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Review

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Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource

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Summary

Phosphorus (P) is limiting for crop yield on > 30% of the world's arable land and, by some estimates, world resources of inexpensive P may be depleted by 2050. Improvement of P acquisition and use by plants is critical for economic, humanitarian and environmental reasons. Plants have evolved a diverse array of strategies to obtain adequate P under limiting conditions, including modifications to root architecture, carbon metabolism and membrane structure, exudation of low molecular weight organic acids, protons and enzymes, and enhanced expression of the numerous genes involved in low-P adaptation. These adaptations may be less pronounced in mycorrhizal-associated plants. The formation of cluster roots under P-stress by the nonmycorrhizal species white lupin (*Lupinus albus*), and the accompanying biochemical changes exemplify many of the plant adaptations that enhance P acquisition and use. Physiological, biochemical, and molecular studies of white lupin and other species response to P-deficiency have identified targets that may be useful for plant improvement. Genomic approaches involving identification of expressed sequence tags (ESTs) found under low-P stress may also yield target sites for plant improvement. Interdisciplinary studies uniting plant breeding, biochemistry, soil science, and genetics under the large umbrella of genomics are prerequisite for rapid progress in improving nutrient acquisition and use in plants.

I. Introduction

Phosphorus (P) is one of 17 essential elements required for plant growth (Bieleski, 1973; Raghothama, 1999). The P concentration in plants ranges from 0.05 to 0.50% dry weight. This element plays a role in an array of processes, including energy generation, nucleic acid synthesis, photosynthesis, glycolysis, respiration, membrane synthesis and stability, enzyme activation/inactivation, redox reactions, signaling, carbohydrate metabolism, and nitrogen (N) fixation. The concentration gradient from the soil solution P to plant cells exceeds 2000-fold, with an average inorganic phosphate (P_i) concentration of 1 μM in the soil solution (Bieleski, 1973; Schachtman *et al.*, 1998). This concentration is well below the Km for plant uptake. Thus, although bound P is quite abundant in many soils, it is largely unavailable for uptake. As such, P is frequently the most limiting element for plant growth and development. Crop yield on 30–40% of the world's arable land is limited by P availability (Runge-Metzger, 1995; von Uexküll & Mutert, 1995). Phosphorus is unavailable because it rapidly forms insoluble complexes with cations, particularly aluminum and iron under acid conditions. The acid-weathered soils of the tropics and subtropics are particularly prone to P deficiency (Sanchez & Uehara, 1980). Application of P-containing fertilizers is usually the recommended treatment for enhancing soil P availability and stimulating crop yields.

II. The phosphorus conundrum

Application of P fertilizer, however, is problematic for both the intensive and extensive agriculture of the developed and developing worlds, respectively. In intensive agriculture, a maize crop yield of 6–9 t ha^{-1} requires crop uptake of 30–50 kg P ha^{-1} (Ellington, 1999; Vance, 2001; Johnston, 2002) with about two-thirds of that removed in the harvested portion of the crop. Small grains yielding 3 t ha^{-1} take up 15–22 kg P ha^{-1}, again with a 70% removal rate. Soybean takes up 20–25 kg P ha^{-1}, with 80–100% removed in the harvested portion (Johnston, 2002). However, even under adequate P fertilization, only 20% or less of that applied is removed in the first year's growth because of retention by the soil (Russell, 1973). This results in P loading of prime agricultural land. Run-off from P-loaded soils is a primary factor in eutrophication and hypoxia of lakes and marine estuaries of the developed world (Runge-Metzger, 1995; Bumb & Baanante, 1996). Another reason for alarm is that by some estimates inexpensive rock phosphate reserves could be depleted in as little as 60–80 yr (CAST, 1988; Vance, 2001). Phosphorus fertilizer use increased four- to five-fold between 1960 and 2000 and is projected to increase further by 20 Tg yr^{-1} by 2030 (Table 1). As noted by Abelson (1999), a potential phosphate crisis looms for agriculture in the twenty-first century. An even greater concern than the excessive use of P fertilizers by intensive agriculture, is the lack of available P fertilizers for extensive agriculture in the tropics and subtropics where the majority of Earth's people live. Lack of fertilizer infrastructure, money for purchase, and transportation make P fertilization unattainable for these areas. Sustainable management of P in agriculture requires that plant biologists discover mechanisms that enhance P acquisition and exploit these adaptations to make plants more efficient at acquiring P, develop P-efficient germplasm, and advance crop management schemes that increase soil P availability.

Table 1 Agriculture production and resource use in the recent past to the near future

Item	1960	2000	2030–2040
Food production (Mt)	1.8×10^9	3.5×10^9	5.5×10^9
Population (billions)	3	6	8 (perhaps 10)
Irrigated land (% of arable)	10	18	20
Cultivated land (ha)	1.3×10^9	1.5×10^9	1.8×10^9
Water-stressed countries	20	28	52
N fertilizer use (Tg)	10	88	120
P fertilizer use (Tg)	9	40	55–60

Data adapted from Vance (2001). Mt, metric tons; Tg, 10^{12} g or million metric tons.

III. Adaptations to low P

Plants have evolved two broad strategies for P acquisition and use in nutrient-limiting environments: (1) those aimed at conservation of use; and (2) those directed toward enhanced acquisition or uptake (Lajtha & Harrison, 1995; Horst *et al.*, 2001; Vance, 2001). Processes that conserve the use of P involve decreased growth rate, increased growth per unit of P uptake, remobilization of internal P_i, modifications in carbon metabolism that bypass P-requiring steps, and alternative respiratory pathways (Schachtman *et al.*, 1998; Plaxton & Carswell, 1999; Raghothama, 1999; Uhde-Stone *et al.*, 2002a,b). By comparison, processes that lead to enhanced uptake include increased production and secretion of phosphatases, exudation of organic acids, greater root growth along with modified root architecture, expansion of root surface area by prolific development of root hairs, and enhanced expression of P_i transporters (Marschner *et al.*, 1986; Duff *et al.*, 1994; Schachtman *et al.*, 1998; Gilroy & Jones, 2000; Lynch & Brown, 2001). Although beyond the scope of this review, the most prevalent evolutionary adaptation by land plants (80% of all species) for acquiring P is through mycorrhizal symbioses (Koide & Kabir, 2000; Smith *et al.*, 2000; Burleigh *et al.*, 2002; Tibbett & Sanders, 2002).

IV. Uptake of P

Phosphorus is taken up by plants in the orthophosphate (P_i) forms $H_2PO_4^-$ and HPO_4^{2-}, which occur in soil solutions at

very low concentrations (0.1–10 μM; Hinsinger, 2001). A pH optimum for P_i uptake of 4.5–5.0 indicates preferential plant uptake of $H_{i2}PO_4^-$ over HPO_4^{2-} (Raghothama, 1999). Although total soil P content typically varies from 500 to 2000 p.p.m., total bioavailable P, as measured by soil extractants may be only a few p.p.m. (Sanyal & DeDatta, 1991). Up to half the soil P can be organic, derived from plant residues and soil organisms. Organic P must be mineralized before it can be taken up by plants (Horst *et al.*, 2001).

The reactions controlling the amounts of P_i in solution include dissolution–precipitation of P-bearing minerals, adsorption–desorption of phosphate on soil surfaces, and the hydrolysis of organic matter (Matar *et al.*, 1992; Comerford, 1998; Hinsinger, 2001). It can be difficult to distinguish adsorbed and precipitated forms of P, but retention or 'fixation' of P by soil components is greatest in the presence of Fe- and Al-hydroxylated surfaces (from Fe and Al oxides and clay minerals) and, at higher pH, calcium carbonate (Matar *et al.*, 1992; Comerford, 1998). Along with the types and amounts of clay and metal oxides, P availability is also controlled by soil solution pH, ionic strength, concentrations of P and metals (Fe, Al and Ca) and the presence of competing anions, including organic acids (Sanyal & DeDatta, 1991; Hinsinger, 2001). Thus, plant roots (and microbes) can alter solution P_i availability by acidification of the rhizosphere, exudation of organic acids, and secretion of extracellular phosphatases (Comerford, 1998; Hinsinger, 2001).

Because of its strong reactions with soil components, P_i is principally supplied to plant roots by diffusion rather than mass flow (Comerford, 1998; Hinsinger, 2001). At the root surface, P_i is rapidly taken up, resulting in a P_i depletion shell of 0.2–1.0 mm around the root (Barber *et al.*, 1963; Holford, 1997). Although the soil solution P_i concentration rarely exceeds 2 μM, that in plant cells is much higher, 2–20 mM (Bieleski, 1973; Schachtman *et al.*, 1998). For the plant to surmount this concentration difference as well as the negative membrane potential, active transport across the plasmalemma is required. Physiological research over the last few decades, including both inhibitor and kinetic studies, laid the foundation for the molecular investigation of transport processes (Epstein, 1953; Smith, 2001). The striking reduction in P_i accumulation of tissues treated with inhibitors reflects the energy requirement for uptake (Bieleski, 1973; Raghothama, 1999). Moreover, kinetic analysis of P_i uptake shows that plants have both a low- and high-affinity uptake system (Bieleski, 1973; Smith *et al.*, 2000). The high-affinity system operating at low P_i concentrations has an apparent K_m ranging from 3 to 10 μM; the low-affinity system operating at high P_i concentrations has a K_m ranging from 50 to 300 μM (Nandi *et al.*, 1987; Furihata *et al.*, 1992). The high-affinity uptake process is induced when P_i is deficient whereas the low-affinity system appears to be constitutive in plants (Raghothama, 1999). Phosphorus moves symplastically from the root surface to the xylem at a rate of about 2 mm h^{-1} (Bieleski, 1973). Xylem flow then transports P_i to the above-ground organs, where symplastic transport carries it to cells within individual tissues. The movement of P_i from the xylem to the cell cytoplasm and from cytoplasm to vacuole is also against a steep electrochemical potential gradient which requires energized transport (Ullrich & Novacky, 1990). Because most P_i is transported as $H_2PO_4^-$, cotransport involves a cation. Acidification of the cytoplasm which occurs upon P_i addition to P-deficient cells suggests H^+ is the cotransport product for the vast majority of plants (Schachtman *et al.*, 1998). However, authors of a recent study on internodal cells of the giant alga *Chara* have reported a Na^+-coupled P_i uptake system that was induced by P_i deficiency (Reid *et al.*, 2000).

Within recent years, functional complementation of yeast mutants defective in P_i transport has been used to isolate and characterize P_i transporters from a diverse array of plants (Muchhal *et al.*, 1996; Leggewie *et al.*, 1997; Daram *et al.*, 1998; Liu *et al.*, 1998; Liu *et al.*, 1998). Molecular characterization of P_i transporters coupled to the discovery of as many as 16 P_i transport genes within the genome of *Arabidopsis* confirms that plants have a multiplicity of P_i transporters functional in specific organs and tissues (Raghothama, 1999; Gilroy & Jones, 2000). The deduced amino acid sequence of plant P_i transporters indicate they have an M_r of ≅ 58 kDa and are comprised of 525–550 amino acid residues. All characterized P_i transporters have 12 transmembrane domains that occur as two groups (6 + 6) connected by a hydrophobic domain of 60 amino acids (Schachtman *et al.*, 1998; Smith *et al.*, 2000). Computer modeling predicts that both *N*- and *C*-terminal domains of high-affinity P_i transporters are found on the inner membrane surface (Fig. 1), while in the low-affinity transporters these domains are on the exterior surface of the membrane. Initial P_i uptake kinetic studies with a yeast mutant complemented with a P_i transporter disappointingly showed an apparent uptake K_m to be greater than 100 μM, suggesting that it was not a high-affinity transporter (Leggewie *et al.*, 1997). This reduced affinity may also reflect that expression was in a heterologous system. However, more recent studies have yielded a K_m of 31 μM for the tomato *LePT1* P_i transporter (Daram *et al.*, 1998). An even higher affinity, apparent K_m of 3.1 μM, was shown when the *Arabidopsis* PHT1 P_i transporter gene was expressed in cultured tobacco cells (Mitsukawa *et al.*, 1997). *In situ* hybridization and immunolocalization of high-affinity P_i transporters transcripts and protein in root epidermal and root hair cells (Daram *et al.*, 1998; Liu *et al.*, 1998; Chiou *et al.*, 2001) suggests their importance for P_i uptake from soil solution. Localization of plant uptake systems in the youngest regions of roots and in the root hairs is most effective for recovery of nutrients from undepleted soil (Smith *et al.*, 2001). As a depletion zone develops, effective uptake of P_i behind the root hair zone requires either mycorrhizal uptake (through hyphae that bridge the depletion zone) or plant adaptations, such as cluster roots, that solubilize and extract additional P_i from localized soil patches.

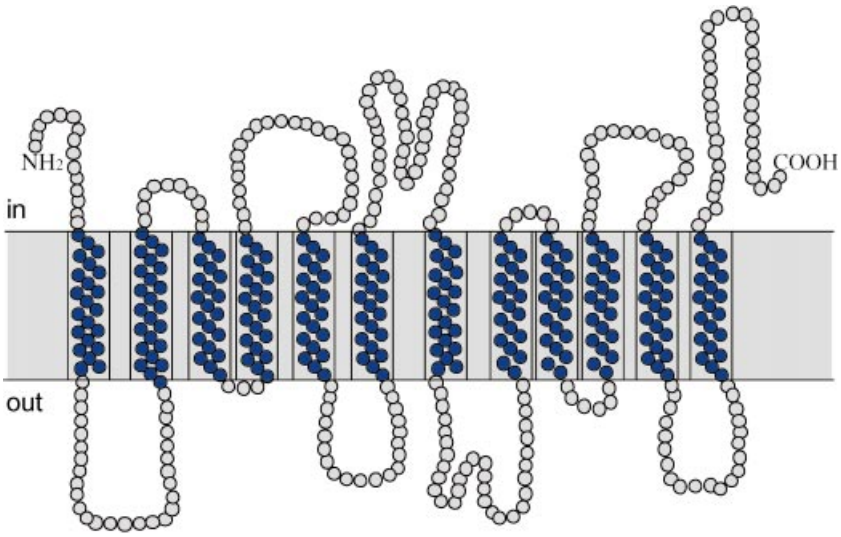

Fig. 1 Predicted topology of the high-affinity inorganic phosphate (P_i) transporter LaPT1 from white lupin based upon homology to other high-affinity P_i transporters.

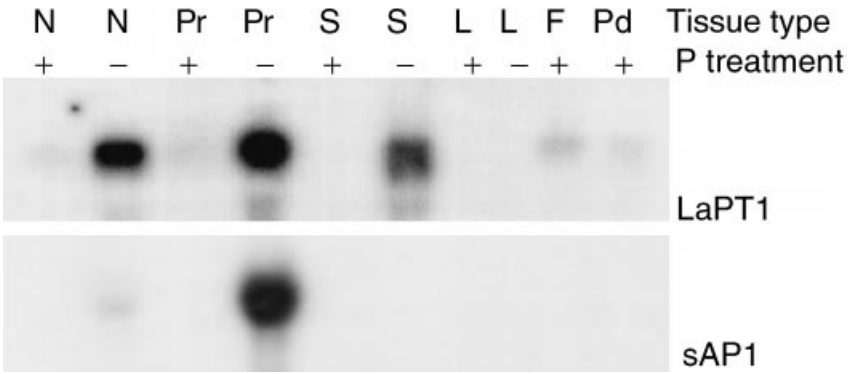

Fig. 2 Transcript abundance of phosphate (P) transporter (LaPT1) and secreted acid phosphatase (sAP1) in phosphate-sufficient and P-deficient white lupin at 14 d after emergence (DAE). Plants grown in presence of sufficient P are designated by (+) and those grown under P stress are designated by (–). Letters above each lane represent: N, normal roots; Pr, proteoid (cluster) roots; S, stems; L, leaves; F, flowers; Pd, pods. Flowers and pods did not form on –P plants. Each lane contains 20 µg of total RNA. (Derived from Liu *et al*., 2001 and Miller *et al*., 2001.)

When placed in +P nutrient solution, uptake of P_i on a gram fresh weight basis by P-deficient cluster roots of white lupin is much greater than that of P-sufficient plants (Keerthisinghe *et al*., 1998; Neumann *et al*., 1999). Moreover, the apparent K_m for P_i uptake of cluster roots is 8.6 µM compared with a K_m of 30.7 µM for P-sufficient controls (Neumann *et al*., 1999). These results suggest that a high-affinity P_i uptake system is induced in cluster roots of P-deficient plants. Liu *et al*. (2001) have recently characterized a high-affinity type P_i uptake gene (*LaPT1*) from cluster roots of white lupin (*Lupinus albus*) that shows highly intensified expression in P-deficient plants. Similar to other plant P_i transporters, the white lupin cluster root P_i transporter has a 1620 bp open reading frame that encodes a protein of 540 amino acids with a M_r of 59 kDa organized into 12 transmembrane domains (Fig. 1). The deduced amino acid sequence of *LaPT1* is 85% similar to previously reported high-affinity type P_i transporters but only 75% similar to low affinity type P_i transporters. Transcripts of *LaPT1* are highly expressed in P_i-deficient cluster roots, normal roots, and stems with little to no expression in P-sufficient plants (Fig. 2). Thus, the enhanced uptake of P_i displayed by P-deficient cluster roots may be directly related to increased expression of a high-affinity type P_i transporter.

V. P deficiency alters root development and function

Shoot and fruit development, because of their agricultural and horticultural importance, have historically received much more attention than that of roots. In recent years, with a flush of interest in nutrient acquisition, studies of root biology have become more attractive. Roots are the primary source of all the mineral elements required for plant growth. As such, root growth and development are highly plastic (Klepper, 1992; McCulley, 1995; Neumann & Martinoia, 2002) and vary greatly depending upon numerous soil factors including: nutrient availability, water content, oxygen concentration, soil density, pH and gravity (Russell, 1973; Lynch, 1995; McCulley, 1995; Charlton, 1996; Raven & Edwards, 2001). Plant roots typically respond to P deficiency through allocation of more carbon to roots, resulting in increased root growth, enhanced lateral root formation, greater exploration of the surface soil, increased length and number of root hairs (Lynch, 1995; Gilroy & Jones, 2000; Liao *et al*., 2001; Lynch & Brown, 2001; Williamson *et al*., 2001), enhanced expression of P transporters (Muchhal *et al*., 1996; Leggewie *et al*., 1997; Smith *et al*., 1997; Liu *et al*., 1998; Liu *et al*.,

2001), and exudation of constituents (organic acids, acid phosphatases) that increase P availability (Dinkelaker *et al.*, 1989; Duff *et al.*, 1991; Tadano & Sakai, 1991; Johnson *et al.*, 1994, 1996a; Gilbert *et al.*, 1998; Gilbert *et al.*, 1999; Neumann & Römheld, 1999).

Coordinated expression of plant genes within a given environment trigger biochemical events that give rise to plant organs and tissues (Torrey, 1986; Boerjan *et al.*, 1992; Celenza *et al.*, 1995; Lynch, 1995; Scheres & Wolkenfelt, 1998; Gilroy & Jones, 2000). Changes in either gene expression or the environment can alter the developmental fate of any given organ. Understanding the genetic and biochemical mechanisms that regulate the development of plant organs are cardinal questions in plant biology. Studies of *Arabidopsis*, common bean (*Phaseolus vulgaris*) and white lupin have proven particularly valuable for revealing genetic and biochemical factors that mediate plant adaptations to P deficiency.

Root architecture

Root architecture (Lynch, 1995; Lynch & Brown, 2001) refers to the complexity of root system spatial configurations that arise in response to soil conditions. It includes root morphology, topology and distribution patterns. Soil P limitation is a primary effector of root architecture (Charlton, 1996; Johnson *et al.*, 1996b; Borch *et al.*, 1999; Williamson *et al.*, 2001) and is known to impact on all aspects of root growth and development. The classic studies of Drew (1975) and Jackson *et al.* (1990) demonstrated the effect of localized supply of soil P on root proliferation in grass species. Elegant experiments with common bean coupled to simulation modeling have shown genotypic adaptations to P deficiency involve changes in root architecture that facilitate acquisition of P from the topsoil (Ge *et al.*, 2000; Liao *et al.*, 2001; Lynch & Brown, 2001). Adaptations that enhance acquisition of P from topsoil are important because of the relative immobility of P in soil, with the highest concentrations usually found in the topsoil and little movement of P into the lower soil profiles. Lynch and Brown (2001) refer to P-deficiency induced modifications in bean root architecture as adaptations for topsoil foraging. Root characteristics associated with improved topsoil foraging for P are a more horizontal basal-root growth angle, resulting in more shallow roots, increased adventitious root formation, enhanced lateral root formation and increased root hair density and length.

Williamson *et al.* (2001) have shown that P availability exerts a marked effect on the root system architecture of *Arabidopsis*. Growth under P-deficient conditions resulted in a redistribution of root growth from the primary root to lateral roots. Reduced primary root elongation under low P conditions was accompanied by increased lateral root density and elongation. Similar to common bean, *Arabidopsis* root biomass was concentrated near the soil surface, suggesting topsoil foraging. Moreover, *Arabidopsis* accessions with enhanced P acquisition have the root architecture modifications mentioned previously and greater root penetration capacity (Narang *et al.*, 2000).

Root hairs

The abundant development of lateral roots associated with P-deficiency induced alterations in root architecture is almost invariably accompanied by increased root hair density and length. Root hairs are tubular-shaped cells specialized for nutrient uptake (Gilroy & Jones, 2000). They arise from root epidermal cells known as trichoblasts and undergo tip growth, thereby extending the root surface area in contact with the soil matrix (Ridge, 1995; Peterson & Farquhar, 1996). Root hairs can form as much as 77% of the root surface area of field crops (Parker *et al.*, 2000). For plants lacking mycorrhizae they are the primary site of nutrient uptake (Jungk, 2001; Schmidt, 2001). Root hair formation and growth is regulated largely by the supply of mineral nutrients, particularly NO_3^- and P (Gilroy & Jones, 2000; Jungk, 2001). In rape, spinach, and tomato, root hair length and number are inversely related to the P concentration in the plant (Jungk, 2001). Similarly, in legumes, P-deficiency results in both increased root hair density and length (Reid, 1981; Jungk *et al.*, 1990). Recent results from our laboratory show that *Medicago truncatula* responds quickly to P-deficiency with increased numbers and length of root hairs (S. Miller and C. P. Vance unpubl. data). Phosphorus uptake in barley cultivars is closely related to variation in root hair abundance and length (Gahoonia & Nielsen, 1997). In *Arabidopsis*, root hair density (Ma *et al.*, 2001) and elongation (Bates & Lynch, 2000) are regulated by P availability. Root hair density was fivefold greater in low-P than in high P-media. Ma *et al.* (2001) found that low P stimulated trichoblast number and the likelihood that trichoblasts would form root hairs. The average length of root hairs on P-deficient *Arabidopsis* was threefold greater than that on P-sufficient plants. Grierson *et al.* (2001) report that at least 40 genes in *Arabidopsis* affect root hair initiation and development. Many of these may be responsive to P-deficiency.

Cluster roots (proteoid roots)

Although much can be garnered from *Arabidopsis* as a model for analysis of the fundamental underpinnings of root development, alternative models must be invoked for determining how P deficiency affects root growth of crop and native species. Native species of Western Australia and South Africa are adapted to grow on the most heavily leached soils in the world (Pate *et al.*, 2001). Species of these regions have evolved specialized structures, cluster roots, for nutrient acquisition from impoverished soils. Beside mycorrhizal associations, cluster roots are regarded as one of the major adaptations for P acquisition (Skene, 1998; Pate & Watt,

2001). Cluster roots are formed in most members of the Proteaceae and in several other plant species adapted to habitats of extremely low soil fertility, including members of the Betulaceae, Casuarinaceae, Cucurbitaceae, Cyperaceae, Eleagnaceae, Leguminosae, Moraceae, Myricaceae and Restionaceae (Louis *et al.*, 1990; Dinkelaker *et al.*, 1995; Skene, 2000; Adams & Pate, 2002). In native habitats, many plant species that form cluster roots are slow-growing, sclerophyllous shrubs and trees that grow on severely P-deficient soils, such as highly leached sands, sandstones and laterites (Dinkelaker *et al.*, 1995; Pate *et al.*, 2001; Lambers *et al.*, 2002). Plant species that form cluster roots usually do not form mycorrhizal associations (Skene, 1998). The cluster roots of Proteaceae occur in close association with decomposing litter. This is especially true for *Banksia*, a genus including trees and shrubs that are dominant in the northern sandplains of south-west Western Australia. (Pate & Watt, 2001). *Banksia* typically forms dense mats of cluster roots beneath the litter layer, exploiting the relatively P-rich A horizon, while vertically descending tap roots provide access to groundwater (Jeschke & Pate, 1995; Pate & Watt, 2001). Growth of these root clusters is seasonal, starting after the onset of winter rain for nutrient uptake from the newly acquired litter during winter and spring (Lamont, 1982; Grierson & Attiwill, 1989; Jeschke & Pate, 1995; Pate & Watt, 2001). During winter, increased concentrations of P and other nutrients were found in xylem sap from cluster roots of *Banksia prionotes*, compared with the remainder of the lateral root system (Jeschke & Pate, 1995). This period of nutrient uptake is accompanied by nutrient storage in the trunk and leaves (Jeschke & Pate, 1995). It is followed by senescence of root clusters when the soil surface dries out during summer. At this stage, minerals stored in trunks and foliage are released for renewed shoot growth during summer (Jeschke & Pate, 1995).

Adaptation to P deficiency via formation of cluster roots is the result of a highly coordinated modification of root development and biochemistry. Cluster roots can comprise single clusters of very densely packed determinate lateral rootlets formed on a parent axis, as found in such Proteaceae as *Hakeae* spp., *Leucadendron laureolum*, *Grevillea robusta* as well as in the legume white lupin (Gardner *et al.*, 1981; Lamont, 1993; Dinkelaker *et al.*, 1995; Skene *et al.*, 1996) (Fig. 3). *Banksia* species, however, form more complex compound clusters (Dinkelaker *et al.*, 1995; Pate & Watt, 2001; Lambers *et al.*, 2002). Several features of cluster root development and morphology are distinguished from that of typical dicot lateral roots. First, lateral roots are randomly initiated from the pericycle of primary roots near the zone of metaxylem differentiation (Torrey, 1986; Charlton, 1996), while cluster roots are initiated in waves (Skene, 2000). Second, lateral roots are initiated singularly opposite a protoxylem point, unlike cluster roots which are in multiples opposite every protoxylem point within the wave of differentiation (Fig. 4). Third, in typical lateral roots, root hair development is highly regulated and occurs from a discrete number of epidermal cells (Ridge, 1995; Malamy & Benfey, 1997; Dolan, 2001), while cluster roots produce a superabundance of root hairs. The accompanying increase in root hair density of clustered rootlets results in an increase of surface area of greater than 100-fold compared with normal roots. Finally, contrasting with the indeterminate growth of lateral roots, cluster root growth is determinate, ceasing shortly after emergence (Fig. 3a). This highly synchronous developmental pattern indicates that cluster root formation is a finely tuned process. Moreover, because root pericycle cells are arrested in the G2 phase of the cell cycle (Skene, 1998, 2000), cluster root initiation must involve concerted release of multiple pericycle cells from the G2 phase in a wave-like pattern along second-order lateral roots.

While mycorrhizal hyphae increase the soil volume that is exploited by roots, cluster roots explore a comparatively small soil volume. The hairy and densely packed lateral rootlets bind tightly to trapped sand and particles of organic matter (Lamont, 1973; Grierson & Attiwill, 1989; Pate & Watt, 2001). The dense aggregates of cluster roots are thought to mobilize sparingly soluble P_i more effectively by concentrating root exudates in localized patches (Gardner *et al.*, 1983; Grierson & Attiwill, 1989). Excretion of organic acids and acid phosphatase from cluster roots has been shown for species of the Proteaceae (Grierson, 1992; Dinkelaker *et al.*, 1995; Lambers *et al.*, 2002). Detailed studies of the carboxylate exudation in *B. grandis* showed exudation of significant amounts of a range of carboxylates when plants were grown on Fe-phosphate or Al-phosphate (Lambers *et al.*, 2002). Interestingly, different carboxylate patterns were recovered from the rhizospheres in Fe-phosphate and Al-phosphate treatments, indicating that the plants perceived a difference in the chemistry of the rhizosphere environment (Lambers *et al.*, 2002). Development of cluster roots was suppressed when plants were grown with higher P supply (K-phosphate) (Lambers *et al.*, 2002).

Although the development and function of cluster roots in Proteaceae have received growing attention, most of the detailed physiological and biochemical work on the functioning of root clusters has been performed with white lupin. This well-characterized legume has proven an illuminating model system for understanding plant adaptations to low P in species lacking mycorrhizal associations (Avio *et al.*, 1990; Watt & Evans, 1999; Skene, 2000; Neumann & Martinoia, 2002). Biochemical changes that can increase the acquisition of scarce P occur within the cluster root zones of P-stressed white lupin (Neumann & Martinoia, 2002) (Fig. 3b). Copious quantities of organic acids, H^+ and acid phosphatase (APase) are exuded to solubilize bound P from inorganic and organic complexes (Marschner *et al.*, 1986; Gerke *et al.*, 1994; Johnson *et al.*, 1994; Dinkelaker *et al.*, 1995; Neumann *et al.*, 1999; Miller *et al.*, 2001). In addition, P_i uptake within cluster

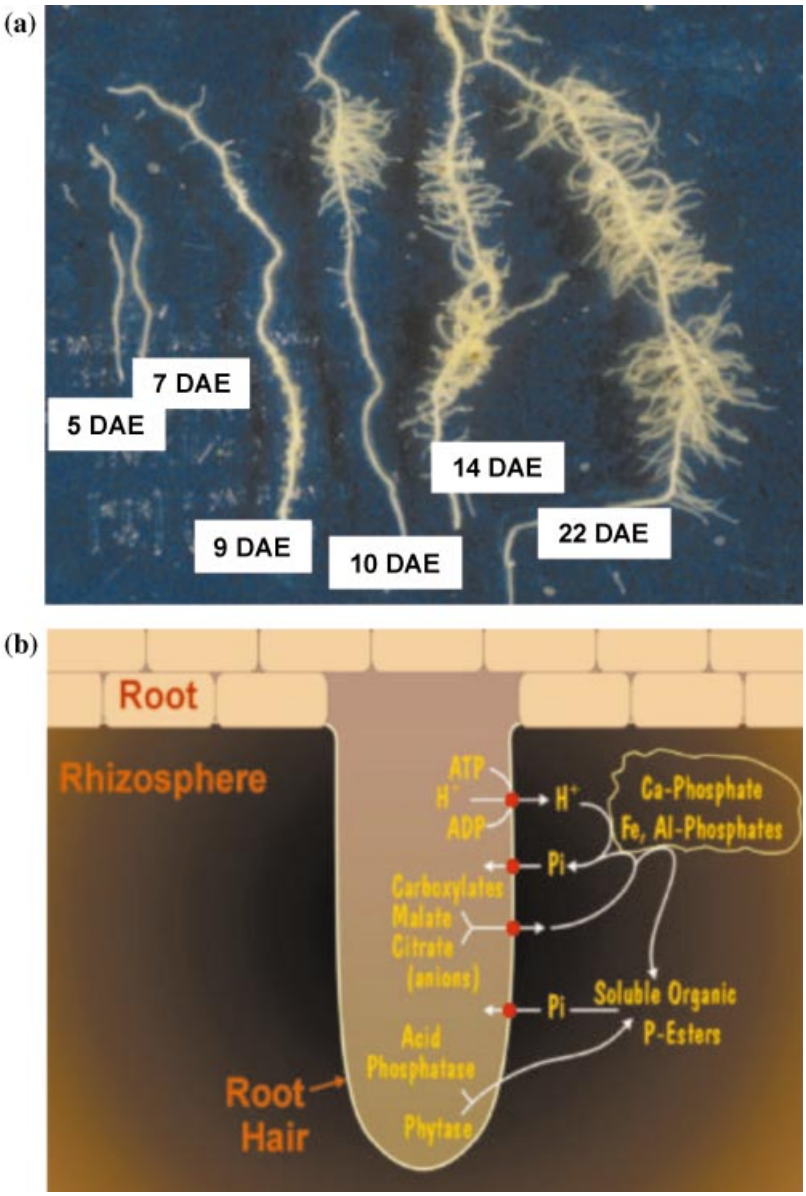

Fig. 3 Cluster roots of white lupin and biochemical mechanism occurring in cluster roots to enhance availability of soil phosphate (P). (a) Cluster roots of P-deficient white lupin at different stages of development. Secondary lateral roots having proteoid segments were collected from 5 to 22 d after shoot emergence (DAE). Note the determinate nature of cluster roots. (Derived from Johnson *et al*., 1996b.) (b) Biochemical adaptations occurring in proteoid roots involve extrusion of protons and carboxylates into the rhizosphere via a plasmalemma H^+ ATPase and anion channels, respectively. Release of protons and carboxylates solubilize P from unavailable bound forms. Acid phosphatases and perhaps phytases exuded from root hairs solubilize P from organic esters. Carboxylates and protons extruded from root hairs may also make organic P-esters more soluble and susceptible to acid phosphatases. Phosphorus released from insoluble ligands and organic P esters is taken up through P transporters in the plasmalemma.

root zones is much greater on a gram fresh weight basis than that of normal roots (Keerthisinghe *et al*., 1998; Neumann *et al*., 1999). Molecular events that give rise to the metabolic changes which increase P acquisition by cluster roots are mediated in part by enhanced expression and accumulation of transcripts encoding P_i transporters (Liu *et al*., 2001), exuded acid phosphatase (Miller *et al*., 2001) and enzymes of carbon metabolism (Uhde-Stone *et al*., 2003a,b). Recently, we have taken a functional genomics approach to investigating adaptation to P deficiency in white lupin by sequencing some 2000 expressed sequence tags (ESTs) from P-deficiency induced cluster roots (Uhde-Stone *et al*., 2003a,b). This approach has provided us with access to several white lupin genes important for adaptation and acclimatization to low P conditions (noted in later sections).

Hormones and root architecture

As might be expected, the internal balance of the plant growth regulators auxin, ethylene, and cytokinin is thought to play a role in P-deficiency induced alterations in lateral root development and architecture, root hair formation and cluster root development. Substantial support for the role of auxin in lateral root development is derived from evidence that: (1) exogenous auxin applications stimulate lateral root formation in many species (Torrey, 1986; Blakely *et al*., 1988; Muday & Haworth, 1994; Charlton, 1996); (2) auxin transport inhibitors block lateral root formation and this block can be alleviated by exogenous auxin (Torrey, 1986; Muday & Haworth, 1994; Reed *et al*., 1998); (3) *Arabidopsis* mutants that overproduce auxin have enhanced lateral root formation

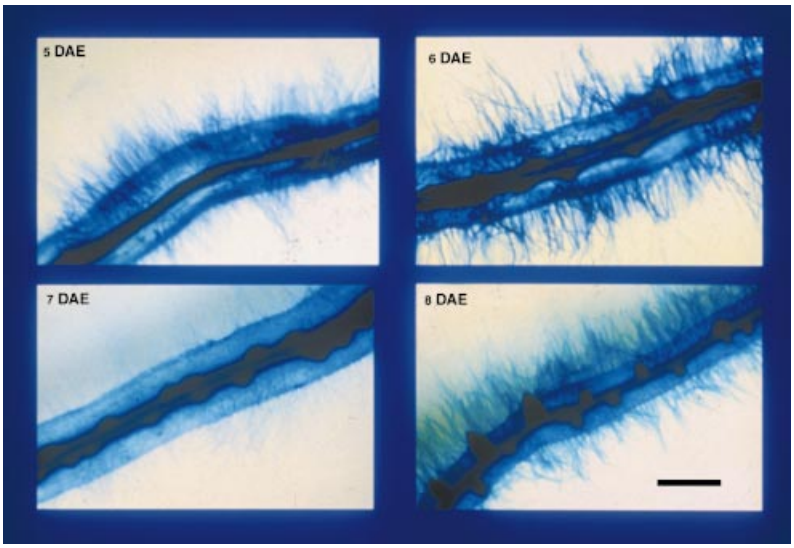

Fig. 4 Initiation and emergence of cluster roots from secondary lateral roots of P-deficient white lupin. Sections (2 cm) of secondary lateral roots representing the proteoid transition zone were collected at 5, 6, 7, and 8 d after shoot emergence (DAE). Root segments were cleared with sodium hypochlorite and stained with methylene blue. Bar, 1 mm. (Derived from Johnson *et al*., 1996b.)

(Boerjan *et al*., 1995; Celenza *et al*., 1995; King *et al*., 1995; Scheres & Wolkenfelt, 1998), while mutants insensitive to auxin have impaired lateral root development (Celenza *et al*., 1995; Hobbie & Estelle, 1995; Rogg *et al*., 2001; Fukaki *et al*., 2002); and (4) mutants in auxin transport have altered lateral root formation (Ruegger *et al*., 1997; Casimiro *et al*., 2001; Marchant *et al*., 2002). Because cluster root formation during P deficiency reflects a striking change in root development, auxins have been implicated in their formation (Lamont *et al*., 1984; Dinkelaker *et al*., 1995). We (Gilbert *et al*., 2000) and others (Skene & James, 2000) have shown that exogenous addition of auxin to P-sufficient white lupin mimics cluster root formation as seen under P-deficient conditions. Moreover, Gilbert *et al*. (2000) demonstrated that auxin transport inhibitors added to P-deficient plants dramatically reduced the formation of cluster roots. The data suggest the cluster root response to P deficiency in white lupin is directly under control of auxin transport. Conversely, auxin insensitive mutants of *Arabidopsis* (*aux1*, *axr1* and *axr4*) which have reduced lateral root formation, showed typically enhanced production of laterals on low P media (Williamson *et al*., 2001). The authors interpret the data to mean that auxin is not directly involved in the induction of lateral roots under low P. Unfortunately, auxin transport inhibitors were not added to the P-stressed mutants, neither was auxin measured; thus interpretation of this work is open to question. An auxin transport inhibitor experiment with *aux1* under P-stress would be particularly informative since *aux1* may act as an auxin transporter (Scheres & Wolkenfelt, 1998).

Although the role of ethylene in lateral root formation is less clear there is convincing evidence that ethylene plays a role in root hair formation and abundance (Kieber, 1997; Michael, 2001). Treatments that inhibit ethylene synthesis inhibit root hair formation and growth, while the stimulation of ethylene synthesis results in an increase in root hair density and length (Tanimoto *et al*., 1995; Masucci & Schiefelbein, 1996; Schmidt, 2001). Of the more than 40 genes affecting root hair development (Grierson *et al*., 2001) at least eight act directly or indirectly on ethylene biosynthesis. Mutations in the ethylene signaling pathway involving the constitutive triple response *ctr1* gene cause plants to respond as though ethylene is continually present and root hair formation as well as density is much greater than wild type. Ethylene overproduction mutants *eto1*, *2*, *3* have much longer and more root hairs than normal, while the ethylene receptor mutant *etr1 has* much shorter than normal root hairs. The ethylene-overproducing mutant *eto3* exhibits a root hair phenotype reminiscent of P-deficiency induced conditions (Schmidt, 2001). Data from auxin insensitive mutants *axr2*, *iaa7*, and *aux1* show root hairs that are reduced in numbers or absent, suggesting that ethylene and auxin are both involved in regulating root hair numbers.

Nutrient stress, particularly P and Fe, are known to stimulate ethylene production (Romera & Alcantara, 1994; Borch *et al*., 1999; Waters & Blevins, 2000; Lynch & Brown, 2001). Schmidt (2001) has proposed that the mechanism of P-deficiency induced changes in root hair density and length involves ethylene through two functionally redundant pathways. Similarly, Cho & Cosgrove (2002) present evidence that root hair initiation and growth are modulated by convergent developmental and environmental pathways, with ethylene/auxin signalling a predominant feature of the environmental stress pathway.

The changes in root development and root hair formation in cluster roots of white lupin are postulated to occur through ethylene signaling (Gilbert *et al*., 1997; Watt & Evans, 1999). Gilbert *et al*. (2000) found a significant increase in ethylene synthesis in white lupin cluster roots. Surprisingly, application of ethylene synthesis inhibitors had little to no effect on cluster roots, but root hair number and length were not

evaluated. In our EST analysis of genes expressed in P-deficient cluster roots we found several involved in ethylene biosynthesis that were highly redundant, including ACC (1-aminocyclopropane-1-carboxylate) oxidase, methionine synthase and S-adenosylmethionine synthetase (Uhde Stone *et al.*, 2003b), but whether ethylene is a primary factor in cluster root formation and function remains to be established.

The role of cytokinins in root growth and architecture, like that of ethylene, is not resolved. Traditionally, cytokinins are thought to inhibit root growth while stimulating shoot growth (Skoog & Miller, 1957). Moreover, low cytokinin/auxin ratios favor root initiation and development in plant tissue culture experiments (Christianson & Warnick, 1985). Phosphate starvation and N deficiency appear to result in decreased cytokinin content. In addition, exogenously applied cytokinin can counteract lateral root growth stimulation induced by nutrient deficiency (Salama & Wareing, 1979; Horgan & Wareing, 1980; Kuiper *et al.*, 1988) and can repress expression of several P_i-starvation responsive genes in *Arabidopsis* (Martin *et al.*, 2000). Cytokinin inhibition of root growth may also be coupled to ethylene action (Cary *et al.*, 1995). In recent years, *Arabidopsis* mutants altered in cytokinin sensitivity (Baskin *et al.*, 1995; Faure *et al.*, 1998; Cary *et al.*, 2001) have been isolated but these mutants have no clear-cut root phenotype. Neumann *et al.* (2000) have shown that exogenous application of kinetin inhibits cluster root formation in P-deficient white lupin. Also, kinetin content was increased in cluster roots compared with normal roots. While we have not measured cytokinin profiles, it is revealing that an abundant EST from cluster roots showing enhanced expression (three- to five-fold) encoded a deduced protein having high similarity to cytokinin oxidase ($E = 10^{-20}$). Cytokinin oxidase is the key enzyme implicated in cytokinin degradation (Morris *et al.*, 1999). Enhanced degradation of cytokinins in cluster root formation and/or development might be expected because low cytokinin levels favor root growth, and P deficiency reportedly results in reduced xylem sap cytokinin levels (Wagner & Beck, 1993; Binns, 1994; Martin *et al.*, 2000). Alternatively, *in planta* regulation of the potentially large quantities of cytokinins that could be released by the mass induction of cluster root meristems may require cytokinin oxidase (Morris *et al.*, 1999; Mok & Mok, 2001).

VI. P deficiency modifies carbon metabolism

Organic acid efflux

Under normal growth and development conditions, plant roots exude a wide variety of organic compounds including: simple sugars, organic acids, amino acids, phenolics, quinones (iso)-flavonoids, growth hormones, proteins, and polysaccharides (Curl & Truelove, 1986; Marschner, 1995). Exudation of organic compounds from roots can alter rhizosphere chemistry, soil microbial populations, competition, and plant growth. Exuded compounds are functionally diverse and can be involved in a wide array of processes ranging from signaling in plant–microbe interactions, to allelopathy and nutrient acquisition (Curl & Truelove, 1986; Marschner *et al.*, 1986; Harrison, 1997).

Under the influence of environmental stress the complement of compounds found in exudates can be significantly altered in either or both quality and quantity (Marschner, 1995; Ryan *et al.*, 2001; Neumann & Martinoia, 2002). For example, when plants are attacked by soil pathogens, the amount and type of phenolics and phytoalexins are altered, depending upon the pathogen. Similarly, rhizobia bacteria and mycorrhizal fungi induce changes in amount and types of compounds released from roots. During nutritional stress (i.e. P limitation), Al toxicity, low Fe availability, and exposure to heavy metals, roots show enhanced synthesis and exudation of several organic acids (anions) (Dinkelaker *et al.*, 1989; Delhaize *et al.*, 1993; Delhaize & Ryan, 1995; Ryan *et al.*, 1995a,b, 1997; Larsen *et al.*, 1998; Neumann *et al.*, 2000). Of the changes in response to plant nutrition, those related to low P and excess Al are among those that have been most thoroughly documented. Convincing evidence now exists for exudation of malate and citrate as a principal mechanism in alleviating the edaphic stress of P-deficiency and Al-toxicity. The release of organic acids allows for the chelation of Al^{3+}, Fe^{3+} and Ca^{2+}, and subsequent displacement of P_i from bound or precipitated forms (Gerke, 1994; Jones, 1998; Hinsinger, 2001; Ryan *et al.*, 2001), and may also cause organic P to become more susceptible to hydrolysis by acid phosphatases (Gerke, 1994; Braum & Helmke, 1995) (Fig. 3B). In Al-toxic soils root exudation of organic acids protects plants by chelating Al^{3+} ions in the rhizosphere, thus excluding their entry into the root. Because several recent excellent reviews (Jones, 1998; Lopez-Bucio *et al.*, 2000b; Hinsinger, 2001; Ryan *et al.*, 2001) have critically evaluated organic acid synthesis and exudation from roots, we will focus on the salient features related to the role of organic acids in alleviating P-deficiency in cluster roots of white lupin, including recent evidence for a potential efflux mechanism.

When white lupin is P deficient, cluster roots synthesize and exude striking amounts of malate and citrate (Gardner *et al.*, 1982; Dinkelaker *et al.*, 1989; Johnson *et al.*, 1994; Keerthisinghe *et al.*, 1998; Neumann *et al.*, 1999). Grown under P-deficiency, cluster roots exude 20- to 40-fold more citrate and malate than P-sufficient roots (Fig. 3b). The amount of carbon exuded in these two compounds can range from 10% to greater than 25% of the total plant dry weight. Surprisingly, P-deficient plants do not appear to suffer any loss in either dry matter accumulation or N fixation until the reproductive stage of growth (Dinkelaker *et al.*, 1989; J. Schulze, G. Gilbert, C. Vance, unpubl. data). Although the internal concentrations of organic acids increase in P-deficient roots, it is not proportional to the amount released as exudates.

This suggests specific selective synthesis and exudation of malate and citrate in response to P-stress.

Measurements of CO_2 fixation and pulse-chase labeling with $^{14}CO_2$ show the carbon required for enhanced organic acid exudation from cluster roots of P-deficient white lupin is derived from both photosynthetic CO_2 fixation and dark CO_2 fixation by cluster roots (Johnson *et al.*, 1994, 1996a). Photosynthetic CO_2 fixation provides about 65% of the carbon exuded, while cluster root dark CO_2 fixation provides about 35%. Rates of dark CO_2 fixation in cluster roots are similar to those occurring in legume root nodules (Johnson *et al.*, 1996a). The increased cluster root *in vivo* CO_2 fixation is accompanied by enhanced specific activity of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), and citrate synthase (CS) (Johnson *et al.*, 1996a,b; Gilbert *et al.*, 1999; Uhde-Stone *et al.*, 2003a).

Similar increases in PEPC activity in response to P deficiency have been noted in chickpea (*Cicer arietinum*), oilseed rape, and *Sesbania rostrata* (Hoffland *et al.*, 1992; Moraes & Plaxton, 2000; Aono *et al.*, 2001). Steady-state amounts of PEPC, MDH and CS mRNAs are much higher in P-deficient cluster roots compared with P-sufficient roots (Johnson *et al.*, 1996a; Uhde-Stone *et al.*, 2003a) suggesting a coordinated induction of genes involved in both developmental as well as biochemical changes.

Enhanced exudation of organic acids may not only occur because of upregulation of selected reactions leading to greater synthesis but also may be due to either reduced degradation or utilization of citrate. The activity of aconitase (AC) is reduced in cluster roots (Neumann & Römheld, 1999; Neumann *et al.*, 1999). Similarly, respiration rates are also reduced in P-deficient cluster roots (Johnson *et al.*, 1994; Neumann *et al.*, 1999). Both phenomena could result in more citrate available for exudation.

Organic acid anion (OA^-) exudation from cluster roots of white lupin coincides with a marked acidification of the rhizosphere (Marschner *et al.*, 1987; Dinkelaker *et al.*, 1989; Neumann & Martinoia, 2002). This is consistent with reports of a correlation of OA^- exudation and the release of protons from white lupin and chickpea roots (Neumann & Römheld, 1999; Sas *et al.*, 2001). Sas *et al.* (2001) have shown that extrusion of protons and organic acids in white lupin was highly dependent upon P supply. On an equimolar basis, H^+ extrusion in P-deficient plants was two- to three-fold greater than organic acid exudation. Similarly, a recent report by Yan *et al.* (2002) showed substantially enhanced proton release from cluster roots of P-deficient white lupin. Proton release occurred primarily from young clusters and decreased significantly as cluster roots aged. During prolonged P deficiency in white lupin plants, OA^- exudation from cluster roots increased while the release of protons decreased, indicating that different mechanisms are involved in proton release and OA^- exudation (Sas *et al.*, 2001). A comparison with tomato (*Lycopersicon esculentum*) showed an increase of the net release of protons from roots of P-starved tomato while OA^- excretion from tomato roots decreased under P deficiency (Neumann & Römheld, 1999). Wheat (*Triticum aestivum*) displayed a decrease in OA^- exudation under P deficiency and no proton extrusion (Neumann & Römheld, 1999).

Proton release into the rhizosphere is a common response of plant cells to counter intracellular acidity (Raven & Smith, 1976). Generally, proton release results from the activity of a plasma membrane H^+ ATPase. This enzyme uses ATP to pump protons out of the cell, thereby creating pH and electrical potential differences across the plasmalemma. Yan *et al.* (2002) reported enhanced catalytic activity of a plasma membrane H^+ ATPase of P-deficient cluster roots in white lupin that might be responsible for the increase in H^+ extrusion. Characterization of the H^+ ATPase from P-deficient cluster roots showed a threefold increase in V_{max} and a fourfold increase in H^+ ATPase enzyme protein compared with P-sufficient roots. Only the youngest P-deficient cluster roots had significant proton extrusion and H^+ ATPase activity. It is noteworthy that Sakano (2001) addresses the potentially problematic generation of protons during OA^- synthesis via glycolysis in his revised pH-stat hypothesis of the plant cell. By contrast to the 'feed-forward' fashion of the nonplant system, plant glycolysis is regulated by a feedback process. This feedback regulation only permits glycolytic activity when the cytoplasm is alkaline enough to stimulate PEPC, an enzyme with an alkaline pH optimum (Sakano, 2001). As Sakano points out, active H^+ extrusion is a possible response of the plant cell to prevent the inhibiting effect of cytosolic acidification.

While neither the specific cell types for nor the mechanism of organic acid exudation have been characterized for cluster roots of white lupin, studies on Al tolerance in wheat (Ryan *et al.*, 1997) and maize (*Zea mays*) (Kollmeier *et al.*, 2001; Pineros & Kochian, 2001) have identified the root apex as the zone of organic acid exudation. Moreover, these patch-clamp and inhibitor studies indicate that Al may activate a malate/citrate-permeable anion channel to facilitate the exudation of large quantities of organic acids. Zhang *et al.* (2001) showed that anion channel frequency and rapidity of activation was greater in an Al^{3+}-tolerant wheat line compared with a near-isogenic Al^{33+}-sensitive line. Neumann *et al.* (1999) showed that exposure of P deficiency induced cluster roots to the anion channel-blockers ethacrynic- and anthracene-9-carboxylic acids inhibited citrate exudation by 40–60%. Moreover, we have recently shown through *in situ* hybridization (Uhde-Stone *et al.*, 2003a) that PEPC and MDH transcripts are localized to cluster root apices and elongation zones, indicating the enzymes necessary for malate and perhaps citrate synthesis occur in the root tips (Fig. 5). Our data coupled with that of Neumann *et al.* (1999) suggest that P deficiency induced rapid exudation of organic acids, similar to Al^{3+}-tolerance, may involve selective anion channel proteins at sites of exudation. Since malate and citrate are fully dissociated in the

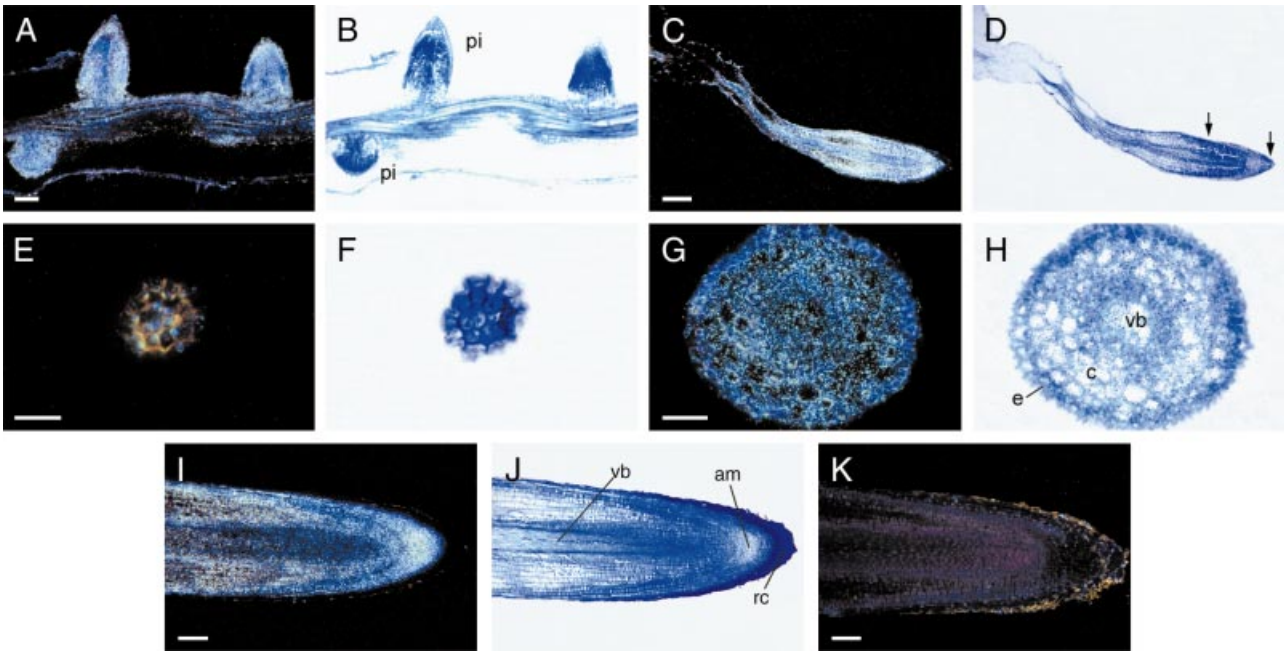

Fig. 5 *In situ* localization of phosphoenolpyruvate carboxylase (PEPC) in P-deficient cluster roots of white lupin. Sections A–J were hybridized with ^{35}S-labeled antisense RNA derived from LaPEPC1 cDNA, whereas section K was hybridized with the corresponding sense RNA probe. The dark-field (a, c, e, g, i and k) and bright-field (b, d, f and j) images correspond to longitudinal and transverse sections through cluster rootlets 9 d after emergence (DAE) (a, b) and 12 DAE (c–h) as well as through 12 DAE +P normal roots (i, j, k). Arrows in d indicate the approximate location of the two transverse sections shown in e–h. The areas that are white in a, c, e, g and i indicate the presence of PEPC mRNA. Sense control (K) showed no reaction. Bars are 50 μm (e, g), 100 μm (a) and 200 μm (c, i, k); pi, proteoid initials; vb, vascular bundle; c, cortex; e, epidermis, am, apical meristem, rc, rootcap. (Derived from Uhde-Stone *et al*., 2003a.)

cytosol and because cell membranes are in essence impermeable to ions, it is not surprising that the release of organic acids from root tips in either P deficiency or Al toxicity might involve some type of channel protein.

Although a large number of cation channels and transporters belonging to a multiplicity of gene families have been identified over the past decade (Cao *et al*., 1995; Maathuis & Sanders, 1999; Barbier-Brygoo *et al*., 2000; Howitt & Udvardi, 2000; Theodoulou, 2000; Maser *et al*., 2001), the molecular events involved in organic acid (anion) excretion remain elusive. While anion channels are probably ubiquitous in plants, few have been characterized (Schroeder, 1995; Barbier-Brygoo *et al*., 2000). Mechanisms of anion transport potentially could include chloride channels (CLC), aquaporins, and multidrug extrusion proteins (MATE). To date, only putative anion channel genes related to the CLC family have been characterized (Hechenberger *et al*., 1996; Lurin *et al*., 1996; Geelen *et al*., 2000). These genes encode messages of approximately 2.4 kb giving rise to proteins of approximately 785 amino acids with M_r of *c*. 85 kDa. The *Arabidopsis CLC-d* gene could functionally substitute for a yeast CLC protein involved in iron-limited growth, while the *CLC-a* gene appeared to be related to nitrate acquisition. Anion channel genes encoding transmembrane proteins involved in organic acid efflux have yet to be characterized.

During our annotation of ESTs from cluster roots we found that several proteins with putative transport-related functions were highly expressed in P-stressed conditions. One of these ESTs was redundant (found 10 times) and showed a ninefold induction in -P cluster roots (Uhde-Stone *et al*., 2003b). This EST displayed high homology to a putative integral membrane protein in *Arabidopsis* belonging to the MATE protein family. MATE proteins are a large family of putative antiporters that are thought to be involved in excretion of a variety of drugs and toxins (Brown *et al*., 1999; Debeaujon *et al*., 2001; Diener *et al*., 2001). Around 55 putative members of the MATE family have been predicted in *Arabidopsis*, two distantly related MATE proteins have been predicted in yeast. To date, only a few MATE proteins have been cloned (Debeujon *et al*., 2001; Diener *et al*., 2001; Rogers & Guerinot, 2002). An *Arabidopsis* MATE has been shown to control response to iron deficiency. It represents either an Fe sensor or transporter of small organic molecules (Rogers & Guerinot, 2002). Analysis of the white lupin P-stress induced MATE revealed a 2.1-kb cDNA encoding a deduced amino acid sequence of 531 amino acids having 10–12 transmembrane helices, depending upon the program used for analysis. These findings make this putative MATE EST an extremely interesting candidate for a role in organic acid excretion. Functional studies in yeast and *Xenopus* oocytes will

allow us to examine the role of the MATE type protein in organic acid excretion.

Alternative glycolytic pathways

Besides increased acquisition of soil P, conservation of internal pools of acquired P_i is considered an important adaptation for growth on low P. As several enzymes of the glycolytic pathway depend on P_i or adenylates as cosubstrates, metabolic processes may be impaired by severe P deprivation. Yet the generation of energy and the production of carbon skeletons continues during conditions of P limitation, as evident in the excretion of significant amounts of organic acids. In response to limited P, many plants show remarkable flexibility in adjusting metabolic rates and utilizing alternative metabolic pathways. Alternative glycolytic reactions can bypass P_i- or ATP-requiring steps of glycolysis under environmental stress conditions such as P_i starvation or anoxia (Duff *et al.*, 1989b; Mertens, 1991; Theodorou *et al.*, 1992; Theodorou & Plaxton, 1996). While ATP and ADP-levels have been reported to decline under P deficiency (Ashihara *et al.*, 1988; Duff *et al.*, 1989b), pyrophosphate (PP_i) concentrations appear to be buffered during P_i stress (Duff *et al.*, 1989b; Dancer *et al.*, 1990). Despite an earlier assumption of cellular bioenergetics that PP_i anhydride bonds were not utilized in cellular metabolism, studies have shown that under certain environmental stresses PP_i can serve as an energy donor in the plant cytosol (Duff *et al.*, 1989b; Plaxton, 1996; Plaxton & Carswell, 1999). A well-documented alternative glycolytic pathway is catalysed by a PP_i-dependent phosphofructokinase (PFP) that, under P deficiency, can bypass the ATP-dependent phosphofructokinase (PFK), generating fructose 1,6-biphosphate (Theodorou *et al.*, 1992; Theodorou & Plaxton, 1996; Plaxton & Carswell, 1999). Other processes that might use PP_i are the cleavage of sucrose by a PP_i-dependent sucrose synthase pathway and the active transport of protons into the vacuole by a PP_i-dependent H^+ pump in the tonoplast (Plaxton & Carswell, 1999). PP_i has been assumed to be a byproduct of secondary metabolism and anabolism, therefore use of PP_i as an energy donor helps to conserve limited ATP pools (Duff *et al.*, 1989b; Theodorou *et al.*, 1992; Plaxton, 1996; Theodorou & Plaxton, 1996; Plaxton & Carswell, 1999).

Another alternative glycolytic pathway known in plants is catalysed by the action of a nonphosphorylating NADP-dependent glyceraldehyde-3P dehydrogenase (NADP-G3PDH) that bypasses P_i-dependent NAD-G3PDH and phosphoglycerate kinase (Duff *et al.*, 1989b; Theodorou *et al.*, 1992; Plaxton & Carswell, 1999). A third bypass of the glycolytic pathway can be catalysed by the combined activities of PEPC, MDH and NAD-malic enzyme (Theodorou & Plaxton, 1993). Phosphorus stress can severely limit the activity of pyruvate kinase (PK), an enzyme requiring P_i and ADP. The PEPC, MDH and NAD-malic enzymes can bypass PK and thus maintain the flow of carbon from glycolysis to the TCA cycle by avoiding the use of ADP but generating free P_i (Theodorou *et al.*, 1992; Plaxton & Carswell, 1999).

The described metabolic adaptations to P_i stress appear to be plant specific. P-deficiency induced PFP activity was found in plants that are adapted to infertile soils and contain very low PFP activity when grown under sufficient P, e.g. *Arabidopsis*, rape (*Brassica napus*), sugar beet (*Beta vulgaris*), and buckwheat (*Fagopyrum esculentum*) (reviewed in Plaxton & Carswell, 1999). Murley *et al.* (1998) reported that P-stress inducible PFP activity is not found in plants that form symbiotic associations with mycorrhizal fungi. In plants that typically contain abundant PFP activity when grown under sufficient P, PFP activity stayed unchanged or declined under P deficiency (in tobacco (*Nicotiana tabacum*) seedlings, bean seedlings and tomato root cultures; Plaxton & Carswell, 1999). Analysis of ESTs from P-deficient cluster roots of white lupin indicated the induction of genes encoding for PFP and the PEPC/MDH bypass of PK, but did not reveal evidence for the involvement of a nonphosphorylating NADP-dependent G3PDH (Fig. 6).

Alternative mitochondrial respiration

Phosphorus limitation impairs mitochondrial respiration in many plants. Considering the adaptive flexibility of plant responses to P deficiency that is displayed in the glycolytic pathway, adaptations which allow alternative mitochondrial electron transport under P starvation have been hypothesized (Rychter & Mikulska, 1990; Theodorou & Plaxton, 1993).

The significant reduction in cellular ADP and P_i levels in plants undergoing P limitation is thought to inhibit the cytochrome (Cyt) pathway of electron transport. Low ADP and P_i can result in a high ATP : ADP ratio which inhibits respiration, a phenomenon well known as adenylate control of respiration (Bryce *et al.*, 1990). Nonphosphorylative pathways that can bypass energy-requiring sites include rotenone-insensitive NADH dehydrogenase (Rasmusson & Moller, 1991) and the alternative oxidase (AOX) (Lambers, 1985; Lance *et al.*, 1985; Day & Wiskich, 1995). The cyanide-resistant AOX catalyses the oxidation of ubiquinol and the reduction of oxygen to water but bypasses the last two energy-conserving sites normally associated with the Cyt pathway. Alternative oxidase activity is subject to regulation by a sulfhydryl–disulfide redox system (Siedow & Umbach, 2000) and by allosteric activation via α-keto acids, especially pyruvate (Millar *et al.*, 1993; Hoefnagel *et al.*, 1995). It was proposed that induction of AOX during P deficiency might be a consequence of elevated PEPC activity.

In roots of *P. vulgaris*, P deficiency led to decreased activity of the respiratory cytochrome pathway, while alternative, cyanide-resistant respiration and the NADH : NAD ratio increased (Rychter & Mikulska, 1990; Rychter *et al.*, 1992). Indication for a possible link between AOX respiration and

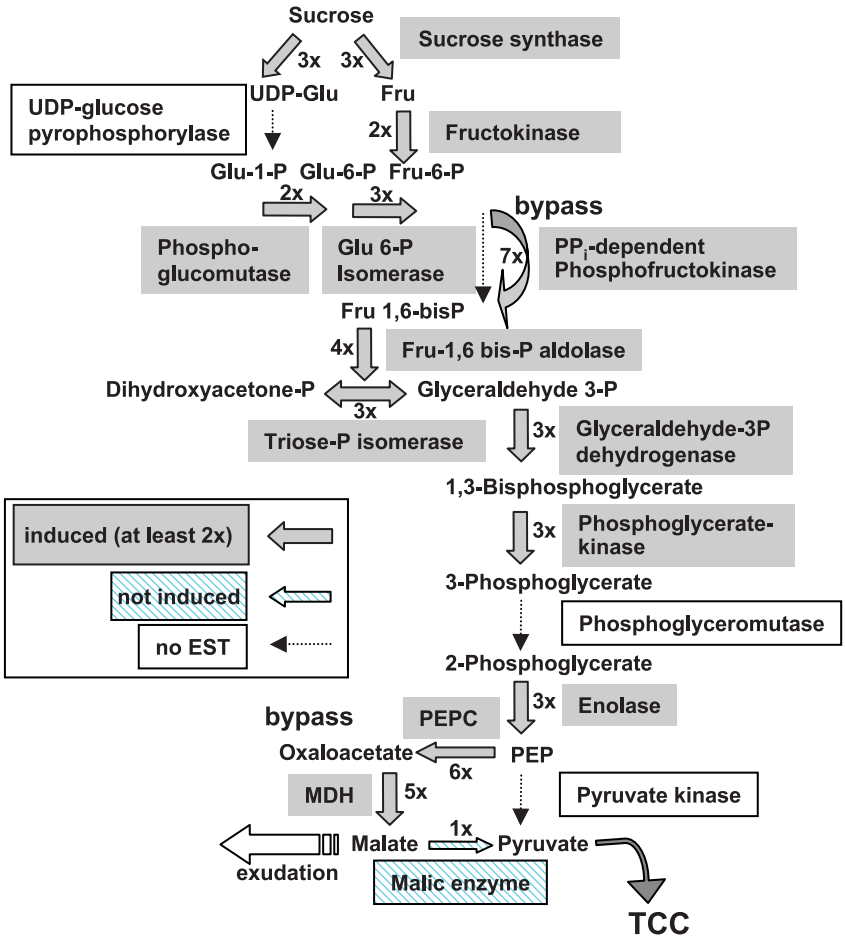

Fig. 6 Schematic representation of the glycolytic pathway. Expressed sequence tags (ESTs) with induced expression in P-deficient cluster roots, compared with P-sufficient normal roots, are represented in gray boxes. The average of gene induction, as determined by two independent macroarrays is indicated at the corresponding arrows (e.g. 2×). Enzymes of the glycolytic pathway that were not found in a collection of 1250 ESTs are shown in white boxes and are represented by dotted arrows. The majority of ESTs with possible function in the glycolytic pathway displayed increased expression in P-deficient cluster roots compared with P-sufficient normal roots. (Derived from Uhde-Stone *et al*., 2003a.)

P starvation has also been found in *Catharanthus roseus* (Hoefnagel *et al*., 1993), *Chlamydomonas reinhardtii* (Weger & Dasgupta, 1993) and tobacco cell cultures (Parsons *et al*., 1999). However, oxidative respiration rates under P deficiency can vary greatly among different plants. Respiratory O_2 consumption in the green alga *Scenedesmus obtusiusculus* was stimulated during the first 96 h of P starvation (Tillberg & Rowley, 1989), while pea (*Pisum sativum*) roots displayed no effect of P starvation on respiration (reviewed by Theodorou & Plaxton, 1996). The respiration rates of *P. vulgaris*, *Selenastrum minutum*, *Lemna gibba* and suspension-cultured *C. roseus*, however, declined under P deprivation (Theodorou & Plaxton, 1996). In P-deficient cluster roots of white lupin, up to 60% reduction in mitochondrial respiration, as measured by O_2 consumption, has been reported (Johnson *et al*., 1994; Neumann *et al*., 1999), accompanied by an increased NADH : NAD ratio. These results indicate that in some plants inhibition of the CN-sensitive cytochrome pathway of respiration might not be fully compensated by AOX activity.

Enhanced glycolysis in P-deficient white lupin was accompanied by significant induction of genes encoding for an alcohol dehydrogenase (Massonneau *et al*., 2001) and a formate dehydrogenase (fdh) (Uhde-Stone *et al*., 2003b), indicating a possible role of fermentative processes during P deficiency. In the green algae *C. reinhardtii*, phosphate starvation resulted in the accumulation of an mRNA with homology to pyruvate formate-lyase, another enzyme of fermentative metabolism (Dumont *et al*., 1993). Taken together, these findings indicate that the increase of glycolytic activity and the resulting organic acid synthesis under P deprivation in white lupin could be, in part, a consequence of severely impaired oxidative respiration.

Secondary metabolism

A switch from primary to secondary metabolism is a common response of plants undergoing nutrient limitation. Phosphorus deficiency typically results in the accumulation of secondary metabolites such as flavonoids and indole alkaloids (reviewed in Plaxton & Carswell, 1999). In rice culture, accumulation of the polyamine putrescine is thought to mediate growth inhibition in response to P depletion (Shih & Kao, 1996). Yamakawa *et al*. (1983) reported that P depletion induced anthocyanin accumulation in *Vitis* cell culture, while P supplementation induced primary metabolism and inhibited anthocyanin synthesis. Increased synthesis of anthocyanins is a frequent response of plants to P deficiency

and presumably functions to ameliorate photoinhibitory damage to chloroplasts (Takahashi *et al.*, 1991).

In addition to the possible involvement of phenolics in senescence, phenolic compounds can be exuded into the rhizosphere in response to P starvation and might act as chelators and/or reductants increasing the release of bound P_i. Examples of phenolics that are exuded into the rhizosphere are piscidic acid from roots of pigeon pea (*Cajanus cajan*) (Ae *et al.*, 1990), alfafuran from alfalfa (*Medicago sativa*) roots (Masaoka *et al.*, 1993), and isoflavonoids from P-deficient cluster roots of white lupin (Neumann *et al.*, 2000).

Sclerophylly, characteristic of many of the Proteaceae, is also thought to be an adaptation involving enhanced phenolic metabolism by many species that occupy low P habitats. Sclerophylls are typically considered to be hard and stiff leaves that are tough (Turner, 1994). They are characterized by a thick cuticle and outer epidermal cell wall with abundant sclerification (Loveless, 1961, 1962). Bundle sheaths and leaf margins in particular show sclerification. The secondary wall of sclerids are heavily lignified, hence the relationship to phenolic secondary metabolism. Loveless (1962) postulated that sclerophylly is an adaptation to conserve nutrients particularly P and N. Turner (1994) concluded that sclerophylly is also related to protection against herbivory.

Enzymes in the phenolic and flavonoid pathways have been reported to be upregulated in P_i-deficient plants (Plaxton & Carswell, 1999). Several white lupin ESTs with high similarity to enzymes involved in secondary metabolism were represented in our cDNA library derived from mature cluster roots of P-deficient plants, including DAHP (3-deoxy-D-arabino-heptulosonate-7-phosphate synthase), phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, isoliquiritigenin 2′-*O*-methyltransferase, 4-coumarate-CoA ligase, caffeoyl-CoA *O*-methyltransferase, cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase (http://www.home.earthlink.net/~whitelupinacelimation). Whether these enzymes are involved in either anthocyanin or lignin biosynthesis remains to be established. By contrast to primary metabolism, secondary metabolism in general does not consume as much P_i but can serve to recycle significant amounts of P_i from phosphate esters (Plaxton & Carswell, 1999; Sakano, 2001). However, secondary metabolism produces an excess of reducing equivalents. The resulting cytosolic acidification could lead to the activation of AOX and other pathways that can relieve the accumulation of reducing equivalents (Sakano, 2001).

Modified thylakoid membranes

Adaptations to P deficiency related to carbon metabolism also include modification of the lipid composition of photosynthetic membranes (Essigmann *et al.*, 1998; Yu *et al.*, 2002). The lipid composition of thylakoids is comprised primarily of: monogalactosyl diacylglycerol, digalactosyl diacylglycerol, phosphaidyl-glycerol and sulfoquinovosyl diacylglycerol. Under P-deficiency, thylakoid membranes display decreased amounts of phospholipids accompanied by large increases in the sulfolipid, sulfiquinovosyl diacyylglycerol (Benning, 1998; Yu *et al.*, 2002). *Arabidopsis* genes encoding the two critical steps in sulfolipid synthesis have recently been isolated and characterized (Essigmann *et al.*, 1998; Yu *et al.*, 2002). The first step is catalysed by SQD, a protein that converts UDP-glucose and sulfite to UDP-sulfoquinovose. The second enzyme required for sulfolipid synthesis is SQD2 which catalyses the condensation of UDP-sulfoquinovose and diacylglycerol to sulfoquinovosyl diacylglycerol (Benning, 1998). Both *SQD1* and *SQD2* have greatly enhanced expression under P-deficient conditions. Moreover, an *Arabidopsis* line with a mutation in *SQD2* is impaired in phosphate-limited growth (Yu *et al.*, 2002). Essigmann *et al.* (1998) proposed that the function of the changes in thylakoid membrane sulfolipid : phospholipid ratio would be to conserve P while maintaining membrane integrity. Anionic sulfolipid could substitute for anionic phosphatidylglycerol, thus reducing the need for membrane-bound P, yet allowing photosynthesis to continue under P-limited conditions.

VII. Acid phosphatase

A universal response by plants to P-deficiency is the synthesis of acid phosphatase enzymes (APases). These enzymes are ubiquitous in plant organs and APase activity can be detected throughout development (Duff *et al.*, 1994). APases are implicated in: providing P during seed germination from stored phytate (Biswas & Cundiff, 1991; Brinch-Pedersen *et al.*, 2002); internal remobilization of P (Duff *et al.*, 1991; del Pozo *et al.*, 1999; Baldwin *et al.*, 2001); release of P from soil organic P-esters by exudation of enzymes into the rhizosphere (Lefebvre *et al.*, 1990; Goldstein *et al.*, 1988; Miller *et al.*, 2001); and the synthesis of glycolate from P-glycolate (Christeller & Tolbert, 1978) as well as glycerate from 3-PGA during photorespiration (Randall *et al.*, 1971). Characteristically, phytases and root-secreted APase have little substrate specificity, while APases involved in carbon metabolism (i.e. phosphoglycolate phosphatase, 3-PGA phosphatase and phosphoenolpyruvate (PEP) phosphatase) have much stricter substrate specificities (Duff *et al.*, 1991; del Pozo *et al.*, 1999; Miller *et al.*, 2001). Both intra- and extracellular APases are prominent in plants, and their activities have traditionally been used as markers for P-deficiency (Duff *et al.*, 1994; Baldwin *et al.*, 2001). Intracellular forms are found in the vacuole or cytoplasm. Although extracellular APases occur in the root apoplast and are frequently released from cell suspension cultures, extracellular types are usually localized in the cell wall, outer surface of root epidermal cells, and the root apical meristem. Intracellular APases appear to be much less stable than extracellular forms, which remain stable for hours to days (Goldstein *et al.*, 1988; Duff *et al.*, 1989a,

1991; Miller *et al.*, 2001). Increased stability would, of course, be a critical characteristic of any APase excreted into the rhizosphere. While intracellular APases induced during P deficiency function primarily in the release of P from senescent tissue for remobilization and in bypassing the P-requiring steps in C metabolism (Duff *et al.*, 1989b; Plaxton & Carswell, 1999), root extracellular APases are more likely to be involved in acquisition from soil (Marschner *et al.*, 1986; Tarafdar & Claasen, 1988). Although the acquisition of P from soil organic matter is equivocal, Miller *et al.* (2001) postulated that secretion of even low-affinity P-cleaving enzymes into soils with high organic P is an effective mechanism to provide additional sources of P for plant growth. Here, we focus on recent progress in understanding biochemical and molecular aspects of APases with special emphasis on exuded APases from cluster roots of P-deficient white lupin.

The literature abounds with reports of APases secreted from roots of P-deficient plants (Goldstein *et al.*, 1988; Li *et al.*, 1997a,b; Gilbert *et al.*, 1999; Hayes *et al.*, 1999; Zhang & McManus, 2000; Hunter & Leung, 2001). However, only recently have APase genes and proteins from *Arabidopsis*, tomato, and lupin cluster roots been fully characterized (del Pozo *et al.*, 1999; Wasaki *et al.*, 1999, 2002; Haran *et al.*, 2000; Baldwin *et al.*, 2001; Miller *et al.*, 2001). del Pozo *et al.* (1999) isolated and characterized a type 5 purple acid phosphatase, *AtACP5*, gene from *Arabidopsis*. The gene contained a 1014-bp open reading frame (ORF) encoding 338 amino acid residues. The protein had a 31 amino acid *N*-terminal extension with characteristics of a membrane targeting signal peptide. Both roots and shoots showed highly induced expression of *AtACP5* under P deficiency and addition of P could reverse induction. Induction of *AtACP5* also occurred in senescent tissues and in response to salt stress. Although AtACP5 was highly expressed in roots under P-stress, the authors could find no evidence for secretion of AtACP5 protein. They concluded that the protein was tightly anchored to the cell wall or plasmalemma. The *AtACP5* promoter, when fused to GUS and used as a reporter, showed high activity in response to P-deficiency, senescence, and salt stress. They concluded that *AtACP5* was involved more in internal P remobilization than P acquisition. Another APase (*LePS2*) implicated in internal remobilization of P has been characterized from tomato (Baldwin *et al.*, 2001). *LePS2* contains an 810-bp ORF encoding a 269 amino acid protein. The protein contains no 5′-signal peptide and is postulated to be in the cytosol. *LePS2* was highly induced in all tissues in P-deficient plants and expression was not induced by other nutrient stresses. Induction of *LePS2* transcripts could also be reversed by addition of P to stressed plants. Both *AtACP5* and *LePS2* were rapidly induced under P-deficient conditions, suggesting that plant molecular responses to P are tightly controlled.

The first truly secreted APase (sAPase) gene to be characterized was from *Arabidopsis* (*AtsAPase*; Haran *et al.* 2000). The *AtsAPase* transcript is 1380 bp corresponding to a 46 kDa protein of approximately 450 amino acids. This protein contains a 34 amino acid *N*-terminal extension predicted to target the mature protein to the endomembrane system. The gene was highly induced in roots of P-deficient plants. When the *AtsAPase* transit sequence was fused to green fluorescent protein (GFP) as a reporter, GFP was detected in root exudates of P-deficient plants. The authors proposed that the APase transit sequence would be useful in strategies to produce and obtain relatively pure proteins of industrial importance from plant roots.

Root APase activity increases dramatically in P-deficient white lupin plants (Ozawa *et al.*, 1995; Gilbert *et al.*, 1999). This large increase in APase activity is associated with the appearance of a novel isoform (Wasaki *et al.*, 1997; Gilbert *et al.*, 1999). Moreover, this novel form induced by P-stress is also secreted into the rhizosphere of cluster roots (Gilbert *et al.*, 1999; Miller *et al.*, 2001; Wasaki *et al.*, 2002). When cluster roots from P-deficient plants are placed on agar containing APase substrate, exudation of APase activity into the rhizosphere can be detected within minutes (Fig. 7). White lupin sAPase is synthesized as a preprotein having an M_r of 52 kDa. During secretion the protein's 31-amino acid presequence is cleaved, giving rise to a 49 kDa processed protein. Similar to other APases, the sAPase is a glycoprotein. Protein blots probed with antibodies for the sAPase showed rapid accumulation of the protein in P-deficient roots accompanied by secretion into the rhizosphere (Miller *et al.*, 2001). The cDNA for white lupin sAPase contained a 1380 bp open reading frame capable of coding a 460-amino acid protein. Expression of sAPase mRNAs was quite specific (Fig. 2) and found predominantly under P-deficiency in cluster roots (Miller *et al.*, 2001; Wasaki *et al.*, 2002). Slight induction was also found in Al stressed roots. Miller *et al.* (2001) isolated the promoter for *sAPase* and showed that the gene was responsive to internal P concentration. In addition, the promoter was functional in alfalfa, showing that the molecular events controlling plant response to P deficiency are conserved between plants. The fact that a 50 bp region within the promoter of white lupin sAPase is 72% identical to a region in the *Arabidopsis* P-induced APase supports this interpretation. White lupin's ability to access P unavailable to other species (Braum & Helmke, 1995) may not only reside in its exudation of organic acids but may also be a consequence of exuded APase activity (Fig. 3b).

VIII. Genetic regulation of P responsive genes

As mentioned previously, numerous molecular events are set in motion in response to P deficiency resulting in biochemical changes that facilitate P conservation and/or acquisition. Raghothama (1999) has suggested that more than 100 genes may be involved in plant response to low-P stress. The fact that many of the molecular and biochemical changes in

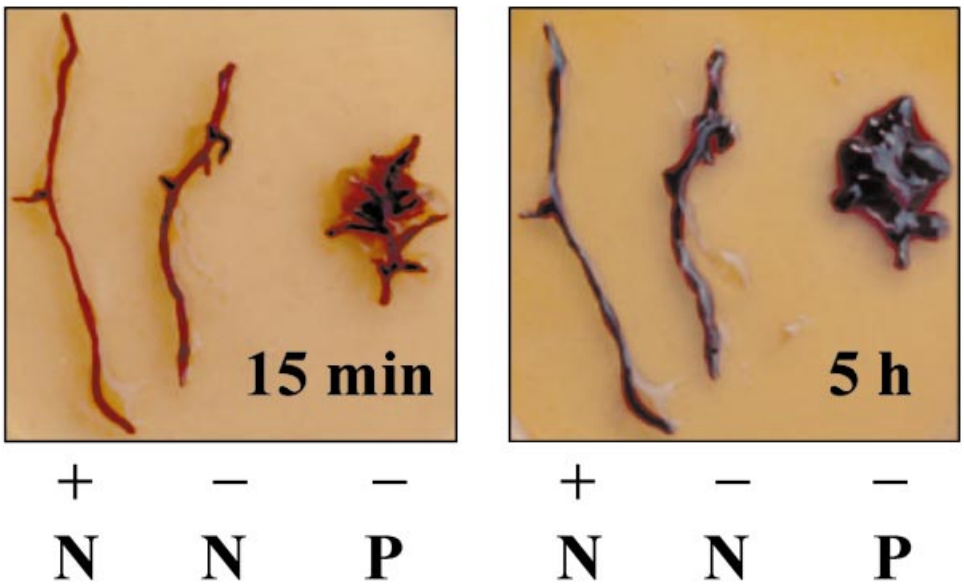

Fig. 7 *In vivo* staining for acid phosphatase activity in roots of normal and cluster roots of white lupin. The root segments were embedded in an agarose–acid phosphatase substrate mixture and photographed at 15 min (a) and 25 h (b). The dark, purple color indicates acid phosphatase activity in the roots and the root exudates. Acid phosphatase is rapidly exuded into the rhizosphere of P-deficient plants; +, plants that are P sufficient; –, plants that are P deficient.

response to P deficiency occur in synchrony suggests that the genes involved are coordinately expressed and share a common regulatory system. Raghothama (1999) and others (Goldstein, 1992; Schachtman *et al.*, 1998) have suggested that regulation of plant response to P stress may reside in a phosphate (*PHO*) regulatory system similar to that found in yeast and bacteria. The *PHO* regulon in bacteria and yeast represents a complex multigene system having both structural and regulatory components (Vogel & Hinnen, 1990; Wanner, 1993; Lenburg & O'Shea, 1996; Oshima, 1997). The P_i signaling in these organisms involves a classic two-component system which includes a sensor protein and a response regulator protein. The sensor element is ascribed to a transmembrane protein comprised of an extracellular module, involved in sensing environmental cues, fused to a protein kinase module, capable of autophosphorylation. The response regulator is cytosolic and also has two modules, one of which has a phosphorylation site, and the other acts as a transcription (*trans*) factor. Induction of -P response genes, in simplest terms, involves the sensor protein receiving the environmental cue and catalysing autophosphorylation. The kinase module of the sensor then phosphorylates the receiver module of the response regulator. In turn, the *trans*-factor module of the response regulator is then rendered capable of binding to specific *cis*-elements in the promoters of P responsive genes. Those specific promoter *cis*-elements in P responsive genes that bind with the *PHO* response regulator *trans*-factor are called *PHO*-boxes.

In recent years, plant two-component signaling systems have been identified that are involved in perception of and response to ethylene, cytokinin and osmoticum (for reviews see Sakakibara *et al.*, 1999; Urao *et al.*, 2000; Hwang & Sheen, 2001; Lohrmann & Harter, 2002). By contrast to the traditional prokaryotic two-component signaling system which comprises two proteins, the two-component system of plants appears to be more complex, involving a multistep process and at least three proteins (Sakakibara *et al.*, 2000; Urao *et al.*, 2000; Chang & Stadler, 2001; Gilroy & Trewavas, 2001; Lohrmann & Harter, 2002). The fundamental system in plants (Fig. 8) involves perception by the input domain of a membrane bound sensor histidine (His) kinase which autophosphorylates an aspartate (Asp) residue in the transmitter domain. The transmitter domain then phosphorylates a His residue on a phosphotransfer (HPt) protein which shuttles into the nucleus and transfers the P to an Asp residue of the response regulator (RR). Upon phosphorylation, the RR undergoes conformational change enabling it to bind to *cis*-elements within the promoter of genes to be activated by the signal molecule. More than 15 His-Asp kinases, five HPt, and 16 RR proteins have been identified in plants (Urao *et al.*, 2000; Lohrmann & Harter, 2002). Although this multistep two-component signaling mechanism has yet to be linked to signaling low and/or high P, it has been implicated in signaling N (Sakakibara *et al.*, 1999) and osmotic (Urao *et al.*, 1999) stress.

A growing body of evidence derived from studies of P-starvation response defective mutants of *Chlamydomonas* (Wykoff *et al.*, 1999) and *Arabidopsis* (Rubio *et al.* 2001) as well as soybean (*Glycine max*) P response genes (Tang *et al.*, 2001) indicates that nuclear localized transcription factors are involved in regulation of plant response to P. Genetic analysis of the *Chlamydomonas* and *Arabidopsis* P response mutants

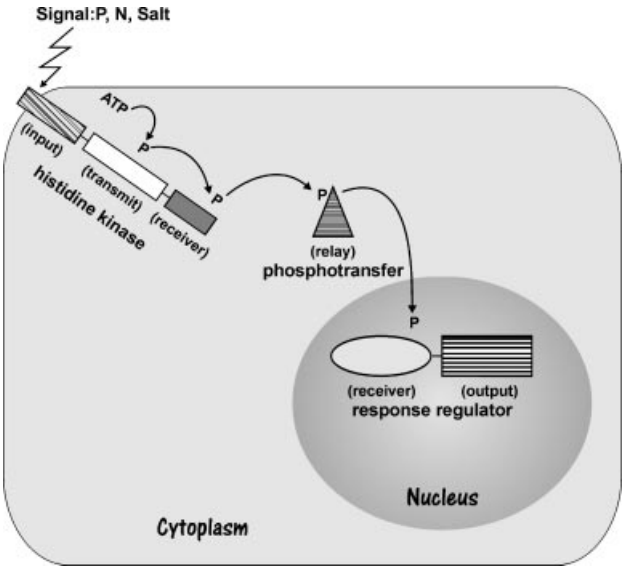

Fig. 8 Postulated two-component signaling system for perceiving and responding to environmental stimuli. This multistep, signal–response system is thought to involve three proteins: (1) a histidine kinase input-receiver protein undergoes autophosphorylation in response to an environmental (i.e. low P, N, O_2) signal; (2) the phosphate (P) is then transferred to a phosphorelay protein which has a nuclear targeting signal; (3) upon entering the nucleus the P is transferred from the phosphorelay protein to the response-regulator (RR) protein which undergoes conformational change allowing it to bind to specific *cis*-elements within the promoter of downstream genes involved in the plant's response to the environment.

showed that the mutations had occurred in MYB coiled-coil transcription factors designated *PSR1* and *PHR1*, respectively. Evaluation of the *Arabidopsis* genome shows the presence of 15 MYB coiled-coil proteins. Although PSR1 and PHR1 transcripts are detected independently of the P status of the plant, both increase under P-deficiency. Both MYB genes are proposed to act downstream from the initial P signaling recognition event. The *Arabidopsis* PHR1 MYB protein was shown to bind to an imperfect palindromic consensus sequence, 5′-GNATATNC-3′, in the promoters of several P starvation-responsive genes including white lupin *sAPase*, *LaPT1*, and *LaMYB1* (Liu *et al.*, 2001; Miller *et al.*, 2001). By comparison, the soybean vegetative storage protein (VSPB) which is downregulated in response to added P was shown to be regulated in part by homeodomain leucine zipper protein (HD-ZIP) which binds to the CATTAATTAG element in the VSPB promoter.

Raghothama (1999) has noted that PHO-box like elements occur in some P-deficiency induced genes and has recently identified a *cis*-element 5′-CACGT(G/T)-3′ in the promoter of several P-response genes. This element is identical to the Pho4 binding domain in yeast P-response genes (Ogawa *et al.*, 1995). We have also recently identified this motif in the promoters of P-deficiency induced APase (Miller *et al.*, 2001) and a MYB transcription factor (J-Q Liu & C. P. Vance unpubl. data), genes which are highly expressed in cluster roots. Interestingly, CACGTG is known as a G-box in plant genes which are regulated by various biotic and abiotic stresses. Also, relevant to identifying genes affecting nutrient acquisition and root development, Zhang and Forde (1998, 2000) have isolated and functionally characterized a MADs box transcription factor, *ANR1*, that controls nitrate-induced changes in lateral root development. Thus, there are likely many transcription factors and *cis*-elements that interact to control nutrient acquisition and root architecture. Many of these may be active during the development of white lupin cluster roots as evidenced by our sequencing of ESTs from these organs revealing *TUBBY*-, *GRAB/NAM*-, *RAS*-, *DIMINUTO*-, and *SCARECROW*-like genes (http://www.home.earthlink.net/~whitelupinacelimation).

IX. Improving P acquisition

Strategies for improving P acquisition and use involve both traditional plant breeding approaches and transgenic technology. Improvement through either approach is dependent upon: (1) identifying a suitable plant trait or gene that potentially affects acquisition and/or use; (2) finding or generating sufficient genetic variation for traits within a germplasm source; and (3) introgression of the trait into agronomically suitable backgrounds. Suitable plant traits having potential to impact P acquisition and use include: root architecture (Yan, 1999; Lynch & Brown, 2001; Williamson *et al.*, 2001), organic acid synthesis and extrusion (Hoffland *et al.*, 1992; Hinsinger & Gilkes, 1997; Neumann & Römheld, 1999), exudation of APases and phytases (Hayes *et al.*, 1999; Miller *et al.*, 2001; Richardson *et al.*, 2001; Brinch-Pedersen *et al.*, 2002); and phosphate transporters (Raghothama, 1999; Gilroy & Jones, 2000; Vance, 2001).

Roots have been notoriously recalcitrant to study and to improve through genetic selection due to the fact that their development is affected by a multitude of genes and their subterranean nature. However, as outlined here and elsewhere in this review, advances in understanding the genetic control of roots, improved methods for root evaluation, and reaffirmation of their importance for improving nutrient use efficiency have led to a renaissance of discovery in root biology that should result in significant improvements to crop plants.

Genetic variability has been documented in a wide range of species (and probably exists in all species) for tap root elongation, basal root growth angle (i.e. rooting depth, lateral and adventitious root development, and root hair numbers; Klepper, 1992; Charlton, 1996). Inclusively these traits comprise root architecture and each has been identified as important to P acquisition (Lynch, 1995; Eshel *et al.*, 1996; Gahoonia & Nielsen, 1997; Care & Caradus, 1999; Yan, 1999; Narang *et al.*, 2000). *Phaseolus vulgaris* has been the prototype for selection of root architecture ideotypes that result in improved

P acquisition (Lynch, 1995; Yan, 1999; Lynch & Brown, 2001). Genetic selection as well as root simulation models with bean have shown enhanced P uptake in genotypes with shallow roots, accompanied by heightened numbers of lateral roots with abundant root hairs. Selection for increased root hair abundance has led to enhanced P acquisition in bean, clover, barley and *Arabidopsis*. Wissuwa & Ae (2001) have identified four quantitative trait loci (QTL) in rice (*Oryza sativa*) associated with increased P uptake under P-deficiency, two of which were related to maintenance of root growth under low P. Similarly, these root architecture traits are also important in the acquisition of N and other nutrients (Klepper, 1992; Zhang & Forde, 1998; Lamb *et al.*, 2000; Vance & Lamb, 2001).

Phosphorus acquisition has also been improved through approaches aimed at increasing organic acid synthesis in and/or exudation from plant roots. This approach is based upon the large body of evidence showing that exudation of citrate and malate from roots effectively solubilizes unavailable sources of P (Marschner *et al.*, 1986). Release of organic acids into the rhizosphere in exudates leads to metal chelation and subsequent desorption of P_i from the soil matrix, with a concomitant increase in availability. Rhizosphere acidification by protons and/or organic acids may be an accompanying trait when root architecture is modified through selection as shown in bean (Yan, 1999), *Arabidopsis* (Narang *et al.*, 2000), rice (Yang *et al.*, 2000) and pigeon pea (Ae *et al.*, 1990). The acidification of the rhizosphere of white lupin cluster roots also exemplifies the concurrent exudation of organic acids accompanying a change in root development. Overexpression of genes involved in organic acid synthesis appears to be a useful strategy to stimulate P acquisition by enhancing exudation of citrate and malate. Koyama *et al.* (1999) developed carrot (*Dacus carota*) cell lines that overexpressed mitochondrial citrate synthase. The overexpressing cell lines exuded more citrate and had improved growth on $AlPO_4^-$ medium compared with untransformed controls. In a similar approach but using a bacterial citrate synthase gene driven by the $CaMV^{35}S$ promoter, Lopez-Bucio *et al.* (2000a) demonstrated that overexpression of citrate synthase resulted in increased secretion of citrate into the rhizosphere and enhanced P accumulation. Our laboratory recently overexpressed a novel form of MDH in alfalfa (Tesfaye *et al.*, 2001). Transgenic plants containing the novel MDH driven by the $CaMV^{35}S$ promoter had increased organic acid synthesis and exudation, which resulted in increased P accumulation compared with either transformed or untransformed controls. Interestingly, in each of the above cases where overexpression of genes involved in citrate and malate production enhanced organic acid synthesis and P acquisition, the transformed plants had greater Al^{3+} tolerance than controls (Lopez-Bucio *et al.*, 2000b; Tesfaye *et al.*, 2001). It is well established that Al^{3+} tolerance in plants is related to organic acid accumulation and/or exudation by roots (Kochian, 1995; Ryan *et al.*, 2001). Moreover, increased organic acid synthesis and exudation has been implicated in tolerance to Pb and other metals (Mathys, 1977; Godbold *et al.*, 1984; Kochian, 1995; Yang *et al.*, 2000). In addition, accumulation and exudation of citrate and malate by roots has been implicated in alleviating Fe deficiency (de Vos *et al.*, 1986; Jones, 1998). Thus, genetic engineering of plants for enhanced organic acid synthesis may have far-reaching effects on plant nutrition and stress tolerance.

Other targets which have potential to improve P_i acquisition include high-affinity P_i transporters (Mitsukawa *et al.*, 1997) and root exuded phytase (Richardson *et al.*, 2001). Overexpression of the *Arabidopsis* high-affinity P_i transporter gene (*PHT1*) in tobacco resulted in cell lines with enhanced growth under P_i-limited conditions (Mitsukawa *et al.*, 1997). Transgenic tobacco lines overexpressing *PHT1* had threefold greater P_i uptake than controls and 50% greater growth under low Pi conditions. Richardson *et al.* (2001) generated transgenic *Arabidopsis* plants that expressed an *Aspergillus* phytase gene and exuded phytase into the rooting medium. The transgenic plants could grow on phytate as the sole source of P whereas control plants could not. We have recently developed alfalfa plants that can overexpress several diverse proteins in roots in efforts to metabolically engineer improved nutrient use acquisition (APase, MDH and metallothioneins). These transgenic plants are currently awaiting characterization.

Evidence is accumulating that selection for and overexpression of selected genes involved in either root development or P acquisition can improve nutrient acquisition. As the arsenal of plant genes involved in these processes expands through whole-genome approaches, we will undoubtedly see more successful examples of plants with better nutrient acquisition and utilization created through traditional breeding and transgenic technology. Because of the urgent need for plant germplasm with improved P-use efficiency, it is imperative that research programs mount multidisciplinary teams that combine traditional plant breeding, biotechnology and plant physiology.

X. Synopsis

The world is on the brink of a new agriculture, one that involves the marriage of plant biology and agroecology under the umbrella of biotechnology and germplasm improvement. Although P fertilizers will continue to play a major role in intensive agriculture, depletion of natural resources, loss of biodiversity, and long-term unsustainability require that alternative strategies be investigated and implemented to buffer against food insecurity and environmental degradation. Furthermore, because improved P acquisition and use by plants has immediate and direct benefit in extensive agriculture in developing countries where access to fertilizers is limited, funding for research at international centers should be a high priority. The following recommendations deserve attention: (1) expand research in root biology;

(2) continue to isolate, characterize and develop a fundamental understanding of individual genes holding promise for application to improving P nutrition; (3) enhance the expression of genes and increase the synthesis of gene products, such as those involved in transport of nutrients and exudation of organic acids, through transgenic technology and incorporate these traits into adapted germplasm; (4) continue and expand collaborations between plant breeders and physiologists to develop germplasm with enhanced P-use efficiency; and (5) reemphasize applied research programs directed toward sustainable approaches to enhancing soil P through crop management (such as intercropping, rotations and incorporation of legumes).

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