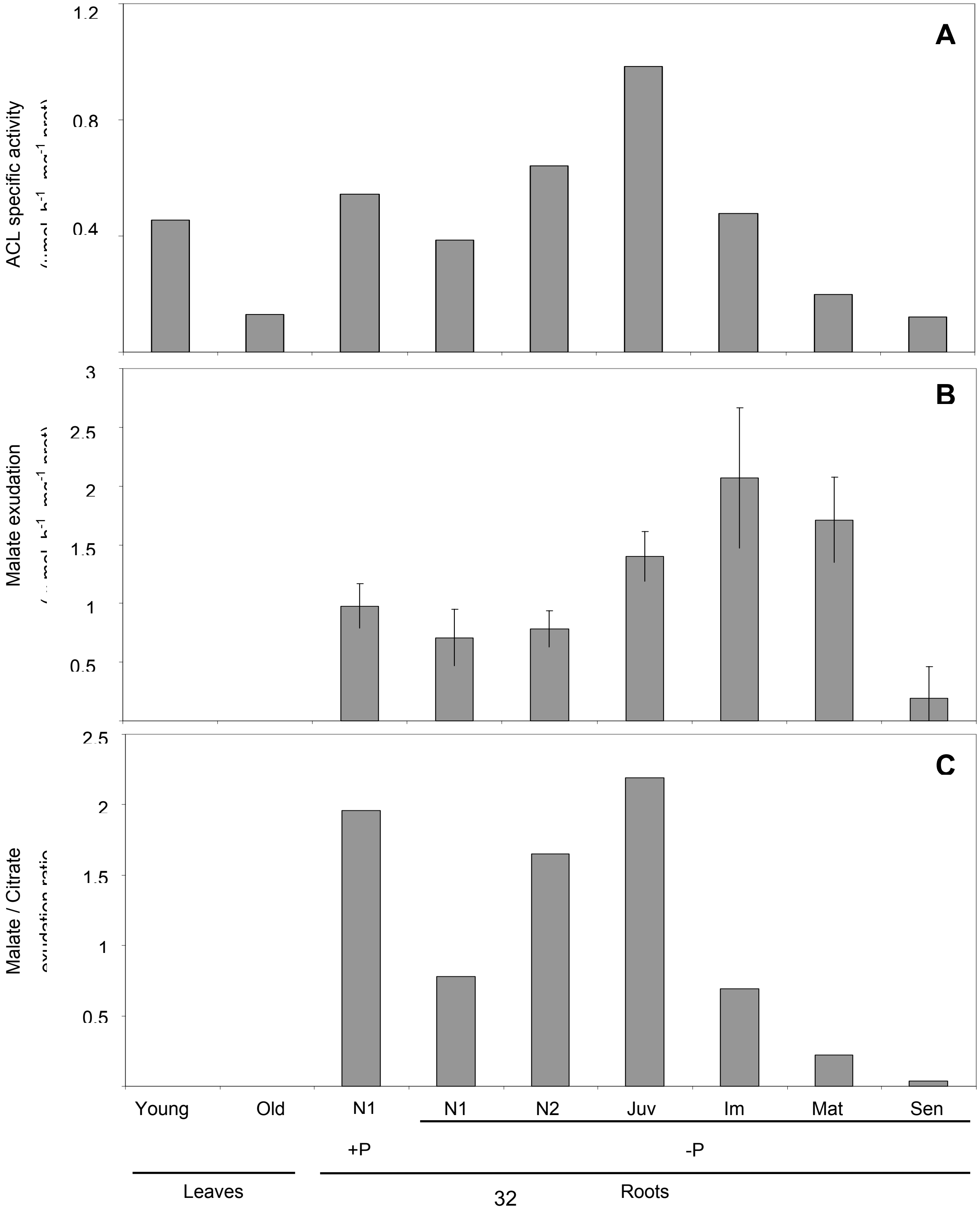
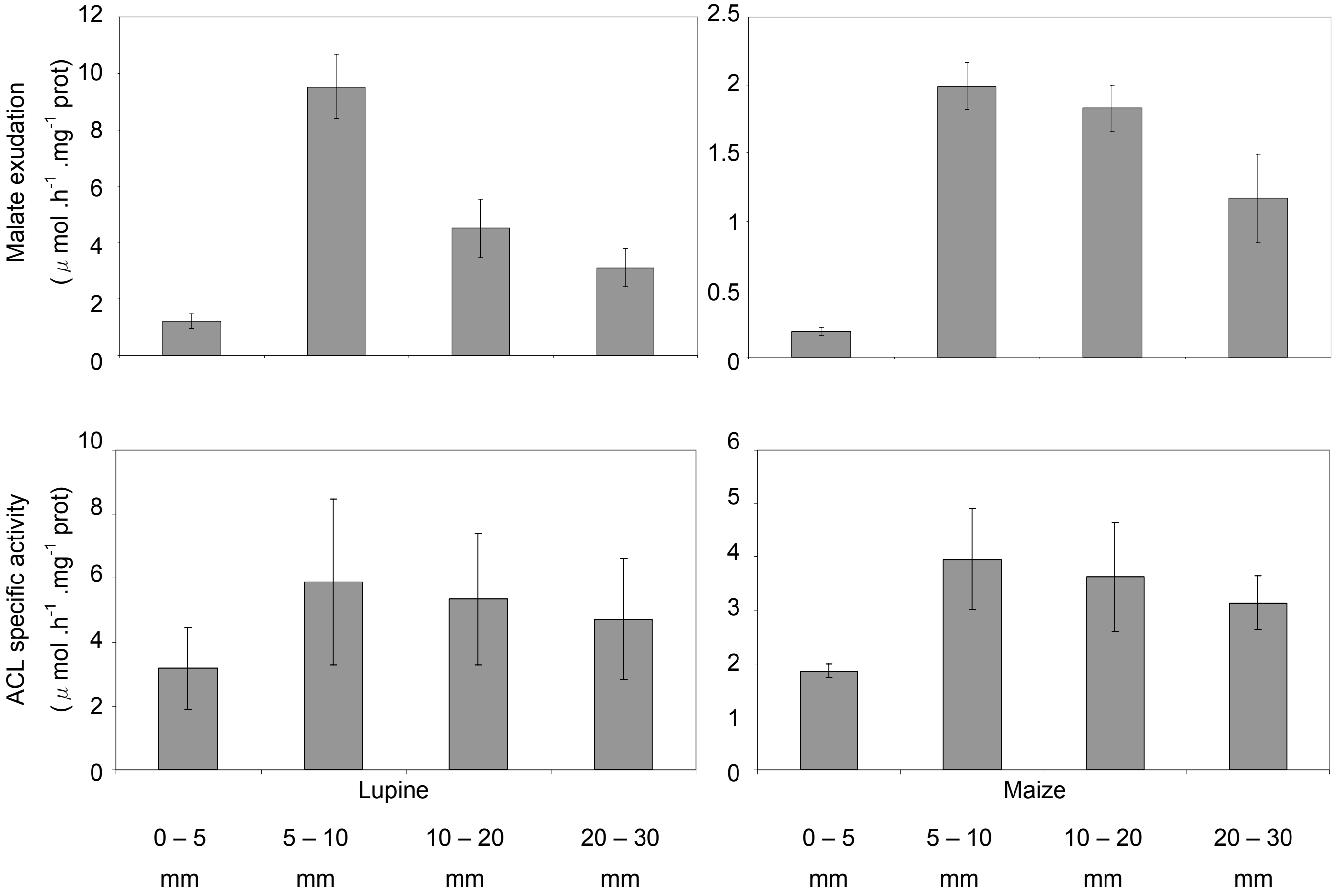


Fig. 5: Malate exudation and ATP citrate lyase activities in different root zones of maize and white lupin. Root zones are indicated using the primary root apex as origin. Data represent the mean ± SD of 3 (lupin) or 4 (maize) independent experiments, each with three replicates.



The correlation between ACL activity and malate exudation is also demonstrated in Figure 6 where all the results presented in this work are plotted. Due to the unequal distribution of malate exudation values (values range between 0 and 10 µmol .h^{-1} .mg^{-1} protein but more than 93% are situated between 0 and 5), it was necessary to transform them (via logarithm) to proceed a regression analysis. The calculation results in a highly significant regression coefficient (R=0.851) and p-value (0.0001 calculated on 15-2 values). The statistical approach demonstrates a very good correlation between ACL activity and malate exudation under the conditions used and in the range of values of ACL activity and malate exudation measured. It can therefore be concluded that increased ACL activity results in higher malate exudation.

Discussion

Root exudation of organic acids plays an important role in a large number of processes, such as deficiency of mineral nutrients, particularly phosphate, exposure to toxic metals, such as aluminium or lead and to hypoxia (Neumann and Römheld, 1999; Ryan and Delhaize, 2001). In addition, organic acid exudation can also selectively stimulate microbial activities in the rhizosphere with impact on the availability of nutrients. At least in some cases, exudation of carboxylates implicates the activation of a carboxylate channel (Kollmeier *et al.*, 2001) and changes in cellular metabolism. In lupin, proteoid roots formed during phosphate starvation exhibit increased PEP carboxylase and citrate synthase activity and concomitant decreased aconitase activity (Neumann *et al.*, 1999). In addition, increased activity of glycolytic enzymes suggests a high demand for carbohydrates for biosynthesis of carboxylates (Massonneau *et al.* 2001). During the development of proteoid roots the pattern of exuded organic acids changes. In young, developing proteoid roots malate is the major carboxylic acid excreted. In contrast, mature proteoid roots excrete mainly citrate, which is more efficient in desorbing phosphate from Al-Fe-P complexes. In a cDNA-AFLP approach we have identified a cDNA coding for a putative ACL highly expressed in juvenile proteoid roots. Since the products of the ACL reaction are acetyl-CoA and oxaloacetate, which is readily reduced to malate we have investigated the role of ACL in organic acid exudation.

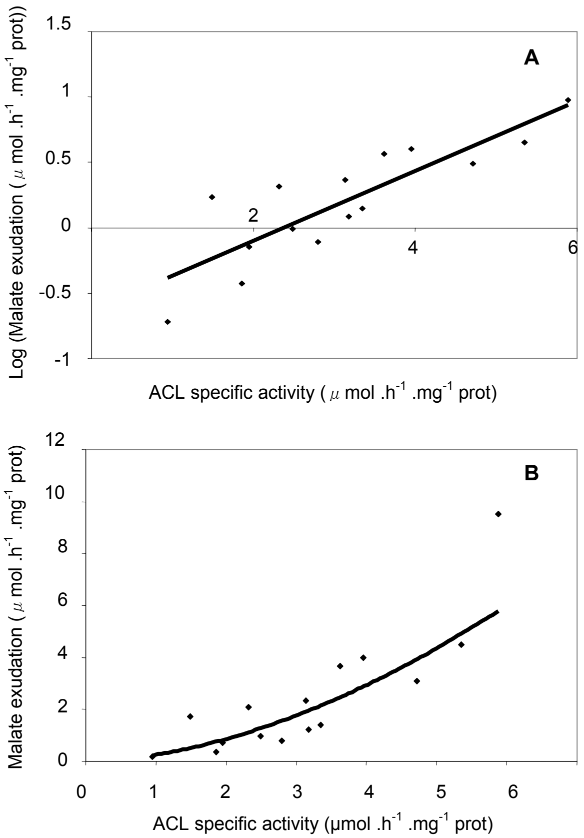
Cloning of the full-length cDNA coding for the putative ACL revealed that in plants the ACL was not constituted by a single but by two different polypeptides corresponding to the N- and C-terminus of the animal ACL. A similar structure of the ACL has been postulated for fungi. However, the functional activity of these two subunits still remains to be proven. A very recent publication demonstrated that *C. limicola* exhibit a similar structure and that both subunits are required for ACL function. Our results demonstrate that this is also true for plants. The ACL structure observed in mammals is also present in *Caenorhabditis elegans* or *Drosophila*.

ACL activity measurements paralleled the expression pattern observed for both genes coding for the ACL, indicating that the transcript levels reflect the activity of the enzyme and that the enzyme is probably regulated at the transcriptional level. The fact that both subunits follow the same expression pattern implies a common regulation. However, the presence of two genes coding for the ACL, may indicate that in plants additional regulatory mechanisms may be involved compared to animals.

ACL activity has been extensively studied in animals and it was shown to be a key step for lipid biosynthesis providing acetyl-CoA. A similar function has been postulated in rape seeds (Ratledge *et al.*, 1997). In this case, ACL activity was localized in plastids, and the activity was correlated with lipid accumulation. In contrast, Kaethner and Rees (1985) localized pea ACL in the cytosol. A hint that ACL may be localized differentially according to the plant species was given by Rangasamy and Ratledge (2001), who found ACL activity predominantly in chloroplasts in rape and spinach, whereas in pea and tobacco, distribution was mainly cytosolic. The sequences of the lupin and *Arabidopsis* genes have no obvious transit peptide (search with PSORT, version 6.4) indicating that at least in these plants ACL is localized in the cytosol. As membranes are considered to be impermeable to acetyl-CoA, it has to be used at its production place in the cytosol, e.g. for synthesis of cytosolic terpenoids such as sterols or synthesis of malonylCoA used for flavonoid or ACC production, or transported to another site either via an acetyl-CoA translocator. An acetyl-CoA translocator has already been cloned in animals (Bora, *et al.*, 1999, Kanamori *et al.*, 1997). However, an acetyl-CoA transporter has not been identified so far in plants.

In lupin and maize, highest ACL activities were found in growing tissues such as young leaves and elongation zones of roots. In maize, the root elongation rate is maximal 6 days after germination and the elongation zone was identified between 3 and 8 mm from the apex (Muller *et al.*, 1998) The correlation of the highest ACL activities with the elongation zone suggest that ACL may be involved in elongation by sustaining lipid biosynthesis. Investigations of ACL function have focused on the acetyl-CoA production and lipid biosynthesis. In contrast, little attention has been paid to the second compound produced during this reaction, oxaloacetate, which is readily reduced to malate.

Fig. 6: Compilation of ATP citrate lyase activities and malate exudations measured in maize and lupin roots. Data are means of 3 or 4 independent replicates. A: The curve $y=0.2543x^{1.7646}$ fits the values ($r^2=0.729$ and p-value=0.0001). B: The regression analysis is conducted on transformed data ($y=0.268x - 0.639$, $r^2=0.723$ and p-value=0.0001)



This is probably due to the fact that in animals the role of malate is not as complex as in plants (Martinoia and Rentsch, 1994). In lupin proteoid roots PEPCase is also highly expressed and thus constitutes a second pathway to produce malate. Part of malate will be used for respiration. Excess of malate can be stored within the vacuole (Martinoia and Rentsch, 1994). This strategy has been demonstrated mainly for leaves. In roots, both vacuolar storage as well as exudation has often been observed (Ryan and Delhaize, 2001). Recalculation of the measurements performed in the present work, revealed a high correlation between ACL activity and malate exudation (Fig. 6). The blot shows two phases: at low ACL activities increase of excreted malate is slow, at higher ACL activities increased ACL activity results in a strongly increased malate exudation. This observation can be explained by the fact, at low ACL activities a major part of malate synthesized is used for respiration whereas at high ACL activities malate is synthesized in excess and a minor part is used for the Krebs cycle. Since in young roots the storage capacity is limited, part of malate will be excreted.

A correlation could also be found between the ACL activity and the ratio of malate to citrate exudation. In mature proteoid roots the ACL activity is decreased, citrate removed from the Krebs cycle is no more readily converted to malate. This may be explained by the extremely low P and energy status of mature clusters (Neumann *et al.*, 1999 and 2000; Masssonneau *et al.*, 2001) limiting the activity of ACL. The cell produces amounts of citrate, which cannot be used in metabolic pathways and are therefore forced to excrete citrate to maintain cellular homeostasis. This is reflected by a shift from preferential malate accumulation in juvenile clusters to almost exclusive citrate accumulation in mature and senescent clusters (Neumann *et al.*, 1999 and 2000). ACL activity, which links malate and citrate, is therefore directly responsible for the switch in the organic acid excreted (malate in juvenile proteoid roots, citrate in mature proteoid roots). Therefore, our observations strongly suggest that ACL is responsible for the preferential malate exudation and is a key enzyme determining which organic acid is excreted in proteoid roots

This may have important consequences from the ecological point of view. Consulting the complex formation constants with metals or mobilization of rock phosphate shows that citrate is much more efficient than malate (Ryan and Delhaize, 2001). Exudation of citrate instead of malate may, therefore, improve P acquisition and the ability to cope with adverse soil chemical conditions such as Al toxicity. However, a mechanism of detoxification to avoid over-accumulation of organic acids in the root tissue may be regarded as original function of intense root exudation of malate and citrate.

Material and methods

Plant material

Growth of white lupin (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, Rastatt, Germany) in presence (+P) or absence (-P) of P source has been previously described (Massonneau *et al.*, 2001). Maize (*Zea mais* L. cv. Delprim; Delley Semences et Plantes SA, Yverdon, Switzerland) was pregerminated for four days and grown hydroponically in complete (+P) medium. Plants were grown at 22°C and 65% relative humudity with a light period of 16 h at 200 µmol m^{-2} s^{-1}.

Harvest of different root parts

The different stages of proteoid roots were harvested as described by Massonneau *et al.* (2001). Normal roots were separated in the apex (1 cm) of slow-growing primary roots (N1) and the apex (1cm) of fast growing secondary roots holding clusters (N2). In order to differentiate the developmental stages of root clusters the root system was immersed in a pH-indicator solution, which indicates acidification in mature cluster regions (Neumann *et al.*, 1999).

Collection and analysis of exudates

Excised roots were rinsed in water and incubated for 1 h in water containing penicillin (Massonneau *et al.*, 2001). Malate contents were determined using the Malate Test Kit (Boehringer, Mannheim, Germany).

Heterologous expression of the ACL in yeast

Saccharomyces cerevisiae W303 (MAT-a ade 2-1 can 1-100 his 3-11 leu 2-3 trp 1-1 ura 3-1) were grown on appropriate selective media and transformed via the Li-acetate protocol (Lundblad, 1997). *La ACLA* and *La ACLB* full length cDNAs in pBluescript were excised with XhoI and NotI and ligated in the pNEV vectors (Sauer N. et Scholtz J., 1994) containing Ura respectively Leu as markers. This system allowed the constitutive expression by the pma-1 promoter (Villalba *et al.*, 1992). Transformed yeast cells were grown to OD 4 to 5 in selective media and subsequently transferred to YPD medium for 1 h 30 before preparing spheroplasts (Lundblad, 1997).

Extraction of soluble proteins and ACL assay

Frozen plant tissues were ground in liquid nitrogen and homogenized with 3 vol.

of extraction buffer (0.1 M Hepes-KOH pH 7.5, 5 mM $MgCl_2$, 2.5 mM DTT, 3 mM Na-DEDTC (diethyldithiocarbamate), 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF, 3 % PVPP K30).

Yeast spheroplasts were lysed osmotically and gently homogenized at 4°C in 1.5 vol. of extraction buffer (complemented with Na-Citrate to a final concentration of 5 mM) using a syringe and needle. After centrifugation (25 min, 12 000 g, 4°C) the supernatant was rapidly used to determine the ACL activities and protein concentrations (DC Protein Assay kit; Bio-Rad).

ACL (EC 4.1.3.8) activity was determined spectrophotometrically at room temperature, using the malate dehydrogenase coupled assay. The assay mixture contained 0.2 M Tris pH 8.4, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 mM Na_3-Citrate, 0.2 mM Coenzyme A, 10 mM ATP (omitted in blanks), 0.2 mM NADH, 0.4 U .ml^{-1} malate dehydrogenase. Values were taken after 30 min and 10 min for plants and yeast respectively. These time points were chosen since prolonged incubation resulted in the inactivation of the enzyme. Blanks were performed by omitting ATP or Coenzyme A resulting in similar values.

cDNA-AFLP

The RNAs from juvenile, mature and senescent cluster roots were compared by cDNA-AFLP technique (Bachem *et al.* 1996). The procedure used in this work is described by Massonneau *et al.* (2001).

Cluster root library construction and screening

The library was constructed from mRNA isolated from juvenile cluster roots. cDNAs synthesis and cloning were conducted using the ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, Amsterdam, The Netherlands, catalog #200400 and catalog # 200450). The screening of *La ACLA* was performed using a PCR approach. Primers were deduced from the sequence of the cDNA-AFLP clone showing homology with the C-terminal part of the rat ACL. Primers 5'GTGCCATTGATGATGCTGCT3' and 5'TGTTTGCCTTCGTGAGAGTG3' amplified a 249-bp fragment. Six independent clones have been completely sequenced and showed approximately the same size, the longest being 2222-bp.

To screen *La ACLB*, degenerated primers have been designed from consensus regions between cotton, alfalfa, soybean, tomato and *A. thaliana*. 5'T GAG YTA GTA RAS ARR GAR CCN TGG3' and 5'ACC ICC ICC NGC NAC CAT NGT CCA3' amplified a fragment from which specific primers have been deduced to screen the library. Six independent clones have been isolated and completely sequenced. They showed approximately the same size, the longest was 1618-bp.

RT-PCR

10^6 copies of pAW109 mRNA (Perkin Elmer, Applied Biosystems, Foster

City, USA) was mixed with 1 µg of DNA-free total RNA and RT-PCR was performed as described by Massonneau *et al.* (2001) using (alpha)^{33}P-labelled dATP. 1/100 of the RT reaction product was used to perform PCR. Two sets of primers were used in the same tube, one specific for pAW (AW112 and AW113, amplifying a 301-bp fragment) and one for *La ACLA* (5’GCTGGAGCTAAGAGTGGTGG3’ and 5’CTGTCACGAGCATCCTTGAA3’ amplifying a 600-bp fragment) or *La ACLB* (5’CGGGTCCATAAACCTCAATG3’ and 5’TGGTCGATGGAAAGCCTTAT3’ amplifying a 603-bp fragment). The annealing temperature was 60°C. After 28 cycles, when strict proportionality between cycle number and amplification was observed for all tissues studied, the PCR fragments were separated on an agarose gel and blotted onto a nylon membrane for autoradiography.

References

Bachem CW, Van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M and Visser RG 1996 Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant J***. 9:** 745-753.

Bora RS, Kanamori A and Hirabayashi Y 1999 Cloning and characterization of a putative mouse acetyl-CoA transporter cDNA. *Gene* **238:** 455-462.

Elshourbagy NA, Near JC, Kmetz PJ, Sathe GM, Southan C, Strickler JE, Gross M, Young JF, Wells TN and Groot PH 1990 Rat ATP citrate-lyase. Molecular cloning and sequence analysis of a full-length cDNA and mRNA abundance as a function of diet, organ, and age. *J. Biol. Chem.* **265:** 1430-1435.

Johnson JF, Allan DL, Vance CP and Weiblen G 1996a Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus*. Contribution to organic acid exudation by proteoid roots. *Plant Physiol*. **112:** 19-30.

Johnson JF, Vance CP and Allan DL 1996b Altered lateral root development and enhanced expression of phospho*enol*pyruvate carboxylase. *Plant Physiol*. **112:** 31-41.

Kanamori, A., Nakayama, J., Fukuda, M.N., Stallcup W.B., Sasaki, K., Fukuda, M. and Hirabayashi, Y 1997. Expression cloning and characterization of a cDNA encoding a novel membrane protein required for the formation of O-acetylated ganglioside: A putative acetyl-CoA transporter. *Proc. Natl. Acad. Sci. U. S. A.* **94:** 2897-2902.

Kanao T, Fukui T, Atomi H and Imanaka T 2001 ATP-citrate lyase from the green sulfur bacterium *Chlorobium limicola* is a heteromeric enzyme composed of two distinct gene products. *Eur. J. Biochem*. **268:** 1670-1678.

Kollmeier M, Dietrich P, Bauer CS, Horst WJ and Hedrich R 2001 Aluminium activates a citrate-permeable anion channel in the aluminium sensitive zone of the maize root apex. A comparison between an aluminium-sensitive and an aluminium-resistant cultivar. *Plant Physiol*. **126**: 397-410.

Lundblad V 1997 *Saccharomyces cerevisiae*. In *Current Protocols in Molecular Biology*. Ed John Wiley ans sons, Inc.

Marschner H 1995 Rhizosphere pH effects on phosphorus nutrition. In: Lee JC, Sharma KK, Subbaro GV, Kueneman EA (eds), Genetic manipulation of crop plants to enhance integrated nutrient management in cropping systems -1- Phosphorus: proceedings of an FAO/ICRISAT expert consultancy workshop. ICRISAT Asia Center India, pp 107-115

Martinoia, E and Rentsch, D 1994 Malate compartmentation-responses to a complex metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **45**: 447-467.

Massonneau A, Langlade N, Léon S, Smutny J, Vogt E, Neumann G and Martinoia E 2001 Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.): relationship between organic acid excretion, sucrose metabolism and energy status. *Planta* **213:** 534-542.

Muller B, Stosser M and Tardieu F 1998 Spatial distributions of tissue expansion and cell division rates are related to irradiance and to sugar content in the growing zone of maize roots. *Plant Cell Environ*. **21:** 149-158.

Neumann G, Massonneau A, Martinoia E and Römheld V 1999 Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta***. 208:** 373-382.

Neumann G and Römheld V 1999 Root excretion of carboxylic acids and protons in phosphorous-deficient plants. *Plant Soil* **211:** 121-130**.**

Neumann G, Massonneau A, Langlade N, Dinkelaker B, Hengeler C, Römheld V and Martinoia E 2000 Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). *Ann. Bot*. **85:** 909-919.

Nowrousian M, Küuck U, Loser K and Weltring K-M 2000 The fungal acl1 and acl2 genes encode two polypepetides with homology to the N- and C-terminal parts of the animal ATP citrate lyase polypeptide. *Curr. Genet*. **37:** 189-193.

Peters WS and Felle HH 1999 The Correlation of Profiles of Surface pH and **Elongation Growth** in **Maize Root**s. *Plant Physiol*. **121**: 905-912.

Ratledge C, Bowater MDV and Taylor PN 1997 Correlation of ATP/citrate lyase activity with lipid accumulation in developing seeds of *Brassica napus* L.. *Lipids* **32**: 7-12.

Ryan PR and Delhaize E 2001 Function and mechanism of organic anion exudation from plant roots. *Annu. Rev. Plant Physiol. Plant Mol. Biol***. 52:** 527-560.

Sauer N and Stolz J 1994 SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. *Plant J*. **6:** 67-77.

Villalba JM, Palmgren MG, Berberian GE, Ferguson C and Serrano R 1992 Functional expression of plant plasma membrane H^+-ATPase in yeast endoplasmic reticulum. *J. Biol. Chem.* **267:** 12341-12349.

Chapter IV

Phosphorus deficiency-induced modifications in citrate catabolism and in cytosolic pH as related to citrate exudation in cluster roots of white lupin

Submitted to Plant and Soil

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Summary

A possible contribution of alterations in metabolic sequences involved in citrate catabolism, to intracellular accumulation and subsequent release of citrate was investigated in cluster roots of phosphorus (P) deficient white lupin (*Lupinus albus* L.). Citrate accumulation during maturation of root clusters was associated with decreased levels of intracellular soluble P_i and ATP and with reduced rates of respiration. Inhibitor studies with KCN and salicylhydroxamic acid (SHAM) suggest a reduced capacity of both the cytochrome pathway and of the alternative respiration with a concomitant decrease of immunochemically detectable protein levels of the alternative oxidase. Reduced respiration seems to be related rather to a general impairment of the respiratory system than to limitation of respiratory substrates such as P_i and adenylates, as indicated by the absence of stimulatory effects of the uncoupler CCCP. The citrate/malate ratio in juvenile root clusters with high rates of respiration and low inherent levels of citrate accumulation was increased by short-term application (4-8 hrs) of azide and SHAM as respiration inhibitors. These findings suggest that P deficiency-induced limitation of root respiration may induce a feedback inhibition of citrate catabolism in the TCA cycle to avoid excessive production of reducing equivalents and over-reduction of the respiratory chain.

During maturation of root clusters, a shift from intracellular malic acid to citric acid accumulation was associated also with downregulation of ATP citrate lyase (ACL), which catalyzes cleavage of citrate into acetyl-CoA and oxaloacetate with a putative function as anapleurotic source for the production of acetyl-CoA under P-deficient conditions. Oxaloacetate is readily converted to malate via malate dehydrogenase. Energy limitation, indicated by low levels of ATP and reduced respiration in mature and senescent root clusters may explain the decreased activity of the ATP-dependent ACL, associated with less consumption and increased accumulation of citric acid in the root tissue.

Inhibition of nitrate uptake and assimilation is a general response to P limitation in many plant species including white lupin. Reduced consumption of the amino acceptor 2-oxoglutaric acid as a product of citrate turnover may therefore contribute to increased citrate accumulation. Accordingly, artificial inhibition of nitrate reduction by localized application of tungstate significantly increased the citrate/malate ratio in juvenile root clusters.

Lowering the cytosolic pH by external application of propionate stimulated citrate and malate exudation in normal lateral roots and in developing root clusters. This effect was reverted by a preincubation with phosphonate to buffer the cytosol. No stimulation of carboxylate exudation was observed in mature and senescent clusters. This may be attributed to cytosolic acidification already present before the application of propionic acid due to excessive accumulation of citrate in these root zones. The results suggest that acidification of the cytosol may be an important factor, triggering the transient release of citrate and protons from mature root clusters in P-deficient white lupin.

Résumé

Une contribution aux altérations métaboliques dans des séquences métaboliques impliquées dans le catabolisme du citrate, entraînant son accumulation intracellulaire puis sa relâche, a été étudiée dans les racines de lupin blanc (*Lupinus albus*, L.) carencé en phosphore (P). L'accumulation de citrate au cours de la maturation des racines protéoïdes a été associée aux niveaux intracellulaires faibles de Pi et d'ATP et à des taux de respiration réduits. Les effets des inhibiteurs KCN et acide salicylhydroxamique (SHAM) suggèrent une capacité réduite à la fois de la voie du cytochrome et de la respiration alternative, avec une diminution concomitante des niveaux protéiques détectables immuno-chimiquement d'oxydase alternative. La respiration réduite semble être davantage reliée à une diminution générale du système respiratoire plutôt qu'à la limitation des substrats de la respiration tels Pi et les adénylates, comme indiqué par l'absence d'effet stimulant du découplant CCCP. Le ratio malate/citrate dans les racines protéoïdes juvéniles avec des niveaux élevés de respiration et faibles d'accumulation de citrate sont augmentés par l'application rapide (4-8 h) d'azide et de SHAM comme inhibiteurs de la respiration. Ces résultats suggèrent que la limitation induite par la carence en P de la respiration racinaire pourrait induire une inhibition par feedback du catabolisme du citrate dans le cycle TCA afin d'éviter une production excessive d'équivalents réducteurs ainsi qu'une sur-production de la chaîne respiratoire.

Lors de la maturation des racines protéoïdes, un déplacement de l'accumulation intracellulaire du malate puis du citrate est associée avec la diminution de l'ATP citrate lyase (ACL), qui catalyse le clivage du citrate en acétyl-CoA et oxaloacétate avec comme fonction possible la production anapleurotique d'acétyl-CoA en condition de carence en P. L'oxaloacétate facilement converti en malate via la malate déshydrogénase. La limitation en énergie, indiquée par les faibles niveaux d'ATP et la respiration réduite dans les racines matures et sénescentes peuvent expliquer la diminution de l'activité ACL, associée à une consommation moindre et une accumulation augmentée de citrate dans les tissus racinaires.

L'inhibition du transport et de l'assimilation de nitrate est une réponse générale à la limitation en P dans beaucoup d'espèces végétales y compris le lupin. La consommation réduite l'accepteur d'amine, le 2 oxoglutarate, comme produit du turnover du citrate, devrait contribuer à augmenter l'accumulation de citrate. En accord, l'inhibition artificielle de la réduction du nitrate par application de tungstate augmente significativement le ratio citrate/malate dans les racines juvéniles.

Diminuer le pH ppar application externe d'acide propionique stimule l'exsudation de citrate et de malate dans les racines normales latérales et dans les racines protéoïdes en développement. Cet effet est aboli par une préincubation avec du phosphonate afin de tamponner le cytosol. Aucune stimulation de l'exsudation de carboxylate n'est observée dans les racines matures et sénescentes. Ce peut être attribué à l'acidification cytosolique déjà présente avant l'application d'acide propionique du fait de

l’accumulation excessive de citrate dans ces racines. Ces résultats indiquent que l’acidification du cytosol peut être un facteur important, induisant la relâche transitoire de citrate et de protons des racines protéoïdes matures dans le lupin blanc carencé en P.

Introduction

White lupin (*Lupinus albus* L.) exhibits an extraordinarily high efficiency to acquire sparingly soluble soil P sources. This can be attributed to the plant's ability to release high amounts of P-mobilizing root exudates such as carboxylates (mainly citrate and malate), protons, phenolics, and acid phosphatase from cluster roots, formed under conditions of limited P supply (Dinkelaker *et al*., 1989; Neumann *et al*., 2000). Citrate is of particular importance due to its high ability to release P from Al-, Fe-, or Ca-phosphates (Jones *et al*., 1998; Neumann and Römheld, 2001). Release of citrate and a concomitant extrusion of protons is mainly restricted to an exudation peak of several days in mature root clusters at extraordinarily high rates of up to 1-2 µmol citrate h^{-1} g^{-1} root fresh weight, released in diurnal intervals (Neumann *et al*., 1999; Watt and Evans, 1999). In the root tissue of developing clusters, a shift from malate to citrate accumulation occurs prior to the exudation pulse, which seems to be induced when the internal citrate concentration reaches a threshold value of 20-30 µmol g^{-1} FW (Neumann *et al*., 1999; 2000). However, despite of similar high internal citrate concentrations, senescent root clusters in later stages of development exhibit only very low rates of exudation (Neumann *et al*., 1999; 2000). Citrate exudation was inhibited by application of anion channel inhibitors. Therefore, a controlled release mechanism, probably mediated by an anion channel, was postulated (Neumann *et al*., 1999), which similarly has been demonstrated in recent patch-clamp studies for Al-triggered organic acid release in apical root zones of maize and wheat (Kollmeier *et al*., 2001; Zhang *et al*., 2001).

Regulatory processes, triggering the release of citrate under P-deficient conditions, are largely unknown. A transport process coupled with H^+ extrusion and increased activity of the plasmamembrane (PM) H^+-ATPase was demonstrated by studies with isolated PM vesicles (Kania *et al*., 2001,Yan *et al*., 2001). Inducing factors for the transient release of citrate from cluster roots may be cytoplasmic acidosis, which has been similarly demonstrated for lactic acid accumulation in hypoxic maize root tips (Xia and Roberts, 1994) or interference with Ca homeostasis as a consequence of excessive intracellular accumulation of citrate (Neumann *et al*., 2000).

Citrate accumulation was shown to be associated with enhanced transcript levels and activities of enzymes involved in carbohydrate catabolism (fructokinase, phosphoglucomutase, and sucrose synthase) and biosynthesis of organic acids (PEP carboxylase, malate dehydrogenase). However, contradictory results have been reported for the activity of citrate synthase in the root tissue of different P-deficient plants (Johnson *et al*., 1994; Takita *et al*., 1999; Neumann *et al*., 1999; Aono *et al*., 2001). The induction of many of these enzymes under P-deficient conditions has been linked with a function in P_i recycling and circumventing P_i-dependent metabolic reaction steps (e.g. sucrose synthase, PEP carboxylase) for a more economic internal P_i utilization at the metabolic level (Plaxton, 1998). On the other hand, reduced root respiration (Neumann *et al*., 1999; Watt and Evans, 1999; Massonneau *et al*., 2001) and decreased activity of enzymes involved in citrate catabolism, such as aconitase (Neumann *et al*., 1999) and ATP-citrate lyase (Neumann and Römheld, 2000; Langlade *et al*., submitted) suggest that also a P deficiency-induced inhibition of citrate turnover may be involved in the selective accumulation of citrate in the cluster root tissue prior to the exudation pulse.

In this study we investigated the possible contribution of P deficiency-induced inhibition of various processes involved in citrate consumption (root respiration, ATP citrate lyase, nitrate assimilation) to intracellular citrate accumulation during cluster root development. The effects of modifying cytosolic pH homeostasis on the release of carboxylate anions were studied, as a putative consequence of excessive accumulation of organic acids in the root tissue.

Materials and methods

Plant material

Seeds of white lupin (*Lupinus albus* L. cv Amiga, Suedwestdeutsche Saatzucht, Rastatt, Germany) were incubated for 6 h in 10 mM $CaSO_4$ solution and germinated in the dark for 4 days on filter paper soaked with 2.5 mM $CaSO_4$. After two days in the light, the plants were transferred to an aerated nutrient solution [2 mM $Ca(NO_3)_2$; 0.7 mM K_2SO_4; 0.1 mM KCl; 0.5 mM $MgSO_4$; 30 mM Fe-EDTA; 10 µM H_3BO_3; 0.5 µM $MnSO_4$; 0.5 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 0.01 µM $(NH_4)_6Mo_7O_{24}$, with addition of 2.5 mM $CaSO_4$ per pot in solid form] in the presence (+P) or absence (-P) of 250 µM KH_2PO_4. The nutrient solution was replaced every 3-4 days. Growth chamber conditions were adjusted to a 16/8 h day/night cycle at a light intensity of 150 µmol m^{-2} s^{-1} and temperature of 25°C with 60% relative humidity.

Root O_2 uptake

Respiration was measured as O_2 uptake in excised root segments (200-400 mg FW) of cluster roots in different developmental stages or 1 cm-apical root zones of lateral roots with a Clark-type O_2 electrode (TriOxmatic EO 200; WTW, Weilheim, Germany) at a temperature of 25°C in 30 ml of an air-saturated 2 mM $Ca(NO_3)_2$ solution (according to Neumann *et al.*, 1999). Respiration inhibitors were used at final concentrations of 0.5 mM and 7.5 mM for KCN and SHAM (Salicylhydroxamic acid), respectively. The optimum concentrations for inhibitor supply were estimated from titration curves according to Møller *et al*. (1988). CCCP (Carbonyl cyanide-3-chlorophenyl-hydrazone) as an uncoupler of oxidative phosphorylation was used at concentrations from 0.2 to 20 µM. Each measurement was performed with separate root samples. Fresh and dry weight of the root material was recorded when the measurements were finished.

Cytochrome pathway (COX) capacity was defined as apparent respiration when the alternative pathway was inhibited by SHAM and alternative pathway(AOX) capacity as the apparent respiration with simultaneous inhibition of the cytochrome pathway by KCN. All data were corrected for residual respiration, measured by application of both, KCN and SHAM.

In vivo inhibitor experiments and determination of organic acids.

Only P-deficient plants were used for the incubation with inhibitors. Cytochrome pathway was inhibited with 1 mM azide and the alternative pathway with 7.5 mM SHAM.

(-)-threo-hydroxycitrate (*Garcinia cambogia* extract; Fluka, Seelze, Germany) was used in a concentration range between 1 to 100 mM to inhibit ATP-citrate lyase, and sodium-tungstate (Na_2WO_4) was applied at concentrations between 300-1000 µM to inhibit nitrate reductase. Azide was used instead of KCN to avoid evaporation of the inhibitor under the conditions of an open and aerated system.

For localized inhibitor application, a small beaker containing the respective inhibitor compound, which was dissolved in aerated nutrient solution without Fe-EDTA, was fixed on the inner wall of a larger pot, containing aerated nutrient solution without inhibitor. From a single plant, 3-5 lateral roots with cluster roots in different developmental stages were placed into the beaker, whereas the remaining root system was incubated in the larger pot. For harvest, the incubated root parts were cut, rinsed in 1 mM $CaSO_4$ solution to remove the adhering inhibitors, and juvenile, mature, and senescent proteoid root segments were frozen separately in liquid nitrogen.

For determination of organic acids, the root segments were homogenized in 5% (v/v) H_3PO_4 [50 mg root FW ml^{-1}] using mortar and pestle, the homogenate was transferred into Eppendorf reaction vials and centrifuged for 10 min at 20 000 g. The supernatant was diluted 10-fold in HPLC buffer (18 mM KH_2PO_4, pH 2.15) and stored at –20°C until HPLC analysis.

Conditions for HPLC

Separation of organic acids was conducted on a reversed-phase column (250 mm x 4 mm ID ; GROM-SIL 120 ODS-5 ST, 5 µm particle size, Grom, Herrenberg, Germany) equipped with a Hypersil ODS guard column (20 mm x 4 mm ID; GROM, Herrenberg, Germany) in the ion-suppression mode. A sample volume of 20 µl was isocratically eluted with HPLC buffer containing 18 mM KH_2PO_4 adjusted to pH 2.15 with H_3PO_4, at a flow rate of 0.5 ml min^{-1}, column temperature of 40.0°C, and a UV detection wavelength of λ=215 nm . Identification of organic acids was performed by comparing retention times and absorption spectra with those of known standards.

Western blot

Extracts for non-reducing SDS-PAGE were prepared from fresh root material. Root tissue was ground in liquid nitrogen using a mortar and a pestle. 500 mg of the ground tissue was suspended in a 1 ml volume of protein sample mixture (62.5 mM Tris-HCl, pH 6.8; 2 % (w/v) SDS; 10 % glycerol, 2 mM EDTA, and 0.002 % bromophenol blue). PMSF (final concentration 1 mM) was added to inhibit proteases and samples were immediately boiled for 5 min. For separations under reducing conditions , 5 % (v/v) 2-mercaptoethanol was added. After cooling on ice, samples were centrifuged for 10 min at 16 000 g and 8°C in a microliter centrifuge to precipitate cell debris. Proteins were separated by SDS-PAGE according to the method of Laemmli (1970) with a 6 % (w/v) polyacrylamide stacking gel and a 12 % (w/v) polyacryamide resolving gel. The separated proteins were subsequently transferred to a nitrocellulose blotting membrane (0.2 µm pore size; Sartorius, Göttingen, Germany) by semi-dry blotting (Khyse-Andersen, 1984) according to Neumann *et al*., (1999).

For immunoblot analysis, the blot was incubated in a 1:75 dilution of a monoclonal antibody raised against alternative oxidase (AOX) of *Sauromatum guttatum*. The AOX antibody was kindly provided by Dr. T.E. Elthon (Elthon *et al*., 1989). Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate was used as a secondary antibody (dilution 1: 17500). Colour development was performed with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma, Deisenhofen, Germany).

ATP citrate lyase (EC 4.1.3.8) assay

Frozen plant tissues were ground in liquid nitrogen and homogenized with 3 vol. of extraction buffer (0.1 M HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 2.5 mM DTT, 3 mM Na-DEDTC (diethyldithiocarbamate), 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF, 3 % PVPP K30). After centrifugation (25 min, 12 000 g, 4°C) the supernatant was rapidly used to determine the ATP citrate lyase (ACL) activities and protein concentrations (DC Protein Assay kit; Bio-Rad). ACL activity was determined spectrophotometrically at room temperature, using the malate dehydrogenase coupled assay. The assay mixture contained 0.2 M Tris pH 8.4, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 mM Na_3-Citrate, 0.2 mM Coenzyme A, 10 mM ATP (omitted in blanks), 0.2 mM NADH, 0.4 U ml^{-1} malate dehydrogenase. Values were taken after 30 min and 10 min for plants and yeast, respectively. These time points were chosen since prolonged incubation resulted in the inactivation of the enzyme. Blanks were performed by omitting ATP or Coenzyme A resulting in similar values.

Acidification of the cytosol

Different parts of lupin roots were collected as described by Massonneau *et al*. (2001). Four to ten root segments were rinsed briefly in distilled water and incubated during 1 hour at 22°C in Eppendorf reaction tubes containing 1 mL of 20 mM propionate, 20 mM MES-KOH or 20 mM HEPES-KOH at pH 4.0 or pH 7.0. Citrate and malate concentrations were determined in these root washings using the citric acid test kit and L-malic acid test kit (Boehringer, Mannheim, Germany). The presence of applied chemicals has been verified not to influence the determination method. For treatments with P_i and Phosphonate (Phi), lupin plants were cultivated during 35 days without P supply and subsequently transferred for 24 h in a -P growth medium supplemented with 0.25 mM of KH_2PO_4 (+P) or H_3PO_3 (phosphonate) buffered to pH 5.2 with KOH (+Phi). Root-induced pH-changes were monitored by application of agar sheets with bromocresol purple as pH indicator onto the root surface, as described by Massonneau *et al*. (2001).

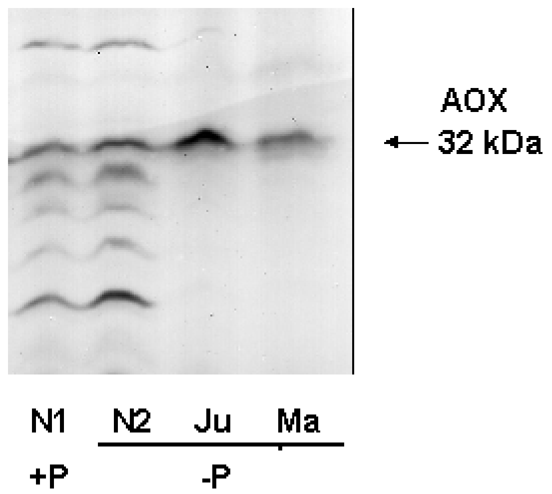
Results and discussion

Root respiration

During maturation of cluster roots, accumulation of citric acid in the root tissue coincides with a decline of root respiration and reduction of intracellular soluble P_i, total RNA and ATP (Neumann *et al*., 1999; Massonneau *et al*., 2001). It has been postulated that redistribution of P_i from older root zones to the juvenile, actively growing parts of the root, may cause a more severe expression of P deficiency in mature and senescent root clusters. This would lead to a P deficiency-induced limitation of root respiration, a downregulation of citrate turnover in the TCA cycle by a feedback mechanism to avoid excessive production of reducing equivalents, and finally to increased accumulation of citric acid in the root tissue. Accordingly, in this study the highest levels of total root respiration were found in 1 cm-apical root zones of lateral roots in P sufficient and P deficient plants and also in juvenile cluster roots. During maturation and senescence of the clusters, respiration declined by up to 60% (Fig.1A). Limitation of root respiration has been frequently reported in plants with limited nutrient supply (Hoefnagel *et al*., 1993). By contrast, Rychter and Mykulska (1990) found no changes of total respiration in roots of P-deficient bean plants but an increased relative contribution of the alternative oxidase (AOX) pathway of root respiration. Although not coupled with ATP production, electron flow through the AOX pathway may enable the operation of the TCA cycle even under conditions when the respiratory cytochrome (COX) pathway and ATP production is limited by P deficiency.

Similarly, in this study, selective inhibition of the COX or the AOX pathway by application of cyanide or SHAM revealed a two -fold increase of the AOX capacity in cluster roots of P-deficient plants compared with apical root zones of the P-sufficient control. However, during cluster root maturation and senescence, the capacity of both, the COX and the AOX pathway decreased again (Fig.1A). Preliminary measurements revealed also declining levels of immunochemically detectable AOX protein during ageing of cluster roots, although, in apical root zones of P-sufficient control plants and in juvenile root clusters, interpretation of the results was complicated by formation of multiple immunoreactive bands probably due to proteolysis of the AOX protein (Fig.2). The results suggest a general impairment of the respiratory system in mature and senescent root clusters.

Figure 2: Western blot analysis of aldehyde oxidase in different root zones of *Lupinus albus* L. grown under P-sufficient (+P) and P-deficient (-P) conditions.



This assumption is further confirmed by the finding that uncoupling of respiration with CCCP (0.2 – 20 µM) did not increase O_2 consumption, irrespective of the investigated root zone or P supply of the plants (Fig.1B). This indicates that respiration was not under adenylate control and therefore not limited by the availability of adenylates or P_i even in P-deficient plants. Structural modifications of membranes (Rychter and Mikulska, 1990), a limitation of endogenous sugar levels (Lambers *et al.*, 1996) or of protein synthesis induced by P deficiency might therefore explain the limited function of the respiratory chain in mature and senescent root clusters. This would explain also a downregulation of the activity of the TCA cycle coupled with reduced turnover and finally accumulation of citric acid in the root tissue.

Accordingly, in juvenile root clusters with high rates of root respiration and malate as dominant organic acid (Neumann *et al.*, 1999), a 2-3 fold increase of the citrate/malate ratio was observed, when respiration in these root zones was inhibited by localized short-term application (4 h and 8 h) of azide or SHAM, respectively (Table 1).

Figure 1: Spatial variation of oxygen uptake in different root zones of *Lupinus albus* L. as related to the level of P supply and application of inhibitors. +P:0.25 mM P_i; -P:0 mM Pi; a:1 cm apical root zone of first order laterals; j:juvemile root clusters; m: mature root clusters; s: senescent root clusters. A: Total respiration and effects of inhibitors:KCN [0.5 mM] (cytochrome pathway) : SHAM [7.5 mM] (alternative pathway). Data corrected for residual respiration, measured by application of both, KCN and SHAM. B: Effect of uncoupling respiration with CCCP in different root zones of *Lupinus albus* L. Means of 5 - 13 independent replicates.

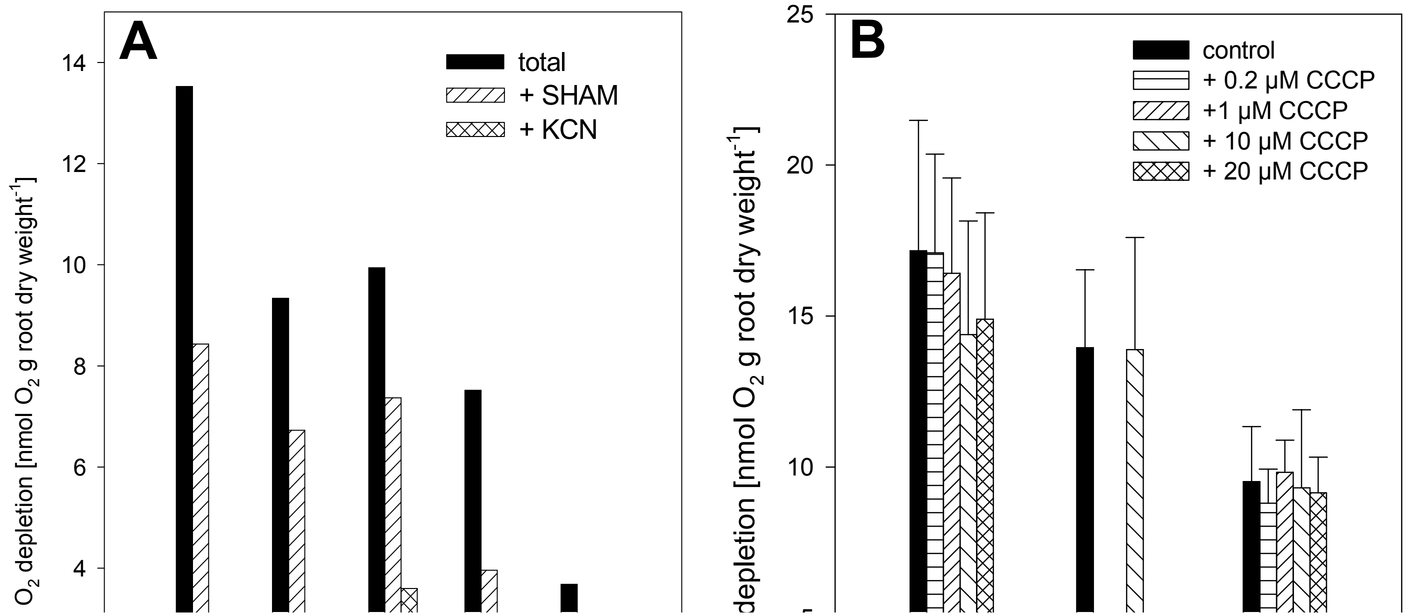


Table 1: Citrate/malate ratios in juvenile root clusters of P-deficient white lupin as a facted by localized application of respiration inhibitors.

TREATMENT / DURATION	RATIO CITRATE / MALATE	
	4 h	8 h
Control	0.51 a	0.39 a
SHAM	0.57 ab	0.68 b
Azide	0.92 b	0.83 b

Decreases in respiratory rates and particularly in COX capacity have been previously reported for ageing plant tissues (Millar *et al*., 1998). Lambers *et al*. (1997) found inhibition of the AOX pathway associated with the production of massive amounts of citrate. This may be attributed to the production of reactive oxygen species and particularly of H_2O_2 as a consequence of a overreduction of the respiratory chain due to a limited AOX activity. H_2O_2 is a potent inhibitor of aconitase (Verniquet, 1991; Gardner and Fridovich, 1992), which mediates the conversion of citrate to isocitrate. Accordingly, a decreased activity of aconitase parallels the accumulation of citrate in cluster roots of white lupin (Neumann *et al*., 1999). Similarly, McIntosh (1994) reported dramatically increased production of H_2O_2 by transgenic tobacco cells lacking AOX, and intracellular citrate accumulation in response to treatments with H_2O_2 (Valerberghe and McIntosh, 1996).Citrate may also act as a regulatory metabolite, which controls the expression of AOX at the transcriptional level (Vanlerberghe and McIntosh, 1996). Thus, a better understanding of the respiratory control in cluster roots under P-deficient conditions and the possible link with root exudation of citrate would require more detailed studies on putative regulatory metabolites (e.g. citrate, pyruvate, H_2O_2 etc.), redox state, and more reliable measurements of respiratory electron flow and partitioning by isotopic ($^{18}O^{16}O$) discrimination techniques (Vanlerberghe, 1997).

ATP citrate lyase

Using the AFLP technique, we identified a large number of genes with putative differential expression during cluster root development in white lupin (Massonneau *et al*., 2001). Among these genes, a putative ATP citrate lyase (EC 4.1.3.8) was identified by cloning and heterologous expression in yeast (Langlade *et al*., submitted). ATP-citrate lyase was upregulated in juvenile cluster roots. The activity of the enzyme showed a positive correlation with exudation and intracellular levels of malate. However, the activity declined with maturation of the clusters associated with increased intracellular accumulation of citrate. ATP-citrate lyase catalyzes cleavage of citrate into acetyl-CoA and oxaloacetate. The function of the enzyme has been linked with the formation of acetyl-CoA as a precursor for the biosynthesis of lipids and terpenoids (Ratledge *et al*., 1997; Muller *et al*., 1998). Under P-deficient conditions, an increased proportion of the glycolytic carbon flux seems to be directed via the PEP carboxylase pathway, probably to circumvent P_i-dependent metabolic reactions of conventional glycolysis and pyruvate catabolism (Johnson *et al*., 1996; Plaxton, 1998). This would be associated with increased biosynthesis of organic acids but at the same time with reduced production of acetyl-CoA, due to a decreased activity of the P_i-dependent pyruvate kinase/pyruvate dehydrogenase pathway. Cleavage of citrate by ATP-citrate lyase in juvenile root clusters might therefore provide an anapleurotic supply of acetyl-CoA, required for the biosynthesis of lipids, phenolics and terpenoids under P-deficient conditions. Conversion of oxaloacetate, as the second reaction product, to malate by enhanced activities of malate dehydrogenase (Johnson *et al*., 1994) may lead to increased accumulation and exudation of malate in juvenile root clusters. Limitation of metabolic energy (ATP, P_i) in later stages of cluster root development (Massonneau *et al*., 2001) may limit the activity of ATP-citrate lyase. thereby contributing to the accumulation of citrate in mature and senescent root clusters (Fig.3; Langlade *et al*., submitted). However, attempts to inhibit the activity of the enzyme in juvenile clusters by localized application of hydroxycitrate failed to induce any changes in the citrate/malate ratio (data not shown). But it is not clear whether the inhibitor compound, which was commercially available only as a partially purified preparation of a crude plant extract, was really taken up by the roots in sufficient amounts to exert specific effects.

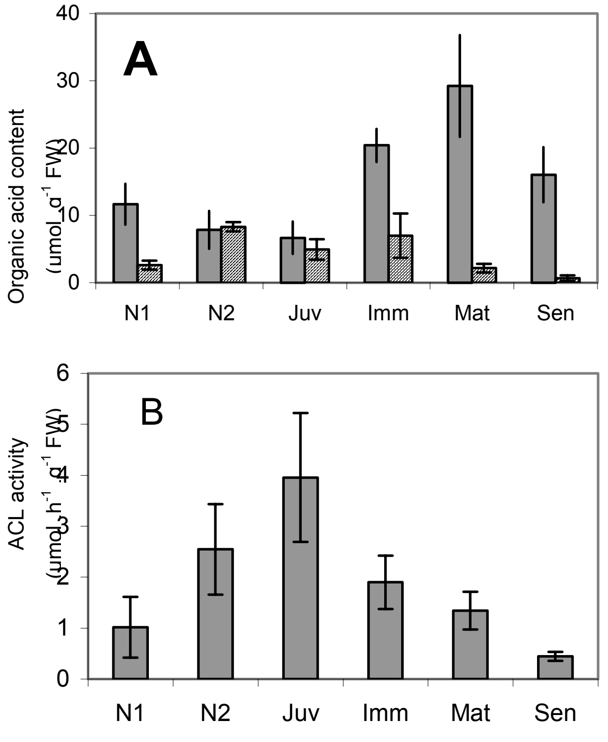


Figure 3: Citrate ■ and malate ▨ concentrations (A) as related to activity of ATP-citrate lyase (B) in different parts of the root system of P-deficient white lupin. Data are means ± SD of three independent replicates.

Nitrate assimilation

In many plant species, including white lupin, P deficiency causes a rapid decline in nitrate uptake (Neumann *et al*., 1999; Pilbeam *et al*., 1993; Rufty *et al*., 1990; Schjorring, 1986; Lee, 1982). This is frequently associated with a decline in the activity of nitrate reductase and with nitrate accumulation in the root tissue, which probably induces feedback inhibition of nitrate uptake (Pilbeam *et al*., 1993; Gniazdowska and Rychter, 2000). Therefore, a lower demand of 2-oxoacids (particularly of oxoglutaric acid) as precursors for the biosynthesis of amino acids may further contribute to a reduced consumption of organic acids and finally to citrate accumulation in the root tissue, when the biosynthesis of organic acids is upregulated under P-deficient conditions (Johnson *et al*., 1996, Neumann *et al*., 1999). Accordingly, artificial inhibition of nitrate reductase by localized application of tungstate significantly increased the citrate/malate ratio in juvenile root clusters of white lupin within an incubation period 12–16 hours (Fig 4).

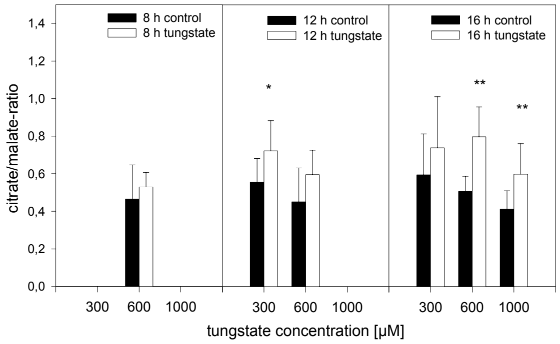


Figure 4: Citrate/malate ratio in juvemile root clusters of P-deficient *Lupinus albus* L. as affected by application of tungstate (Na_2WO_4) as inhibitor of nitrate reductase. Means ∀ SD of 9-25 replicates.

Although, unspecific effects of inhibitors, particularly after long-term application cannot be excluded, this result may give at least a first indication that impairment of nitrate assimilation under P-deficient conditions can also have an impact on the intracellular accumulation of organic acids. Further investigations would require direct measurements of nitrate uptake and assimilation as well as ^{14}C labelling studies to trace changes of the fate of carbon skeletons in different stages of cluster root development.

Carboxylate exudation and intracellular pH

The transient pulse of citrate exudation in mature root clusters suggests a controlled release mechanism for citrate anions. This assumption is further supported by the effectiveness of anion channel inhibitors (Neumann *et al*., 1999). Similarly, anion channel-mediated root exudation of malate and citrate has been recently demonstrated by patch-clamp studies with wheat and maize under aluminum stress (Kollmeier *et al*., 2001; Zhang *et al*., 2001). In white lupin, citrate release is charge-balanced by a concomitant extrusion of protons, which is responsible for rhizosphere acidification. Accordingly, upregulation of plasmalemma H^+-ATPase was detected in plasma membrane vesicles isolated from mature root clusters (Kania *et al*., 2001; Yan *et al*., 2001). However, the signal which triggers the transient release of citrate is still unknown. Anion channels can be modulated by carboxylate anions themselves, as demonstrated for vacuolar anion channels of CAM plants (Cerana *et al*., 1995) or for stomatal guard cells (Hedrich and Marten, 1993). Decreased levels of ATP, characteristic also for mature and senescent root clusters, have been shown to activate anion channels permeable for NO_3^- in (Thomine *et al*., 1997; Frachisse *et al*., 1999). Increased accumulation of lactic acid in the hypoxic maize root tips induces overacidification of the cytosol (Xia and Roberts, 1994) at similar concentration levels which have been reported for citric acid accumulation in mature root clusters of white lupin (Neumann *et al*., 2000).To assess whether modifications of the intracellular pH may be involved in the induction of carboxylate exudation in roots of P-deficient white lupin, we used weak organic acids such as propionic, acetic or methyl trichloroacetic acid (Methyl-TCA, not metabolized) for external application to the root medium at pH 4. According to the ion trap principle, the protonated acids easily permeate the plasmalemma of the root cells and accumulate in the cytosol, due to complete dissociation at pH 7.0-7.5. This is associated with a decrease of the cytosolic pH. During an incubation period of 1 h with propionic acid, release of both, citrate and malate, was dramatically increased particularly in apical root zones of normal lateral roots, in juvenile root clusters, and in premature cluster roots, which have reached their final length but usually exhibit only low rates of carboxylate exudation (Figs.5A,B). Similar results were obtained with acetic or methyl-TCA (data not shown). Buffering the pH of the incubation medium to 4.0 and 7.0 with MES or HEPES in absence of propionate did not alter the rates of carboxylate exudation, demonstrating that increased exudation is not simply a result of a modified external pH.(Figs. 5A,B). These findings suggest that a drop of the cytosolic pH induced by intracellular organic acid accumulation may be in fact an important factor triggering the release of carboxylates in roots of P-deficient plants. In contrast, there were no consistent effects in mature and senescent clusters (Figs.5A,B). This may be explained by a low cytosolic pH in these root zones already before propionate application, induced by the high levels of intracellular citric acid accumulation. To test whether the induction of carboxylate exudation was really a pH effect, roots of P-deficient plants were preincubated for 24 hrs with phosphonate (H_3PO_3, Phi), which is taken up but not metabolized by plant roots, to achieve pH buffering of the cytosol (Carswell *et al*., 1996; 1997).

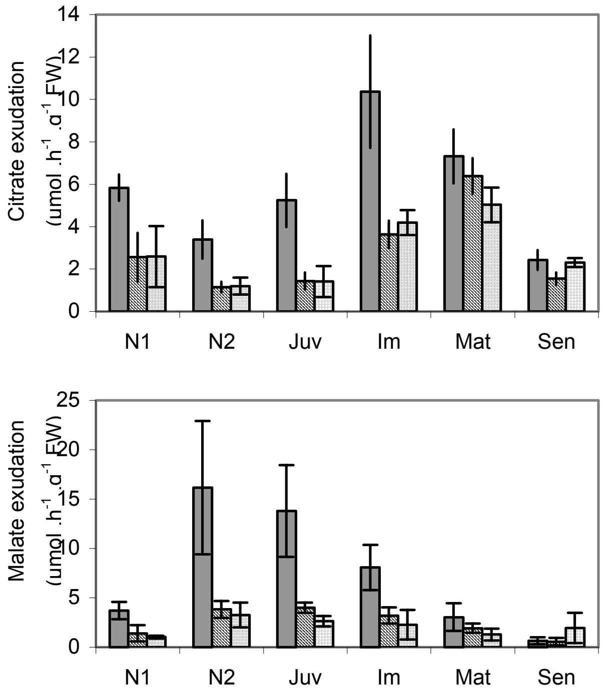


Figure 5: Citrate and malate exudation in different root zones of white lupin in presence of propionic acid (pH 4.0) ■, MES (pH 4.0) ▨ or HEPES pH 7.0 ░. Data are means ± SD of three independent replicates.

Accordingly, neither carboxylate exudation (not shown) nor proton extrusion was induced by propionate application in roots of phi treated plants (Fig.6). Similar responses were observed when roots of P-deficient plants were preincubated for 24 h with phosphate (P_i) instead of Phi (Figs 6). In the phosphate treatments, ATP levels similar to those of P-sufficient plants (data not shown) indicate rapid P_i metabolization and recovery from P-deficient conditions, counteracting cytosolic acidification. No such effects were detected in Phi treated plants. A direct confirmation for a role of the intracellular pH in triggering root exudation of carboxylates would require NMR studies for direct pH determination in the cytosol of different root zones and a membrane-physiological approach with patch-clamp experiments or PM vesicles to characterize pH dependency of the transport mechanism. It is also not clear which factors are responsible for the rapid decrease of citrate exudation in senescent root clusters despite of high internal concentrations of citric acid. Possible explanations could be restabilization of the cytosolic pH after the pulse of proton and carboxylate exudation or energy limitation of the PM H^+-ATPase due to the extremely low levels of respiration and ATP in these root zones.

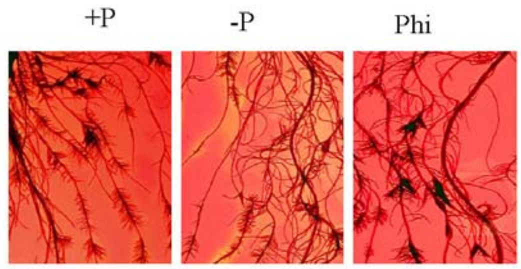


Figure 6: Effects of treatments with Pi (+P), and phosphonate (Phi) on root-induced pH changes in P-deficient white lupin (-P) detected by application of agar sheets with bromocresol purple as pH indicator.

Conclusions

A detailed understanding of physiological mechanisms involved in the regulation of root exudation as an adaptive response for acquisition of sparingly soluble nutrients or detoxification of toxic elements is a prerequisite for attempts to manipulate tolerance of crop plants towards adverse soil chemical conditions by root-induced changes in rhizosphere chemistry.

Citrate is among the most potent root-born chelators involved in P mobilization and Al detoxicication, provided it is released in sufficient amounts (Jones, 1998; Neumann and Römheld, 2000). As a base for more detailed investigations, this study presents further evidence for the hypothesis that downregulation of various metabolic sequences involved in citrate catabolism may be an important factor for accumulation of citric acid in cluster roots of P-deficient white lupin, which preceeds the intense pulse of citrate exudation. Moreover, there is a first indication that acidification of the cytosol induced by excessive intracellular accumulation of citric acid could be part of the signal chain, which induces the transient release of citrate and protons into the rhizosphere.

References

Aono T, Kanada N, Ijima A and Oxaizu H 2001 The response of the phsophate uptake system and the organic acid exudation system to phosphate starvation *in Sesbania rostrata. Plant Cell Physiol* **42**:11, 1253-1264.

Carswell C, Grant BR, Theodorou ME, Harris J, Niere JO and Plaxton WC 1996 The fungicide phosphonate disrupts the phosphate-starvation response in *Brassica nigra* seedlings. *Plant Physiol* **110**: 105-110.

Carswell MC, Grant BR and Plaxton WC 1997 Disruption of phosphate-starvation response of oilseed rarpe suspension cells by the fungicide phosphonate. *Planta* **203**: 67-74.

Cerana R, Giromini L and Colombo R 1995 Malate-regulated channels permeable to anions in vacuoles of *Arabidopsis thaliana*. *Aust J. Plant Physiol*. **22** 115-121.

Dinkelaker B, Römheld V and Marschner H 1989 Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (*Lupinus albus* L.). *Plant Cell Environ* **12** 285-292.

Elthon TE, Nickels RL and McIntosh L 1989 Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. *Plant Physiol*. **89** 1311-1317.

Gardner P R and Fridovich I 1992 Inactivation-reactivation of aconitase in *Escherichia coli*. *J Biol Chem* **267**:13, 8757-8763.

Gniazdowska A and Rychter AM 2000 Nitrate uptake by bean (*Phaseolus vulgaris* L.) roots under phosphate deficiency. *Plant Soil 226* 79-85.

Franchisse JM, Thomine S, Colcombet J, Guern J, and Barbier-Brygoo H 1999 Sulfate is both a substrate and an activator of the voltage-dependent anion channel of *Arabidopsis* hypocotyl cells. *Plant Physiol* **121** 253-262.

Hedrich R and Marten I 1993 Malate-induced feedback regulation of plasma membrane anion channels could provide a CO_2 sensor to guard cells. *EMBO J*. **12** 897-901.

Hoefnagel MHN, van Iren F and Libbenga KR 1993 In suspension cultures of Catharanthus roseus the cyanide-resistant pathway is engaged in respiration by excess sugar in combination with phosphate or nitrogen starvation. *Physiol. Plant*. *87* 297-304.

Johnson JF, Allan DL and Vance CP 1994 Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. Plant Physiol. **104** 657-665.

Johnson JF, Allan DL, Vance CP and Weiblen G 1996a Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus* – Contribution to organic acid exudation by proteoid roots. *Plant Physiol*. **112** 19-30.

Johnson JF, Vance CP and Allan DL 1996b Phosphorus deficiency in *Lupinus albus* – Altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant Physiol*. **112** 31-41.

Jones DL 1998 Organic acids in the rhizosphere – a critical review. *Plant Soil* **205** 25-44.

Kania A, Neumann G, Cesco S, Pinton R and Römheld V 2001 Use of plasma membrane vesicles for examination of phosphorus deficiency-induced root excretion of citrate in cluster roots of white lupin (*Lupinus albus* L.). *In* Plant Nutrition: Food security and sustainability of agro-ecosystems thruough basic and applied research. XIV International Plant Nutrition Colloquium. pp 546-547. Kluwer Academic Publishers.

Khyse-Andersen J 1984 Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *Biochem. Biophys. Meth*. **10** 203-209.

Kollmeier M, Dietrich P, Bauer CS, Horst WJ and Hedrich R *2001 Aluminum activates a citrate-permeable anion channel in the aluminum-sensitive zone of the maize root apex. A comparison between an aluminum-sensitive and an aluminum-resistant cultivar.* Plant Physiol*. **126** 397-410.*

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4: *Nature* **227**

680-685.

Lambers H, Scheurwater I and Atkin OK 1996 Respiratory patterns in roots in relation to their funktioning. *In* Plant Roots: The Hidden Half. Eds. Waisel Y, Estel A, Kafkafi U. pp 323-362.

Lambers H, Scheurwater I and Millenaar F 1997 Variation in Carbon Utilization in Root Respiration and Exudation as Dependent on a Species‘ Potential Growth rate and Nutrient Supply. *In* Radical Biology: Advances and Perspectives of the Function of Plant Roots. Eds. H E Flores, J P Lynch, D Eissenstat. American Society of Plant Physiologists. pp 116-130.

LangladeN, Messerli G, Smutny J, Neumann G, Martinoia E and Massonneau A 2002 ATP citrate lyase: cloning, heterologous expression and its role in organic acid excretion (submitted).

Lee RB 1982 Selectivity and kinetics of ion uptake by barley plants following nutrient deficiency. *Anal. Bot.* **50** 429-449.

McIntosh L 1994 Molecular biology of the alternative oxidase. *Plant Physiol*. **105** 781-786.

Massonneau A, Langlade N, Leon S, Smutny J, Vogt E, Neumann G and Martinoia E 2001 Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.): relationship between organic acid excretion, sucrose metabolism and energy status. *Planta* **213** 534-542.

Millar AH, Atkin OK, Menz RI, Henry B, Farquhar G and Day DA 1998 Analysis of respiratory chain regulation in roots of soybean seedlings. *Plant Physiol*. **117** 1083-1093.

Moeller IM, Berczi A, van der Plas LHW and Lambers H 1988 Measurement of the activity and capacity of the alternative pathway in intact plant tissues: identification of problems and possible solutions. *Physiol. Plant*. **72** 642-649.

Neumann G, Massonneau A, Martinoia E and Römheld V 1999 Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* **208** 373-382.

Neumann G, Massonneau A, Langlade N, Dinkelaker B, Hengeler C, Römheld V and Martinoia E 2000 Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). *Anal. Bot*. **85** 909-919.

Neumann G and Römheld V 2001 The release of root exudates as affected by the plant's physiological status. *In* Rhizosphere: Biochemistry and organic substances at the soil-plant interface. Eds. Pinton R, Varanini Z and Nannipieri P. pp 41-93. Marcel Dekker.

Pilbeam DJ, Cakamk I, Marschner H and Kirkby EA 1993 Effect of withdrawal of phosphorus on nitrate assimilation and PEP carboxylase activity in tomato. *Plant Soil* **154** 111-117.

Plaxton WC 1998 Metabolic Aspects of Phosphate Starvation in Plants. *In* Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes. Eds. J P Lynch and J Deikman. pp 41-93. American Society of Plant Physiologists.

Rufty TW, MacKown CT and Israel DW 1990 Phosphorus Stress Effects on Assimilation of Nitrate. *Plant Physiol*. **94** 328-333.

Rychter AM and Mikulska M 1990 The relationship between phosphate status adn cyanide-resistant respiration in bean roots. *Physiol. Plant*. **79** 663-667.

Schjorring JK 1986 Nitrate and ammonium absorption by plants growing at a sufficient or insufficient level of phosphorus in nutrient solution. *In* Fundamental, ecological and agricultural aspects of nitrogen metabolism in higher plants. Eds Lambers H, Neetson J J and Stulen I. pp 53-58. Dordrecht: Martinus Nijhoff.

Takita E, Koyama H and Hara T 1999 Organic acid metabolism in aluminum-phosphate utilizing cells of carrot (*Daucus carota* L.). *Plant Cell Physiol*. **40** 489-495.

Thomine S, Lelievre F, Boufflet M, Guern J and Barbier-Brygoo H 1997 Anion-channel blockers interfere with auxin responses in dark-grown *Arabidopsis* hypocotyls. *Plant Physiol*. **115** 533-542.

Vanlerberghe GC and McIntosh L 1996 Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. *Plant Physiol*. **111** 589-595.

Verniquet F, Gaillard J, Neuburger M and Douce R 1991 Rapid inactivation of plant aconitase by hydrogen peroxide. *Biochem. J*. **276** 643-64.

Watt M and Evans JR 1999 Linking development and determinancy with organic acid efflux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO_2 concentration. *Plant Physiol*. **120** 705-716.

Watt M and Evans JR 1999 Proteoid roots. Physiology and development. *Plant Physiol*. **121** 317-323.

Yan F, Zhu Y, Müller C and Schubert S 2001 Adaptation of plasma membrane H^+-ATPase of proteoid roots of white lupin (*Lupinus albus* L.) to phosphorus deficiency. *In* Plant Nutrition: Food security and sustainability of agro-ecosystems thruough basic and applied research. XIV International Plant Nutrition Colloquium. pp 186-187. Kluwer Academic Publishers.

Xia JH, Roberts JKM 1994. Improved cytoplasmic pH regulation, increased lactate efflux, and reduced cytoplasmic lactate levels are biochemical traits expressed in root tips of whole maize seedlings acclimated to a low-oxygen environment. *Plant Physiol* **105**: 651-657.

Zhang W-H, Ryan PR and Tyerman SD 2001 Aluminium activates malate-permeable channels in the apical cells of wheat roots. *Plant Physiol* **125** 1459-1472.

CHAPTER V

LATCP1: ROLE IN CLUSTER ROOT FORMATION

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Summary

Cluster roots or proteoid roots are important adaptations for nutrient acquisition, found in a wide range of plant families. These bottlebrush-like structures consist of tight groupings of size-determinated rootlets.

While much is known about the physiology and biochemistry of their mineral acquisition, our knowledge about the developmental processes is still scarce. Thus a Representational Difference Analysis have been conducted to identify genes involved in cluster root development.

Among bands differentially expressed, one clone has been further investigated. This gene (*LaTCP1*) encodes a putative transcription factor including a TCP domain. It shares a high homology with At TCP3 and 4 of unknown function, but TCP domain containing proteins such as TB1 of maize, CYC and DICH of *Anthirinium*, are responsible for axillary structure development (petals and branches) and are proposed to modify organ growth. Expression studies demonstrate La TCP1 is root specific, weakly expressed in the whole root system, but specifically induced in emerging rootlets, more specifically in meristems of juvenile cluster roots.

The mRNA sequence shows Auxin Response Elements and domains present in auxin regulated proteins. Furthermore, in lateral roots, an auxin gradient takes place from the tip to the basis, leading to a concentration range in juvenile cluster roots susceptible to induce La TCP1 and rootlets formation. Our findings involve this gene in the initial events of cluster root development and suggest by its homology to TB1 and CYC La TCP1 has a key role in apparition of cluster roots as an adaptation to very poor soils.

Morphology defines cluster roots and constitutes an important feature to increase rhizosphere modifications, but this trait has been poorly investigated so far. This work constitute a first step in knowledge of the molecular aspect of cluster roots development, which would allow a better comprehension of their systematic dispatching and to highlight a unique aspect of plant development.

Résumé

Les racines protéoïdes constituent une adaptation importante en vue de l'acquisition de nutriments, trouvée dans un grand nombre de familles végétales. Ces structures en forme d'écouvillon consistent en des groupements serrés de racines à la taille déterminée.

Alors que la physiologie et la biochimie de l'acquisition des minéraux par les racines protéoïdes sont largement décrites, notre connaissance de leur processus de développement est très limitée. Nous avons donc réalisé une RDA (Representational Difference Analysis) pour identifier des gènes impliqués dans le développement des racines protéoïdes.

Parmi les bandes obtenues et exprimées différemment, un clone a été examiné plus profondément. Le gène correspondant (*LaTCP1*) code un possible facteur de transcription contenant un domaine TCP. Il possède une forte homologie avec At TCP3 et 4 de fonction inconnue. Cependant des protéines incluant un domaine TCP telles TB1 du maïs, CYC et DICH d'Anthirinium, sont responsables du développement de structures axillaires (pétales et branches) et ont été proposées comme modifiant la croissance de ces organes. L'étude de l'expression démontre que La TCP1 est spécifique des racines, faiblement exprimée dans tout le système racinaire, mais spécifiquement induite dans les racines émergentes et plus particulièrement dans les méristèmes des racines protéoïdes juvéniles.

La séquence de l'ARNm contient des éléments de réponse à l'auxine (ARE) et code une protéine contenant des domaines présents dans des protéines régulées par l'auxine. De plus, dans les racines latérales, un gradient d'auxine existe depuis l'apex jusqu'à la base, entraînant une fourchette de concentration dans les racines juvéniles susceptible d'induire La TCP1 et la formation de racines protéoïdes. Nos résultats impliquent La TCP1 dans les évènements initiaux de l'apparition des racines protéoïdes et suggèrent par son homologie à TB1 et CYC que La TCP1 joue un rôle-clé dans l'apparition des racines protéoïdes comme adaptation aux sols très pauvres.

Leur morphologie définie les racines protéoïdes et constitue un aspect important de l'amélioration des modifications de la rhizosphère, mais ce caractère a été délaissé jusqu'à présent. Ce travail constitue un premier pas dans la connaissance de l'aspect moléculaire du développement des racines protéoïdes, ce qui permettrait une meilleure compréhension de leur répartition systémique et d'éclairer un aspect unique du développement des plantes.

Introduction

Cluster roots are bottlebrush-like clusters of rootlets with limited growth along lateral roots of many species of Proteaceae and several other species belonging to Casuarinaceae, Eleagnaceae, Betulaceae, Mimosaceae, Myricaceae, Moraceae and Fabaceae (Neumann and Martinoia, 2002). Cluster rooted plants are particularly well adapted to habitats of extremely low soil fertility such as leach sands, sandstones and laterites present in Western Australia and South Africa, still they are present on all continents. Cluster roots (also called proteoid roots) are related to the intense mobilization of nutrients such as P, which is the major limiting element of plant development on these soils. To manage this purpose cluster roots densely explore a restricted volume of soil and thanks to their morphology completely modify its chemical properties. Due to their particular structure, development and physiology, they are postulated by (Skene, 2000) to be the third major root adaptation for nutrient acquisition after mycorrhizae and N_2-fixing structures.

Cluster root physiology has been recently reviewed by Neumann and Martinoia (2002), Neumann *et al.* (2000) and Skene (2000). In white lupine, along the lateral root, the cluster root follows a precisely described spatial and temporal development (Massonneau *et al.*, 2001, Watt and Evans, 1999 a,b). During the first 1-2 days rootlets are emerging and growing in a simultaneous manner and in a very dense arrangement (between 50 and 1000 rootlets $.cm^{-1}$ root axis). They stop their growth synchronously to reach a predetermined length. Following this, they exhibit the third day an exudative burst (protons, organic acids, phenols and phosphohydrolases) and a higher capacity to transport P_i into root tissues (Hagström *et al.*, 2001 and Neumann and Martinoia 2002). Their metabolism, exudation, secretion, P-transport and reallocation capacities are highly coordinated and they appear to be tightly adapted organs to acquire P in the short time gap allowed Mediterranean rains.

Morphology defines cluster roots and is of importance in their function. But there are some principal differences between plant species and in root cluster morphology, such as length of axis, length and density of rootlets and root hairs, and whether they are simple (unbranched) or compound (branched) (Dinkelaker *et al.*, 1995). All these attributes depend also on plant age and environmental factors. The proportion of clusters of the total root system generally increases with age and may reach 40% to 50% and even 80% in *Leucandendron argenteum* (Dinkelaker *et al.*, 1995). Axis length may range from 10 to 200 mm for one grouping and rootlet length from 3 to 35 mm. In white lupin, which became the model plant to study cluster roots due to its economical importance and its growth fast, cluster roots are simple, the axis is 30 to 40 mm long and rootlets size is 10 ± 5 mm long.

Formation of cluster roots appears to be mainly induced by shortage of P and, in some species by Fe deficiency (*Lupinus albus* and *Consentinii*, *Ficus benjamina*, *Casuarina glauca*, Dinkelaker *et al.*, 1995, Arahou and Diem, 1997). Foliar applications experiments and split root systems demonstrated internal P_i status key-regulates initiation step (Marschner *et al.*, 1987; De Vos *et al.*, 2001).

Some hormonal studies (Gilbert *et al.*, 2000, Skene and James 2001, Neumann *et al.*, 2000), have shown the role of auxins and cytokinins in cluster roots formation. They highlight a promoting effect of auxins application in the culture medium or on foliage and the antagonistic effect of the cytokinin zeatin and also auxin transport inhibitors applied in the growth medium. In accordance, expression analysis revealed a down-regulation of a homologue of IAA (indolacetic acid) glycosyl transferase, and an up-regulation of a zeatin glycosyl transferase, in cluster roots. A role of ethylene has also been postulated but has yet to be defined. Presently, the induction and termination of clustered rootlets is thought to be the consequence of a complex and highly coordinated interaction of different hormones, involving auxin in key steps.

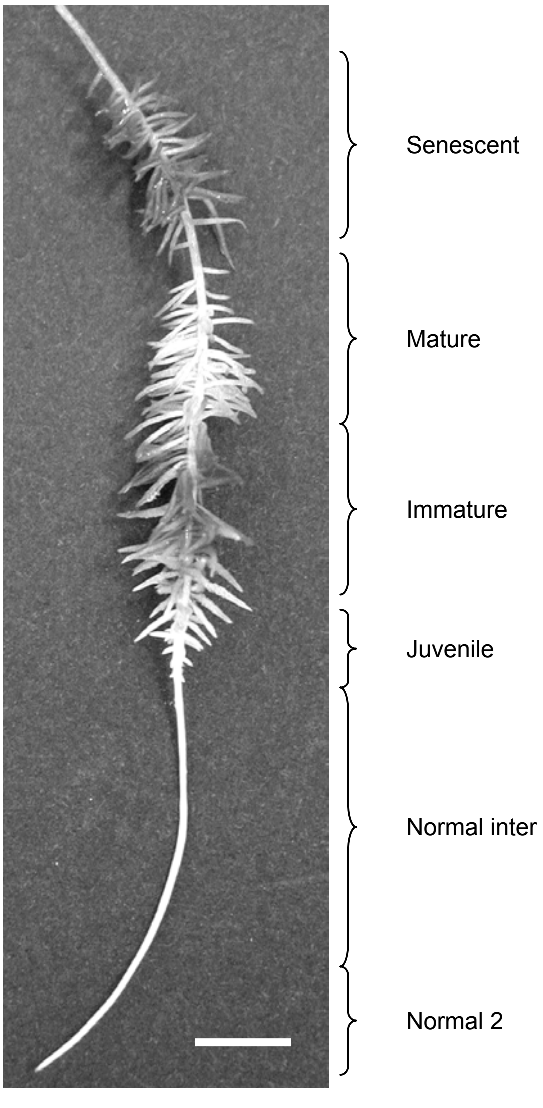
Cluster roots appear to be a powerful model to study several plant biology questions like mineral nutrition, root development or evolutionary adaptation. Hundreds of rootlets (individually very similar to normal root tips) develop simultaneously, in a predictable pattern and all root development stages are present on the same plant at the same time, under identical growth conditions. Comparison of well-defined stages of root development (Massonneau *et al.*, 2001) therefore leads to the identification of genes important for specific developmental or physical stages.

In this context and to give these questions their first cues, it appeared essential to investigate the genetic specificity of cluster roots and more particularly of their initiation step, making the assumption there is a genetic difference with normal roots occurring in the initiation events. We showed in this work, La TCP1 is specifically induced in cluster roots developed under P or Fe deficiency and as other TCP family members is meristem specific.

Results

One of our interests is to understand how plants respond to mineral deficiency in changing their surrounding. In order to cope with phosphorus or iron deficiency some plants are able to form cluster roots, which consist in tight groupings of size-determinated rootlets developing on secondary roots. This unique structure allows plants to explore and drastically modify a restricted volume of rhizosphere by excreting large amounts of organic acids and phenolic compounds.

Fig 1: Different root types harvested on a lateral root of white lupin holding a cluster root. The bar is 1 cm.



This adaptation enables these plants to grow in pioneer environment without mycorrhizal associations. While our comprehension on the physiology and metabolism of cluster roots strongly increased during the last years, very few is known about the development of cluster roots and to our knowledge no molecular approach has been undertaken to identify genes involved in this developmental process. In order to identify genes involved in the early response of cluster root development, we performed a Representational Difference Analysis (RDA) between emerging cluster roots (juvenile) and apex of normal roots (N_2) (fig 1). Two bands could be visualized after two rounds of subtractive hybridization and amplification. The main band consisted in a 545 bp fragment, which was cloned, 25 independent colonies were analyzed and 80% of them corresponded to the same cDNA fragment. The predicted polypeptide showed a high homology with the TCP domain of AtTCP4 and all TCP domain-containing proteins.

The TCP domain is a motif found in proteins regulating plant growth and development, consequently we investigated further the role of this gene in cluster root development. The other band corresponds to malate synthase glyoxysomal and RT-PCR showed this band is highly induced in juvenile cluster roots compared to N2. Since we were interested in developmental processes, its study were delayed.

From the partial cDNA sequence of the major band, we deduced primers to screen a cDNA library synthesized from juvenile cluster roots. The sequencing of six independent clones revealed a cDNA sequence of 2921 bp encoding a predicted protein of only 407 aa (Accession number: AJ426419).

Analysis of La TCP1 *sequence and comparison with other TCPs*

The primary structure of La *TCP1* shown in figure 2, reveals several characteristics. mRNA sequence of La *TCP1* includes very long 5'UTR and 3'UTR, 1260 bp and 440 bp respectively, and the ORF is 1221-bp long. It encodes a predicted 407 aa protein, very similar to *Arabidopsis* AtTCP4 (AP000370.18, 51% of identity and 62% of similarity) and AtTCP3 (At1g53230, 49% of identity and 60% of similarity).

Thus, nearly 60% of La *TCP1* mRNA consist in untranslated regions. These characteristics strongly suggests that the untranslated regions exhibit a regulatory role, similarly as described recently at the transcriptional and post-transcriptional levels in plants (Hua *et al.*, 2001). This hypothesis is strengthened by the presence of five regions in the mRNA, which contain putative auxin response elements (AREs), as described by Guilfoyle *et al.* (1998). One domain of particular interest in the 5'UTR, contains a longer one (Fig. 2, -794 to –766 from ATG) with several repeated and slightly degenerated TGTCTC surrounding a conserved one. On the other hand, Q-rich regions are present in the predicted sequence of La TCP1 with an enrichment of serine and leucine. This has been shown to be part of auxin response factors (ARFs) (Ulmasov *et al.,* 1999), Q-rich regions acting as activation domains. This strongly suggests a relation between auxin and La TCP1 expression on one hand and on the other hand an activator action on auxin-dependent genes.

The TCP domain present in La TCP1 contains all components of TCPs described by Cubas *et al.* (1999) but the R domain is missing. The basic helix loop helix structure visualized in figure 4, could thus allow the regulation of transcription in binding DNA and interacting with homologues and other proteins (Kosugi and Ohashi, 1997 and Suzuki *et al.*, 2001). La TCP1 is very similar to At TCP3 and 4 (fig 3) which have been shown to belong to cyc/tb1 family of TCP domain containing proteins (Cubas, 2002). But similarity between these proteins is not limited to the TCP domain. Their carboxyl terminal domains are also nearly identical, which could be of interest in their functioning. At TCP4 hold Q-rich stretches, whereas AREs are not present in its putative promoter (1500 bp in 5' cis region, 56881 to 58381 of BAC referred as AP000370). On the other hand At TCP3 does not possess Q-rich regions in its predicted aminoacid sequence but 4 AREs are present in its putative promoter (50341 to 51841 of BAC referred as AC008007). These slight differences would make these orthologous proteins different in their function.

To confirm relation between auxin and La TCP1 expression, we determined auxin content in root tissues. In figure 3, auxin concentration is low in non-growing tissues (N1, immature, mature and senescent cluster roots), whereas a gradient occurs from secondary root apex to juvenile cluster roots. N2 auxin content is the highest and greatly varies between both measurements. The variability of auxin levels in lateral roots apices could explain the variations of the distance between apex and juvenile cluster roots. Nevertheless, gradients reach a similar level in juvenile cluster roots. This suggests cluster roots auxin induction would be effective in a particular concentration range (between 2 and 4 nmol $.g^{-1}$ DW), which would induce La *TCP1* transcription.

Fig 3: Auxin contents in roots of P-deficient white lupins. ■ and ▲ correspond to two different harvests. Values are the means and s.d. of three independent replicates except for ▲ and Im, Mat and Sen, where aberrant values were eliminated.

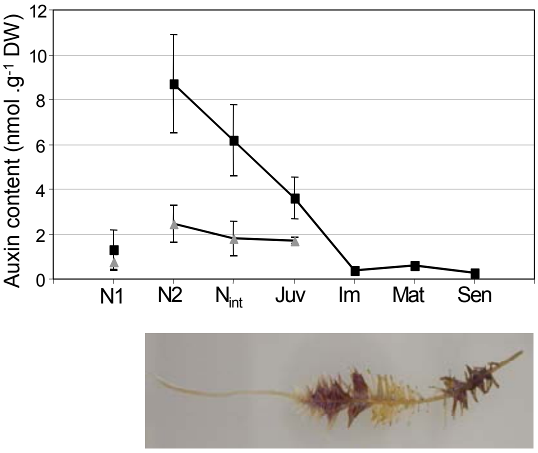


Fig 2: Sequence of La *TCP1* cDNA and its predicted polypeptide traduction. AREs (TGTCTC) and degenerated sequences are shaded. Asparagine (Q) are bold in peptide sequence

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CACGAGCTCGCGTGGCAGAGACACTGGCTTGAGATAAAAAAGAACCAAAGTTTCTAACTTCTAACTAGCAAGTAAAAATT   80
ATAAATTTCCAAAGGAATATAAAAAAATGAAGAAGAGCCAAATCGAGAAAGAGGAAAAATAATAAGAGGGTAGGGTGAAA   160
GTTGAAAGGAAGCAAGTTTTGTGTTATTGGTGATTGAATGAGTTATTATTGATGGGTTGGTGTTGTTTATGTATGTTTAT   240
AGTGTAGTTATTAGTACAAGAAGAAGAGCAGAAAAGGGCAGTGATGAAATCACAGAGATCTTTTAACACCTCCATTCTGA   320
CCAAACACTGCTATCATCATCAAGGGGCTCCCCTTCTTCCTATAAGTCCAGAATGAATTCCTCTTCCCTATAATAGTGAG   400
TGAGAGAGATAGAGGCTTAAATTTATTAACTTACTACTGTTACCTCTTTTTCTTTTCTCTGCCTCATCTTGTGTCTGTTT   480
CTCTGTGTGTTGTGATATGGAAATTTTGCTGCAAATCTTACTAGAGGACACTTCCTCCACTTGGGTGGTTCCCTAAGCCT   560
TGTTTGGCCAAGTGGGTATTAACCAAGCAAAGGCCCCAACCCAATTGTAATTCCATTCAGTTCGGTTAGTCTAGTGCATA   640
AAAGAGCTCAATTTTTAGTATTAAAAAGGCAAAAAGTGGTGTGAAGAAAGAGAGTGTGTGGAAGTGAGAAAATACTGAAA   720
TAGAGAGAAATAGAAAGAGATGGGTTAGGCTGAAAAGAGAGTAGGTATAACAACAACATTGTTGGTCTAGTTTAGTGTTC   800
ATGAGTTAGGGGATTTTTGTGTGTGTGGTGATGGAAGTTGGAAAATTGAATGGCAGGAACAAGTTTTGGGAGAAGCAAAA   880
ACAAGTATCAGAATTGTGGCAGTGGCAGAAAGAGCAGGAACTACAAGAACAAGAAGAGAGCAAAAGCGGGAAGAAGCCAA   960
AGTAGGTTGGAGTTTAAAGAAACAAAAACAACAAGCCAGCAAGAGAAAGCATTCATTAAAAGCTGGAAGAGGAAAATCAC   1040
AACAACAAGGGTCTGAGTCTGGATCCTCAGACGAATCATCATCATCATCATCATCATCAGCATCAGCATCAGCAGCAACA   1120
ACAACAACAACAATCRCTATGTAGGTTTCAACAMAGACTTGCAAYCCCACTTCCAAAAAAACGATCTTTTCTCCTTCCAC   1200
CGCCTCACCCACTTCCTCCCTCGTCATCACCACCTTTCACAATCGAATATGCAAGAAAAATGGGAGAACAACAACAACAA   1280
                                                            M  G  E  Q  Q  Q  Q
CAAACACCAACAACAAGAGGGATGAGGAGTACTCCAACAACAGGAGTTGGAGAAATCGTCGAAGTACAAGGCGGTCACAT   1360
Q  T  P  T  T  R  G  M  R  S  T  P  T  T  G  V  G  E  I  V  E  V  Q  G  G  H  I
TGTCCGGTCCACTGGTCGAAAGGACCGTCACAGCAAAGTATGTACCGCCAAAGGACCAAGGGACAGACGGGTGCGCCTCT   1440
 V  R  S  T  G  R  K  D  R  H  S  K  V  C  T  A  K  G  P  R  D  R  R  V  R  L  S
CAGCCCACACCGCCATCCAATTCTACGACGTCCAGGACCGCCTCGGCTACGACCGCCCCAGCAAGGCCGTCGATTGGCTA   1520
  A  H  T  A  I  Q  F  Y  D  V  Q  D  R  L  G  Y  D  R  P  S  K  A  V  D  W  L
ATCAAAAAAGCCAAGACCGCCATCGACCAACTGGCCGAGCTCCCTGCTTGGAACCCGACCGCTACAATACAACAGCAGCA   1600
I  K  K  A  K  T  A  I  D  Q  L  A  E  L  P  A  W  N  P  T  A  T  I  Q  Q  Q  Q
GCAGCAGCAACAGCAACAGCAAATAAACGACATCCTCCGCCGCGAATCACATGCGACGGAGGATGGACTAATTGGTGGTT   1680
 Q  Q  Q  Q  Q  Q  Q  I  N  D  I  L  R  R  E  S  H  A  T  E  D  G  L  I  G  G  S
CTAGTAGCCGCAGTGCAAATGCGGCTACTACAATCGTGGTGTCTAGTGGTGGAAGGATCTCAGAAGATTTTCATCAACAG   1760
  S  S  R  S  A  N  A  A  T  T  I  V  V  S  S  G  G  R  I  S  E  D  F  H  Q  Q
CAATTTGGTGAAGAGAATAATAACAGTAACAAATATAACAGTGGTGGTTCTGGTTTTCTTCCACCATCTATGGATACTGA   1840
Q  F  G  E  E  N  N  N  S  N  K  Y  N  S  G  G  S  G  F  L  P  P  S  M  D  T  D
CATTGCGGATACGATTAAGTCCTTTTTCCCTATGGTTGCTGCTGCAGAAACGACGACGACGTCGTTTCATAATTATTCAA   1920
 I  A  D  T  I  K  S  F  F  P  M  V  A  A  A  E  T  T  T  T  S  F  H  N  Y  S  T
CACCGCCGGATTTGTTGTCAAGAACTACAAGCGCTGCTGCAAATCATCACAACCACCAGCAAGATCTACGGCTCTCGTTA   2000
  P  P  D  L  L  S  R  T  T  S  A  A  A  N  H  H  N  H  Q  Q  D  L  R  L  S  L
CAATCGTTTCAAGACCCTGTTCTGCTTCACCACCATGGTGAGGAACAAGAACATGACCATGGTCGGTTTCATAGAATGGT   2080
Q  S  F  Q  D  P  V  L  L  H  H  H  G  E  E  Q  E  H  D  H  G  R  F  H  R  M  V
GGCTTGGAATGCTGCTATTGCAGATGCTGCTAACAGTGGACATGGCGGTGGATTTGTCTTTAACTCGCCGCCGCCATCCA   2160
 A  W  N  A  A  I  A  D  A  A  N  S  G  H  G  G  G  F  V  F  N  S  P  P  P  S  T
CGGTGGCGCCGGCTGCGATGTTTGGCCATCATGGCCAGTATTTTTCTCAGAGGGGACCCCTTCAGTCCAGTAACACTCCT   2240
  V  A  P  A  A  M  F  G  H  H  G  Q  Y  F  S  Q  R  G  P  L  Q  S  S  N  T  P
TCGATTCGTGCATGGATAGACCCAAATTCCTTTGCTGCGGCGGCAACTGTTGCCGCGGCCTCCCACCCCCATTATCACCA   2320
S  I  R  A  W  I  D  P  N  S  F  A  A  A  A  T  V  A  A  A  S  H  P  H  Y  H  H
TCACTACCTATCACCGGCGATCCATCAGGCTTCTGTCTCTGGATTCGCCACACCTTCAAGTGGCTTCTCCGGCTTCCGCA   2400
```

```
 H  Y  L  S  P  A  I  H  Q  A  S  V  S  G  F  A  T  P  S  S  G  F  S  G  F  R  I
TTCCAGCACGAATTCAGGGTGAAGAGGAACACGACGGCGTTTCAGACAAGCCGTCCTCTGCTTCCTCTGATTCTCGCCAT   2480
  P  A  R  I  Q  G  E  E  E  H  D  G  V  S  D  K  P  S  S  A  S  S  D  S  R  H
TGATGGAAACACATCTCAAAAATCCAGACTTTCCTTTTCCTTCAGCAACAAGGATTATTCATTCAAGAGCAAGGTGGATT   2560
AGGTGTTAAGTTTGGATTTAAGATGCTGCTTCCTTTTCAATGCCCTAATATAATATGCCTTGGGAATGAAGAAAATCTGG   2640
GTTGAATTTTCAGGTTTTCCAATCCCATGGTTTCTTCTGCTTTTAATCTTATGTTCTATGTTTTTCAATCTTTGACTAGG   2720
ACTACAAGTACTATGTCATATTTTCATGTTTGTTTCAGCTATGTTGGATTCTTATCTTTTCCTATGTTACAATTTGTAGT   2800
AGCTCTGTCTCCTTTGTACTTCTATTTCTGGTTCCTTCTAGTGGTTGGGAAAAACATGAAACACAAAATAACAGTGCTAC   2880
TAGTTTGTTTAATTTATCTTATAAAAAAAAAAAAAAAAAAA                                          2921
```

Fig 4: Alignment of La TCP1, At TCP3 and At TCP4 obtained with the PILE UP program (GCG Center) and edited using Genedoc (Version 2.6.001, Karl Nicholas).

```
                                                                    TCP domain

LaTCP1  :  MGEQQQQQTPTTRGMRSTPTT-----GVGEIVEVQGGHIVRSTGRKDRHSKVCTAKGPRDRRVRLSAHTAIQFYDVQDRLGYDRP  :   80
AtTCP3  :  ~~~~~~~~~~~~~~MRHHQSATENGGGCGEIVEVQGGHIVRSTGRKDRHSKVCTAKGPRDRRVRLSAPTAIQFYDVQDRLGFDRP  :   71
AtTCP4  :  ~~~~~~~~~~~~~~MRHRSTSDAADGGCGEIVEVQGGHIVRSTGRKDRHSKVCTAKGPRDRRVRLSAHTAIQFYDVQDRLGFDRP  :   71


LaTCP1  :  SKAVDWLIKKAKTAIDQLAELPAWNPTATIQQQQQQQQQQQINDILRRESHATEDGLIGGSSSRSANAATTIVVSSGGRISEDFH  :  165
AtTCP3  :  SKAVDWLITKAKSAIDDLAQLPPWNPA----------------DTLRQHAAAA-----ANAKPRKT--KTLISPPPPQPEETEHH  :  133
AtTCP4  :  SKAVDWLIKKAKTSIDELAELPPWNPA----------------DAIR---LAA-----ANAKPRRTTAKTQISPSPPPPQQQQQQ  :  132


LaTCP1  :  QQ---QFGEENNNSNKYNSGGSGFLPPSMDTD-IADTIKSFFPMVAA---AETTTTSFHNY--STPPDLLSRTTSAAANHHNHQQ  :  241
AtTCP3  :  R-----IG-------EEEDNESSFLPASMDSDSIADTIKSFFPV--ASTQ--------QSYHHQ-PP---SRGNT------QNQD  :  186
AtTCP4  :  QQLQFGVGFNGGGAEHPSNNESSFLPPSMDSDSIADTIKSFFPVIGSSTEAPSNHNLMHNYHHQHPPDLLSRTNS------QNQD  :  211


LaTCP1  :  DLRLSLQSFQD--PVLLHH----------------HGE-------------EQEH---DHGRFHRMVAWNAAIADAA-NSGHGG  :  290
AtTCP3  :  LLRLSLQSFQNGPP-----------FPNQTEPALFSGQSNNQLAFDSSTASWEQSHQSPEFGKIQRLVSWNN--VGAAESAGSTG  :  258
AtTCP4  :  -LRLSLQSFPDGPPSLLHHQHHHHTSASASEPTLFYGQS-NPLGFD--TSSWEQ--QSSEFGRIQRLVAWNSGGGGGATDTGNGG  :  290

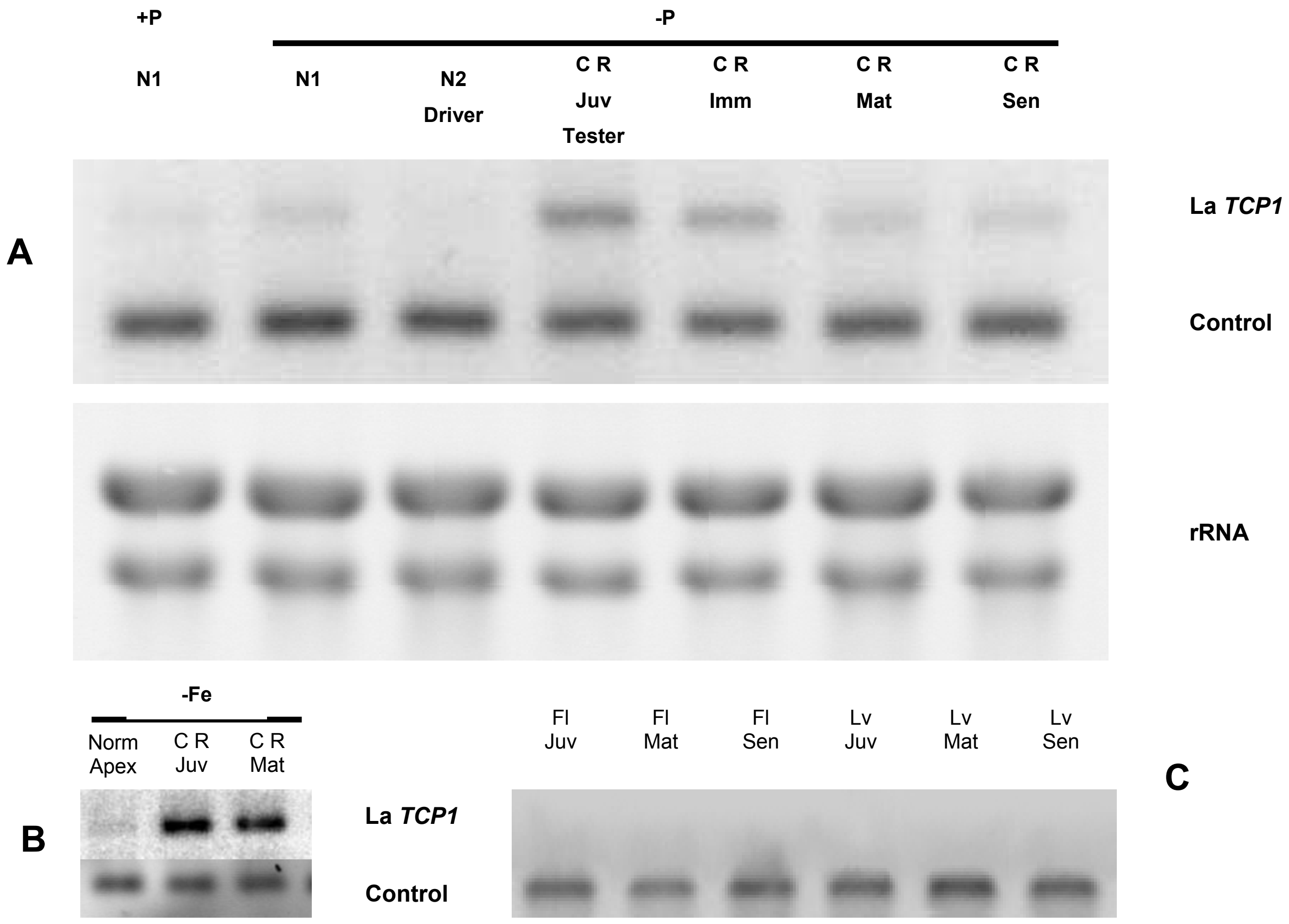

LaTCP1  :  GFVFNSPPPSTVAPAAMFGHHGQYFSQRGPLQSSNTPSIRAWIDPNSFAAAATVAAASHPHYHHH--YLSPAIHQASVSGFATPS  :  373
AtTCP3  :  GFVFASP----SSLHPVYSQS-QLLSQRGPLQSINTPMIRAWFDPHHHHHHHQQSMTTDDLHHHHPYHIPPGIHQSAIPGIAFAS  :  338
AtTCP4  :  GFLFAPPTPSTTSFQPVLGQSQQLYSQRGPLQSSYSPMIRAWFDPHHHH----QSISTDDLNHHH--HLPPPVHQSAIPGIGFA-  :  368


LaTCP1                :                    SG--FSGFRIPARIQG--EEEHDGVSDKPSSASSDSRH                   :       407
```

Analysis of La TCP1 *mRNA expression*

La *TCP1* expression has first been checked under ±P conditions. Figure 4A shows that La *TCP1* is present in all root tissues at low expression level. To ensure specificity of the signal, RT-PCR fragments amplified in juvenile and immature tissues have been cloned and sequenced; they corresponded to

La *TCP1*. However this non-quantitative 35 cycles RT-PCR, showed a significant upregulation of mRNA level in juvenile and immature cluster roots. This clearly confirmed RDA results in amplification of genes expressed in a very different manner. Nevertheless, the essential information is that La *TCP1* is not related to growth and lateral root development, but specifically to cluster root initial events because it is not significantly expressed in growing apex of lateral roots (N2). Since cluster roots can be induce in white lupin by iron deficiency too, we checked whether La *TCP1* is not induced by phosphorus starvation but linked to cluster root formation by analyzing La *TCP1* under Fe stress. In accordance to P deficiency induced cluster roots, expression levels shown in figure 4B confirmed La tcp1 was specifically induced in cluster roots.



All homologues of La TCP1 studied so far, PCF1 and 2, TB1, CYC, DICH, At TCP2 and 3 (Kosugi and Ohashi, 1997, Doebley *et al.*, 1997, Luo *et al.*, 1999 and Cubas *et al.*, 1999) are expressed in shoot meristems. Thus, we performed RT–PCR expression studies on leaves and flowers, differentiating between young, mature and senescent stages of their development to avoid dilution of the transcript, which would have lead to a negative result. Absolutely no signal was obtained in these tissues, La TCP1 is thus certainly root specific, and upregulated in cluster roots formation.

Another characteristic of TCPs is their specific expression in shoot apex and axillary meristems (TB1, CYC, DICH, At TCP1, 2 and 3). The detailed expression pattern of La *TCP1* in P deficient roots shows a high expression during emergence and growth of rootlets (juvenile) and at the meristem differentiation step when rootlets stop to grow (immature). Localization of La *TCP1* expression was thus of particular interest to precise this question. Consequently, we performed *in situ* hybridizations in juvenile and immature cluster roots, the sole tissues showing strong expression.

La *TCP1* is expressed in the very first emergence of rootlets (in fig 6 D and E) where the rootlet has not yet emerged from the lateral root. Very low expression in immature rootlets specified the expression timing of La *TCP1*. The signal observed in -P cluster roots could be explained by the high amplification of 35 cycles RT-PCR and by a contamination of growing rootlets in immature cluster roots during harvest. Nevertheless, La *TCP1* is expressed specifically in cluster rootlet meristems, during their early initiation steps. This is in accordance with expression patterns of previously studied TCPs expressed in axillary meristems (sepals, petals in flowers, branches and leaves).

Since fixation of these tissues is very delicate, mainly in non-meristematic tissues with very large vacuoles, we controlled our hybridizations using the sense probe (negative control, fig 6A) and an anti-sense probe of a highly and constitutive gene, a homologue to L13a ribosomal protein (positive probe, fig 6B). We could observe a strong signal in meristematic zones of early emerging rootlets (fig 6 C, D, E) within 24h. During the same time, no signal was visible in immature rootlets (fig 6 F, G), but after weeks of reaction a slight signal appeared (fig 6H). This confirms that fixation protocol for these tissues allowed the detection of La *TCP1* expression.

Transgenic* Arabidopsis *overexpressing LaTCP

Arabidopsis thaliana have been transformed with pCAMBIA1301 CaMV 35S : La *TCP1* construction instead of GUS, allowing a constitutive over-expression of La TCP1. Twenty-one independent transformed plants have been isolated in the first generation. The analysis of these plants and of the following generation, on the genomic and expression levels, showed that all these plants possessed the correct construct but did not expressed La *TCP1*. In accordance to this, T2 homozygote plants checked by segregation of hygromycin resistance in T3 generation, did not exhibit special phenotypes at the root, flower or branching levels. This silencing result found on an important number of independent lines, indicates a very important role of homologues of La TCP1 certainly in meristem activity and shows their spatial and temporal expression regulation is crucial for plant viability.

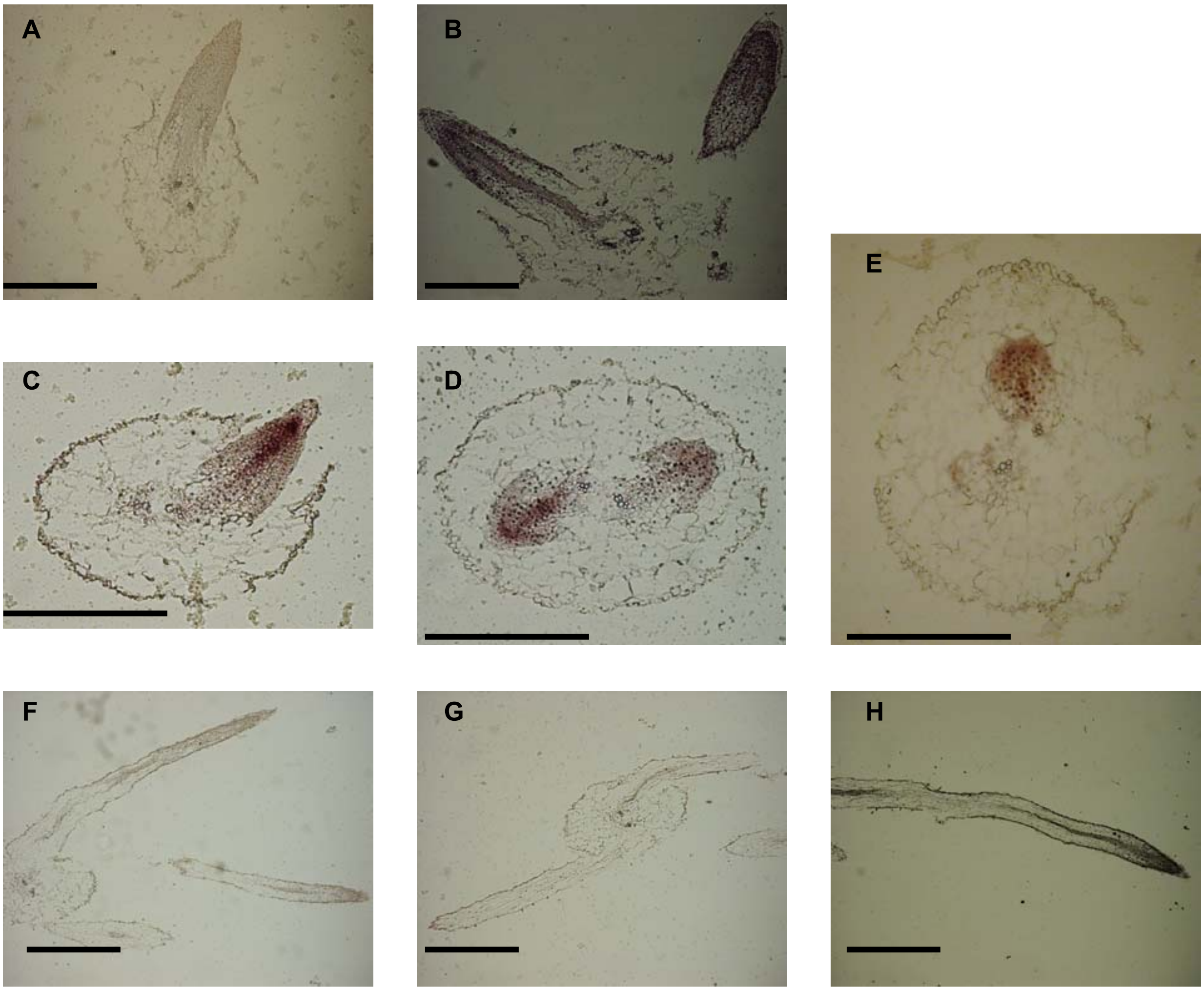


Fig 6: Localization of La TCP1 in –P cluster roots of white lupin by *in situ* hybridization. Sections of juvenile (A-E) and immature (F-H) cluster roots were hybridized with sense labeled-RNA of La TCP1 (A), with antisense labeled-RNA of L13a ribosomal protein homologue (B) and with antisense labeled-RNA of La TCP1 (C-H). Phosphatase reaction was stopped after 24h except in H after 4 weeks. Bars represent approximately 500µm (A-E) or 1 mm (F-H).

Discussion

Cluster roots are important root adaptation; these bottlebrush-like structures intensely mobilize nutrients (P, Fe and other metals) exhibiting a complete set of strategies to acquire notably P from all sorts of soil sources via a highly coordinated development. The morphological advantage of clustered structure is to increase root surface (multiplied by root hairs) not to improve absorption because of the overlapping of the rhizosphere zones, but to increase the capacity of plant to modify the chemical properties of a soil patch allowed by this overlapping. This aspect is significant, if we consider the concentrations of organic acids around roots (more than 580 µM around cluster roots and down to 0.1 µM around other roots, Ryan and Delhaize, 2001) and the millimolar citrate concentrations theoretically required to effectively desorb phosphate from complexes in the soil. Research has increased in cluster root area during the last decade to understand the physiological mechanisms involved in P and metal mobilization and acquisition, but the developmental aspects of cluster roots had not been investigated in detail so far.

Molecular specificity of cluster root is not *a priori* evident because cluster rootlets are identical to lateral roots, and the difference only consists in the clustered arrangement and their determinated size. Indeed, these points could be considered as extreme adaptations of preexisting capacities of plants to induce lateral roots and to differentiate meristems. However, our results show that cluster roots express specific genes, which are very likely to play a central role in the development of this specific structure. Consequently, cluster roots do not consist in a compilation of nutrient deficiency responses already present in other plants, but in special structures with a specific molecular program leading to a cascade of developmental stages that exhibit highly efficient strategies improving plant nutrition.

Two characters define cluster roots, their morphology and their specific physiology. The interdependence relation between both traits has to be systematically investigated, but several evidences claim in favor of dissociated pathways. Gilbert *et al.* (2000) demonstrated auxin-induced cluster roots do not exhibit upregulation of PEPC and MDH as their P-induced counterparts. In accordance, several studies (reviewed by Neumann and Martinoia, 2002) showed P-mobilization and acquisition responses were not restricted to cluster roots even if they are more strongly represented, indicating more a quantitative than a qualitative difference. The identification of a molecular program (with La *TCP1* as a first step) related to the morphological trait could allow the distinction between specific cluster root related phenomenon and P-induced or Fe-induced responses, and consequently an accurate comprehension of the pathways which undergo cluster root development.

La *TCP1* belongs to a family involved in the evolution of morphological characters in Angiosperms. TCP genes control meristematic activity and thus determine the presence of an organ in response to hormonal status. TB1 is responsible for differences in apical dominance and ear development between teosinte and maize. The pole phenotype of maize is indeed due to a higher expression of TB1 in axillary buds preventing their outgrowth and leading to ear development. Furthermore, nucleotide polymorphism in *TB1* showed the selection of farming affected the gene's regulatory regions and not the protein-coding region (Wang *et al.*, 1999). In parallel, in the laboratory of E. Coen the floral asymmetry was studied, focusing on *CYC*. They demonstrated that *CYC* and *DICH* (two members of TCP family) are responsible for a series of steps in developing meristems controlling dorsoventral asymmetry of petals (Luo *et al.*, 1999). They characterized a natural *CYC*-homologue mutant of *Linaria vulgaris* where fundamental symmetry of the flower is changed from bilateral to radial. This mutated gene is transcriptionally silenced because of extreme methylation, a change in a TCP expression regulation leads once again to a variation of morphology. Other studies on TCP members as AtTCP1-3 or PCF1,2 do not explore their morphological function even if they show their expression in flowers and leaves respectively, implying their role in meristematic activity and development of these organs.

TCP genes (*CYC*, *DICH* and *TB1*) regulate morphological traits that are related to auxin, like apical dominance or lateral organs initiation and development in apical meristems (Reinhard *et al.*, 2001). Cluster roots are lateral organs and moreover, La *TCP1* possesses AREs in the regions surrounding the translated sequences and ARFs characteristics as Q-rich regions in the protein sequence. As auxin induces cluster roots in the presence of phosphate (Neumann *et al.*, 2000), La TCP1 is proposed to act as a relay between the auxin signal and the production of rootlet meristem. The presence of both ARE and ARF characters, suggests the following mechanism. Present at very low levels in all roots, particular auxin concentrations may induce La TCP1 and/or activate it in a primary/early auxin response event (as an ARF). Then, in a positive feedback control, LaTCP1 could auto-stimulate its own production via AREs in its regulatory regions. This would lead to the accumulation of the transcript observed in juvenile rootlets and the subsequent La TCP1 could act in the initiation events of meristem production and development.

Due to the enormous data obtained from genomic studies, it is of fundamental importance to relate genes and genomic sequences to particular function and phenotypes. In this study, we strongly correlate induction of La *TCP1* mRNA expression to initiation of cluster roots and we assume this gene possesses a key role in their development. Orthologous genes like AtTCP3,4 and OsTCP3 cannot be linked to this function, since *Arabidopsis thaliana* and *Oryzum sativa* are not able to produce cluster roots, but may help to understand the ancestral function of the La *TCP* gene. Additionally the high similarity of *Arabidopsis thaliana* and *Oryzum sativa* proteins with La TCP1 reinforce the idea that amino-acid sequence does not constitute the sole functional element, but that regulatory regions also play a central role. In the case of *TCP*s, as it has been shown for *TB1* and *CYC* and as we postulate for La *TCP1*, the regulation of the expression in a new spatial and temporal pattern leads to a new phenotype and in the latter case to the appearance of a new specific root structure.

This raises the question of evolution of cluster roots phenotype in *Lupinus* family. The *Lupinus* genus is composed of some 500 New World species and 12 old world species. An essential point is only Old World species develop cluster roots. Between the three clades dividing these species, it appears that only two produce cluster roots even if a controversy exists on *L. luteus* ability to form these structures. This suggests cluster root adaptation would occur during Tertiary (when repeated pluvial events and glaciations would have leached and impoverished soils) in a clade with a narrow genetic base and in a closely related group of species. Study of nucleotide diversity of La *TCP1* and regulating regions, appears now to be a key aspect to answer evolutionary developmental biology question of cluster root adaptation within *Lupinus* genus but also in other families and genus.

Materials and Methods

Plant material

Growth of white lupin (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, Rastatt, Germany) in presence (+P) or absence (-P) of P source has been previously described (Massonneau *et al.*, 2001). Fe deficient white lupines were grown one week after sowing in +Fe conditions and then transferred to a -Fe medium for one week before harvest.

For auxin induction of cluster roots, four weeks-old white lupines were exposed to indolacetic acid at 0.1 mg $.l^{-1}$ during the indicated time. The complete growth medium, supplemented with IAA, was changed every 24h.

Plants were all grown at 22°C and 65% relative humidity with a light period of 16 h at 200 µmol $.m^{-2}.s^{-1}$.

Harvest of different root parts

The different stages of proteoid roots were harvested as described by Massonneau *et al.* (2001) on five week-old plants. Normal roots were separated between the apex (1 cm) of slow-growing secondary roots placed in the deep part of the root system (N_1), the apex (1cm) of fast growing secondary roots in the upper part of the root system, holding clusters (N_2) and the segment of normal root between N_2 and juvenile cluster roots (N_{int}) for auxin determination assays. In order to differentiate the developmental stages of root clusters, the root system was immersed in a pH-indicator solution, which indicates acidification in mature cluster regions (Neumann *et al.*, 1999). Juvenile cluster roots are emerging and growing; immature cluster roots are fully-grown and are not acidifying, they are placed between juvenile and mature cluster roots. Mature cluster roots are also fully-grown and strongly acidify their surrounding; senescent cluster roots are brownish and non-acidifying clusters placed between mature cluster roots and the primary root.

In Fe deficient plants, only apex (5mm) well separated from cluster rootlets, and the following cluster, divided in growing rootlets (juveniles) and fully-grown ones (matures), were harvested.

Auxin assays

Auxin tissue contents were performed by gas chromatography mass spectrometry as described previously (Müller and Weiler, 2000).

Representational Difference Analysis

A RDA according to Hubank and Schatz, (1994), was conducted between juvenile cluster roots and growing apex of normal growing roots (N2), using the following modifications: primer-linkers were HPLC purified and purification steps were performed using Amicon PCR-Microcon columns.

Gel-extracted bands were cloned in pGEM-T Easy vector (Promega) following manufacturer's protocol. Preparations of plasmids were digested using two 4-bp cutting enzymes and restriction length polymorphism was compared to inserts length to define families of clones representing the bands emerging from the RDA. Approximately one third of clones per family was sequenced and identified.

Construction of the cDNA library from juvenile cluster roots and screening

The library was constructed from mRNA isolated from juvenile cluster roots. cDNAs synthesis and cloning were conducted using the ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, Amsterdam, The Netherlands, catalog #200400 and catalog # 200450). The screening of *LaTCP1* was performed using a PCR approach. Primers were deduced from the sequence of the RDA clone corresponding to the major family and including a TCP domain. Primers 5' ATTTGCTGTTGCTGTTGCTG3' and 5' CACCACCTTTCACAATCGAA3' amplified a 415-bp fragment. Six independent clones have been completely sequenced and showed approximately the same size, the longest being 2921 bp.

RT-PCR

10^6 copies of pAW109 mRNA (Perkin Elmer, Applied Biosystems, Foster City, USA) was mixed with 1 µg of total DNA-free RNA and reverse transcription was performed as described by Massonneau *et al.* (2001). 1/100 of the RT reaction product was used to perform PCR. Two sets of primers were used in the same tube, one specific for pAW109 (AW112 and AW113, amplifying a 301-bp fragment) and one for *LaTCP1* (same primers than for library screening). 35 cycles were performed using an annealing temperature of 60°C. PCR products were separated on agarose gel and visualized with ethidium bromide. Furthermore, controls with the constitutive ribosomal protein L13a were performed in the same conditions using $^{5'}$TGCTAGGTCGTCTTGCTTCA$^{3'}$ and $^{5'}$GAGCAAGAGCATGTTCACCA$^{3'}$.

In situ *hybridisation analysis*

The methods for digoxygenin labeling of RNA probes, tissue preparation, and *in situ* hybridization were described previously (Vernoux *et al.*, 2000). The probe used to detect the La *TCP1* transcript was synthesized using the complete cDNA of LaTCP1 in the plasmid pBluescript SK(-) (Stratagene) predigested by Not I and transcribed by T7 RNA polymerase (sense probe), or by Kpn I and transcribed by T3 RNA polymerase (antisense probe). To detect the ribosomal protein transcript, the probe was synthesized using a fragment of cDNA obtained from the RDA inserted the plasmid pGEM-T Easy (Promega). The plasmid was predigested by Apa I and transcribed by SP6 RNA polymerase. It shows 92% of identity on 132 amino-acids with the ribosomal protein L13a of *Lupinus luteus* (O49885). All probes preparation and detection were performed using Roche enzymes, labeling and detection kits.

Transgenic plants

Agrobacterium tumefaciens-mediated transformation of *Arabidopsis thaliana* (WS2) by flower dipping was performed using conventional protocols. Plasmids were constructed by substitution of GUS coding sequence by La *TCP1* coding seqence in pCAMBIA 1301 vector.

References

Arahou M and Diem HG 1997 Iron deficiency induces cluster (proteoid) root formation in *Casuarina glauca*. *Plant Soil* **196**:71-79.

Cubas P 2002 Role of TCP genes in the evolution of morphological characters in Angiosperms. In *Developmental genetics and plant evolution* (ed, Q. Cronk, R. Bateman and J. Hawkins), in press, Taylor and Francis Ltd, London.

Cubas P, Lauter N, Doebley J and Coen E 1999 The TCP domain: a motif found in proteins regulating plant growth and development. *Plant J.* **18**(2): 215-222.

De Vos M, Shane MW, Cawthray GR, Veneklaas EJ and Lambers H 2001 Internal phosphorus concentration modifies the initiation, growth and functioning of cluster roots in Hakea prostrata R. Br. In *Plant Nutrition. Food security and sustainability of ago-ecosystems through basic and applied research* (Horst WJ *et al.* eds) pp 578-579, Kluwer.

Dinkelaker B, Römheld V and Marschner H 1989 Citric acid excretion and precipitation of calcium citrate in rhizosphere of white lupin (*Lupinus albus* L.). *Plant Cell Envir.* **12**: 285-292.

Doebley J, Stec A and Hubbard L 1997 The evolution of apical dominance in maize. *Nature* **386**: 485-488.

Dinkelaker B, Hengeler C and Marschner H 1995 Distribution and function of proteoid roots and other root clusters. *Bot. Acta.* **108**: 183-200.

Gilbert GA, Knight JD, Vance CP and Allan DL 2000 Proteoid root development of phosphorus deficient lupin is mimicked by auxin and phosphonate. *Ann. Bot.* **85**:921-928.

Guilfoyle T, Hagen G, Ulmasov T and Murfett J 1998 How does auxin turn on genes? *Plant Physiol.* **118**: 341-347.

Hagström J, James WM and Skene KR 2001 A comparison of structure, development and function in cluster roots of *Lupinus albus* L. under phosphate and iron stress. *Plant Soil* **232**: 81-90

Hua X-J, van de Cotte B, Van Montagu M and Verbrugge'n N 2001 The 5' untranslated region of the *At-P5R* gene is involved in both transcriptional and post-transcriptional regulation. *Plant J.* **26**(2): 157-169.

Hubank M and Schatz DG 1994 Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* **22**: 5640-5648.

Kollmeier M, Dietrich P, Bauer CS, Horst WJ and Hedrich R 2001 Aluminum activates a citrate-permeable anion channel in the aluminum-sensitive zone of the maize root apex. A comparison between an aluminum- sensitive and an aluminum-resistant cultivar. *Plant Physiol.* **126**:397-410.

Kosugi S and Ohashi Y 1997 PCF1 and PCF2 specifically bind to *cis* elements in the rice proliferating cell nuclear antigen gene. *Plant Cell* **9**: 1607-1619.

Lamont B 1972 The morphology and anatomy of proteoid roots in the genus *Hakea*. *Aust. J. Bot.* **20**: 155-174

Luo D, Carpenter R, Copsey L, Vincent C, Clark J and Coen E 1999 Control of organ asymmetry in flowers of *Antirrhinum*. *Cell* **99**: 367-376.

Marschner H, Röhmeld V and Cakmak I 1987 Root-induced changes of nutrient availability in the rhizosphere. *J Plant Nutr* **10**: 1175-1184.

Massonneau A, Langlade N, Léon S, Smutny J, Vogt E, Neumann G and Martinoia E 2001 Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.): relationship between organic acid excretion, sucrose metabolism and energy status. *Planta* **213**, 534-542.

MüllerA and Weiler EW 2000 Indolic constituents and indole-3-acetic acid biosynthesis in the wild-type and a tryptophan auxotroph mutant of *Arabidopsis thaliana*. *Planta* **211**: 855-63.

Neumann G and Martinoia E 2002 Proteoid roots – an underground adaptation for survival in extreme environments. *Trends Plant Sci* in press.

Neumann G, Massonneau A, Langlade N, Dinkelaker B, Hengeler C, Römheld V and Martinoia E 2000 Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). *Ann. Bot.* **85**, 909-919.

Reinhardt D, Mandel T and Kuhlemeier C 2000 Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell* **12**: 507-18.

Ryan PR and Delhaize E 2001 Function and mechanism of organic anion exudation from plant roots. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **52**: 527-560.

Skene KR 2000 Pattern formation in cluster roots. Some developmental and evolutionary considerations. *Ann. Bot.* **85**: 901-908.

Skene KR 2001 Cluster roots: model experimental tools for key biological problems. *J. Exp. Bot.* **52**:479-485

Skene KR and James WM 2000 A comparison of the effects of auxin on cluster root initiation and development in *Grevillea robusta* Cunn. Ex R. Br. (Proteaceae) and the genus *Lupinus* (Leguminosae). *Plant Soil* **219**:221-229.

Suzuki T, Sakurai K, Ueguchi C and Mizuno T 2001 Two types of putative nuclear factors that physically interact with histidine-containing phosphotransfer (Hpt) domains, signaling mediators in His-to-Asp phosphorelay, in *Arabidopsis thaliana*. *Plant Cell Physiol.* **42**(1): 37-45.

Ulmasov T, Hagen G and Guilfoyle TJ 1999 Activation and repression of transcription by auxin-response factors.Proc *Natl Acad Sci U.S.A.* **96**: 5844-5849.

Vernoux T, Kronenberger J, Grandjean O, Laufs P and Traas J 2000 PIN-FORMED 1 regulates cell fate at the periphery of the shoot apical meristem. *Development*. **127**: 5157-5165.

Wang R, Stec A, Hhey J, Lukens L and Doebley J 1999 The limits of selection during maize domestication. *Nature* **398**: 236-239.

Watt M and Evans J 1999a Linking development and determinancy with organic acid efflux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO_2 concentration. *Plant Physiol*. **120**: 705-716.

Watt M and Evans J 1999b Proteoid roots. Physiology and development. *Plant Ph*ysiol **121**: 317-323.

CONCLUSION AND PERSPECTIVES

This work focuses on the physiology and development of cluster roots developed by white lupin under phosphorus deficiency. Phosphorus is present under two forms in the soil, the organic and the inorganic phosphate. Since only a minor part of inorganic phosphate is free and available to plants, plants evolved different mechanisms to transform both pools into free inorganic phosphate. Cluster roots, which are produced when P is scarce, are specialized organs able to mobilize very efficiently every pool of phosphate (Figure 1).

These root structures are defined by their original morphology. They are composed of dense groupings of size-determinated rootlets along a parent root. This morphological particularity allows the plant to explore a limited soil region in an extremely intensive manner. Furthermore, it strengthens the accumulation effect necessary for root exudates to liberate Pi from organic and inorganic pools of phosphate.

The physiology of cluster roots during their development appears to be directed towards two main aspects: an intense acidification of the rhizosphere, which solubilize Ca-Pi and a dramatic exudation of malate and citrate, which solubilize Ca-, Al- and Fe-Pi. In addition, cluster roots mobilize also organic phosphate via the secretion of an acid phosphatase.

Malate and citrate exudation by cluster roots during their development exhibit several characteristics studied in details in this work. During cluster root development, the nature of the major organic acid exuded changes from malate (in juvenile rootlets) to citrate (in mature and senescent rootlets). Secondly, cluster roots exude extremely important amounts of carbon skeletons as malate and citrate in the rhizosphere. This raises the question of the origin of these skeletons. A third characteristic of organic acids exudation in cluster roots is the important exsudative burst occurring during the mature stage, which correlates with the rhizosphere acidification. These three main points structured this work but complementary aspects such as transport of citrate and phosphate were also taken into account.

Finally, since cluster roots are defined by their morphology, it appeared essential and complementary to investigate their morphological development. More precisely this work constitute a first brick in the understanding of the molecular processes triggering their initiation.

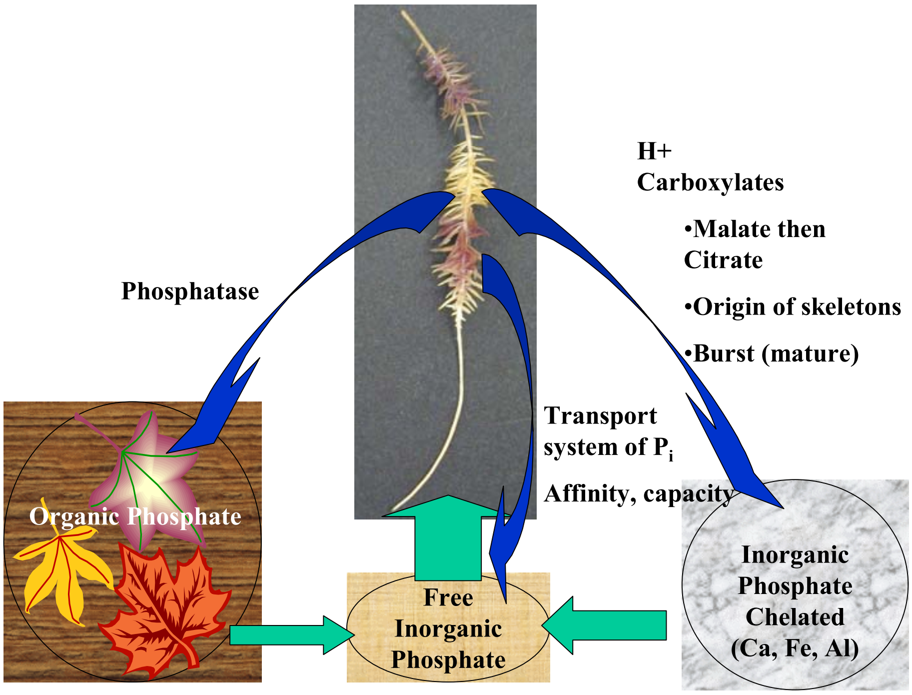


Figure 1: Cluster roots of white lupin mobilize the 3 pools of P present in the soil via secretion of phosphatase and exudation of protons and carboxylates. The affinity and capacity of the transport system of inorganic phosphate liberated from organic and chelated inorganic pools is also increased. Characteristics of organic acid exudation developed in this work namely the transition of the main carboxylate exuded, the origin of carbon skeletons and the exudative burst in mature are précised.

Transition from malate to citrate as the major organic acid exuded

During their growth, rootlets constitute high sinks for carbon skeletons due to their higher metabolic activity. The growth activity requires synthesis of acetyl-CoA for the biosynthesis of membrane lipids by ATP citrate lyase, which thus transforms citrate into malate. ACL is composed by two peptides, both necessary for activityas shown by heterologous expression in *Saccharomyces cerevisiae*. This activity determines the nature of organic acids exuded, explaining the predominance of malate in juvenile cluster root exudates and of citrate at the mature stage. Once rootlets have reached their final length (about 2 to 4 days after emergence), ACL activity declines and citrate becomes the main organic acid released (chapter 3).

Origin of organic acids C skeletons

While in growing tissues P_i content is relatively high, a translocation of P occurs rapidly to feed newly formed roots. This reallocation of sparingly available P is crucial and very efficient, since 97% of the total RNA present in juvenile cluster roots of white lupin is absent in senescent cluster roots. This leads to highly reduced concentrations of P_i and nucleotides in fully-grown rootlets causing substantial changes in metabolism and inhibiting respiration. Under these conditions, plants switch their metabolism to pathways which are less dependenton P. Glycolytic pathway enzymes, which are sensitive to P_i or nucleotide levels (invertases), can be bypassed by alternative pathways (sucrose synthase). This allows a simultaneous C overflow to occur. Carbohydrates catabolism, via a transcription and *in vitro* activities upregulation, of sucrose synthase, phosphoglucomutase and fructokinase (figure

2), and anapleurotic carbon fixation (phosphoenolpyruvate carboxylase) produce an accumulation of carboxylates, which cannot be respired.

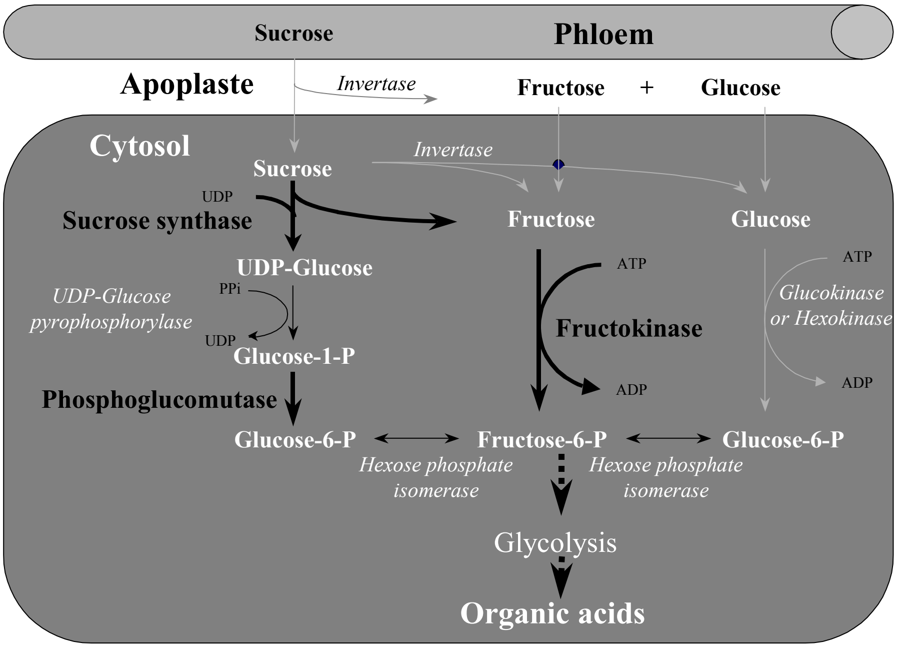


Figure 2 : First steps of sucrose hydrolysis. Sucrose Synthase pathway is induced during the development of cluster roots and explains partially the origin of organic acids accumulated and exuded in the mature stage. This PPi-

Other metabolic alterations leading to citrate accumulation and citrate exudative burst

Due to the activation of citrate synthase and the slight inhibition of aconitase, citrate concentration can reach up to 30 µmol per gram of fresh root. This over accumulation of citrate in cluster roots of *Lupinus albus* permits maximal exudation rates of 7 µmol per hour and per gram of fresh root, possibly as a detoxifying process. Moreover NADH/NAD+ ratio is predicted to increase and the alcohol dehydrogenase could avoid over reduction of cell cytosol. In lupins grown hydroponically and without P, use of pH indicator show a restricted cluster root zone where surrounding acidification occurs. Acidification of cytosol is proposed to activate plasmalemma H^+-ATPases in order to regulate internal proton concentration, turning the colour of the applied pH indicator. Simultaneously to this acidification, this root type, called mature cluster root (described in chapter 2), is the place of the highest citrate exudation whereas its internal citrate content is similar to those of immature and senescent cluster roots. Moreover, acidification of the cytoplasm induces very high exudation rates in all cluster roots. This suggests that the pH has an important regulation role in the exsudative burst.

Citrate transport process

The organic acids exudation is not strictly correlated to internal concentration (discussed by Ryan and Delhaize, 2001), malate, citrate and other organic acids can accumulate to high concentrations in the vacuole but exudation of these molecules ultimately involves a last regulatory step, their transport from cytosol to the external compartment. Three possible pathways can lead to organic acid release: their trans-membrane diffusion, exocytosis and transport via membrane proteins. Very little is known about diffusion and exocytosis in organic anions release, but convincing evidence indicate that anion channels are involved in organic acids exudation from plant cells and notably in cluster roots (Neumann *et al.*, 1999; Kollmeier *et al.*, 2001).

Neumann and co-workers, in 1999, have shown that anion channels inhibitors such as anthracene-9-carboxylic acid partially inhibits citrate efflux in *Lupinus albus* cluster roots. The author confirmed this result (data not shown) limiting it to mature cluster roots. This shows clearly anions channels support a part of the citrate exudation. No plant organic acids channels have been cloned yet and the release mechanisms are largely unknown unless they constitute a very promising area for improvement of plant P-deficiency and Al-toxicity tolerance.

Free inorganic phosphate acquisition

Once root exudates have solubilized phosphorus, P_i must be actively transported into the root cells. Despite energy limitation, mature and even senescent cluster roots of *Lupinus albus* are able to take up significant amounts of P_i at higher rate than normal roots and to counteract a important chemical gradient (chapter 1 and Neumann *et al.*, 1999).

Cluster roots morphology

On the other hand, evolutionary questions are raised considering the apparition of this adaptation in a wide range of distantly related families. Indeed neither every genus in the pre-cited families, nor every species in these genuses, produce cluster roots, but cluster-rooted plants belong to a limited number of ecotypes. *Lupinus* genus phylogeny has been related to cluster root occurrence, pointing towards a single origin leading to cluster root formation ability in only some species of the 12 Old World ones. However, Skene (2000) soundly enlighten that cluster roots developed among the whole plant kingdom should not be identical in respect to their two key aspects, physiology and morphology. In this context and to give these questions their first cues, we identified a putative transcription factor (La TCP1) specific to meristems of emerging cluster roots. Since La TCP1 is possibly related to auxin response and belongs to a family involved in morphological traits appearance during evolution, La TCP1 is proposed to be responsible for cluster roots formation in white lupin.

Perspectives

This work answers a few questions but mostly raises new perspectives of research. Citrate synthesis in cluster roots is an extraordinary process in regards to its accumulation levels in immature to senescent cluster roots. Origins of C skeletons already described (from photosynthesis source via sucrose and from anapleurotic source via PEPC) are likely to furnish C for citrate exudation. However, another source is possibly involved and under investigation at the moment. Senescence is thought to start in the immature stage already, shown by the reallocation of nutrients and the smoothening (related to methyl esterase induction, Diploma Thesis of G. Messerli) of the tissues. Related to senescence, glyoxylic cycle is thought to be induced and could thus provide C skeletons for citrate synthesis.

In addition, organic acids metabolism once excreted into the rhizosphere is poorly understood. A detailed knowledge of the microbial populations in the cluster root environment could reveal that bacteria play a role in plant nutrient acquisition or/and in cluster root induction (proposed by L. Weisskopf in her Diploma Thesis). At the same time plants should avoid a too strong microbial development, which could weaken the accumulation effect of organic acids. In accordance to this, a glucanase and a chitinase are induced in cluster roots (Diploma Thesis of G. Messerli), however they could also be due to non-specific induction related to senescence. Interactions between cluster roots and microbial populations are of fundamental importance to understand the global functioning of these unique root structures.

Finally, I would like to point out the very promising and exciting area unveiled by La *TCP1* characterization. Since La TCP1 is related to formation of cluster roots and only induced in these tissues, it constitutes a very powerful tool to investigate the specificity of cluster roots. The understanding of its specific induction and of its promoting elements, the characterization of proteins interacting with it and of its transcriptional regulator activity will be of great interest in order to dissect the mechanisms leading to cluster root formation. Furthermore, expression analysis in non-cluster-rooted *Lupinus* species or close relatives such as *Melilothus* may confirm if LaTCP1 is a key factor in cluster root formation. On the evolutionary level, comparison of homologues and orthologues of La *TCP1* between *Lupinus* species could confirm mutations in the promoting elements lead to a new expression pattern and gave rise to a new morphological trait, as it has been shown for *TB1* in maize and teosinte. In addition, research of La *TCP1* homologues in other cluster-rooted species (such as in the well-described *Hakea* genus) could indicate if this gene has also been responsible for cluster root appearance in these very different species, making all cluster roots a common structure, among these very divergent plants.

References

Kollmeier M, Dietrich P, Bauer CS, Horst WJ and Hedrich R 2001 Aluminum activates a citrate-permeable anion channel in the aluminum-sensitive zone of the maize root apex. A comparison between an aluminum- sensitive and an aluminum-resistant cultivar. *Plant Physiol* **126**:397-410.

Neumann G, Massonneau A, Martinoia E and Römheld V 1999 Physiological adaptations to phosphorus deficiency during proteoids root development in white lupin. *Planta* **208**: 373-382.

Ryan PR and Delhaize E 2001Function and Mechanism Of Organic Anion Exudation From Plants Roots. *Annu. Rev. Plant Physiol. Plant Mol.Biol* **52**: 527-560.

Skene KR 2000 Pattern formation in cluster roots. Some developmental and evolutionary considerations. *Ann. Bot.* **85**:901-908.