Chapter 6
PLANTS WITHOUT ARBUSCULAR MYCORRHIZAE*

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INTRODUCTION

Although mycorrhizal symbioses (described elsewhere in this volume) are the most important adaptation for angiosperms to acquire scarce phosphorus (P), many plant families contain species that either do not form or rarely form this pivotal association (Skene 1998; Miller *et al*. 1999; Cripps and Eddington 2005; Miller 2005). This review will address adaptations and mechanisms for acquisition and use of scarce P in plants lacking effective mycorrhizal symbioses. The primary focus will be on root adaptations in species that develop specialized-complex roots (cluster and dauciform) in response to P deficiency. Although not producing cluster or dauciform roots in response to P deficiency, Arabidopsis will also be considered because it does not form mycorrhizal symbiosis and is a model species for evaluating plant adaptation to P deficiency.

Plants have evolved two broad strategies for improved P acquisition and use in nutrient-limiting environments: (1) those aimed at conservation of use; and (2) those directed toward enhanced acquisition or uptake (Vance *et al*. 2003; Ticconi and Abel 2004; Misson *et al*. 2005; Morcuende *et al*. 2007). Processes that conserve the use of P involve decreased growth rate, increased growth per unit of P uptake, remobilization of internal P, modifications in carbon (C) metabolism that bypass P-requiring steps, alternative respiratory pathways, and alterations in membrane biosynthesis requiring less P (Plaxton and Carswell 1999; Uhde-Stone *et al*. 2003a,b; Wasaki *et al*. 2003; Misson *et al*. 2005; Lambers *et al*. 2006). In comparison, processes that lead to enhanced P uptake include modified root architecture and greater root growth, prolific development of root hairs leading to expanded root surface area, enhanced expression of Pi transporter genes, and increased production and exudation of phosphatases and organic acids (Marschner *et al*. 1986; López-Bucio *et al*. 2002; Shane and Lambers 2005). These numerous adaptive responses to P-deficiency are not mutually exclusive and all may occur within a single species.

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ROOT ARCHITECTURE

Root architecture (Dinkelaker *et al*. 1995; Lynch and Brown 2001; Williamson *et al*. 2001; López-Bucio *et al*. 2003; Lambers *et al*. 2006) 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 (Dinkelaker *et al*. 1995; Borch *et al*. 1999; Williamson *et al*. 2001; López-Bucio *et al*. 2003; Lambers *et al*. 2006) and is known to impact all aspects of root growth and development. Phenotypic and genotypic adaptations to P deficiency involve changes in root architecture that facilitate acquisition of P from the topsoil (Lamont 1982; Lynch and Brown 2001; López-Bucio *et al*. 2003). 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 modification of root architecture as adaptations for topsoil foraging. Root characteristics associated with improved topsoil foraging for scarce P are a more horizontal basal-root growth angle resulting in more shallow roots, increased adventitious root formation, enhanced lateral root proliferation, and increased root hair density and length. Such modifications in root architecture in response to P deficiency are well documented in Arabidopsis and in those species forming either cluster or dauciform roots.

ROOT DEVELOPMENT AND FUNCTION ARE ALTERED BY P DEFICIENCY

Cluster root species

Distribution

While Arabidopsis is a compelling model species for the study of plant adaptation to P deficiency, a wide group of species respond to the scarcity of P with profound changes in root architecture resulting in cluster roots (Dinkelaker *et al*. 1995; Diem *et al*. 2000; Pate and Watt 2001; Neumann and Martinoia 2002; Lamont 2003; Vance *et al*. 2003; Shane and Lambers 2005; Lambers *et al*. 2006). Phosphorus-deficiency induced cluster root formation involves the localized formation of massive numbers of determinate secondary/tertiary rootlets having prolific root hair formation (Figures 6.1, 6.2). In the literature they can be referred to as cluster, proteoid, bottlebrush, and/or dauciform roots (Lamont 2003; Vance *et al*. 2003; Lambers *et al*. 2006). Originally thought to be present in only native species of the Proteaceae in Australia and South Africa, cluster roots are now known to have widespread occurrence (Table 6.1) in members of the Betulaceae, Moraceae, Casuarinaceae, Myricaceae, Eleagnaceae, Cucurbitaceae, Cyperaceae, Restionaceae, and Fabaceae

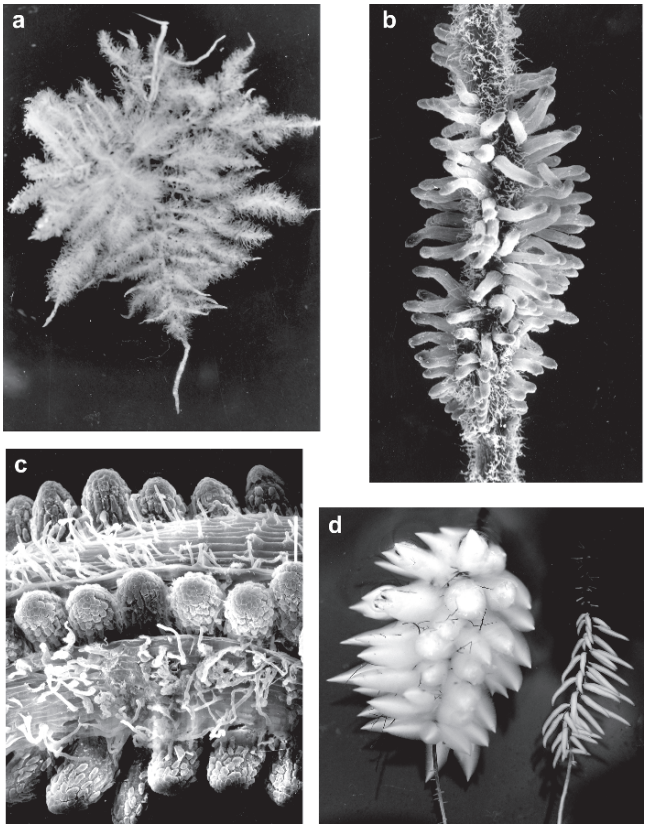

Fig. 6.1 Cluster roots of members of the family Protaceae (**a**, **b**, **c**) and dauciform roots of the Cyperaceae (**d**). **a**. Compound cluster root of *Banksia cirsioides*. This compound cluster root is 6 cm in length (Permission and courtesy of Byron Lamont). **b**. Young cluster root of *Leucodendron laureolum* prior to root hair development on rootlets (Scanning electron microscopy. Permission and courtesy of Byron Lamont). **c**. Cluster root of *L. laureolum* emerging through epidermis (Scanning electron microscopy. Permission and courtesy of Byron Lamont). **d**. Dauciform roots of *Caustis* sp (Cyperaceae) with prolific root hair development. (Permission and courtesy of Michael Shane.)

(Lamont 1993, 2003; Skene 1998; Lambers *et al*. 2006). Many species in these families are particularly adapted to growth in nutrient-impoverished soils through the evolution of cluster roots for acquisition of scarce nutrients. Members of the Cyperaceae and Restionaceae are frequently primary colonizers of marshes and wetland. All cluster root species are well adapted to low P growth environments (Lamont 1993, 2003; Lambers *et al*. 2006). Beside mycorrhizal associations, cluster roots are regarded as one of the major adaptations for P acquisition (Dinkelaker *et al*. 1995; Skene 1998; Diem *et al*. 2000; Adams and Pate 2002; Lamont 2003; Vance *et al*. 2003; Shane and Lambers 2005).

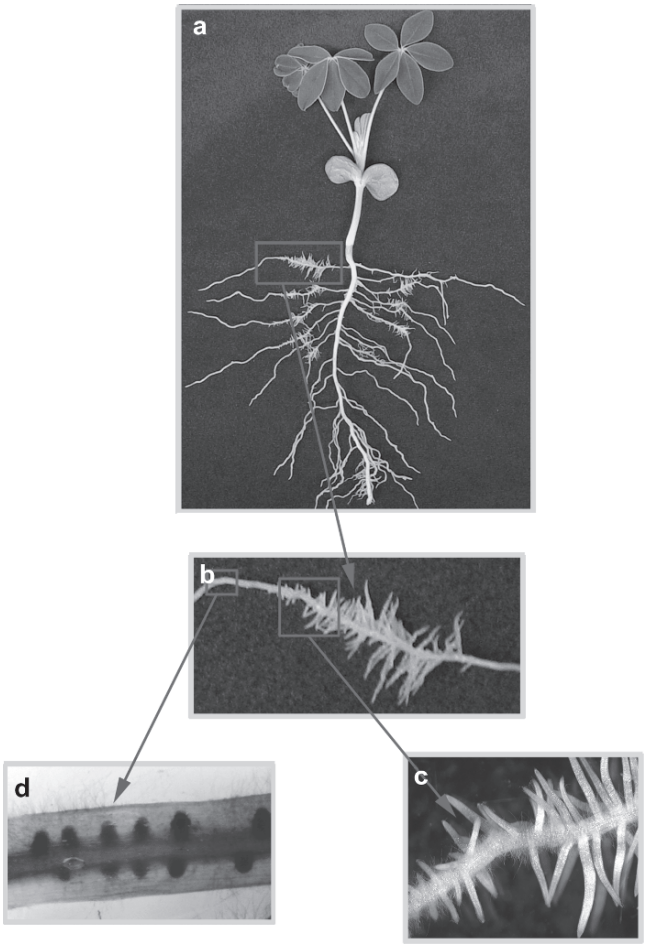

Fig. 6.2 White lupin plant grown under P-deficient conditions 14 days after emergence. **a**. Note cluster root zones on first-order lateral roots. **b**. Cluster root zone boxed is enlarged to show developmental zones. Premature (**c**) and pre-emergent (**d**) zones are shown in boxes at higher magnification. (Prepared by Dr. B. Bucciarelli.)

Development

Recent reviews (Lamont 2003; Shane and Lambers 2005; Lambers *et al*. 2006) have documented the striking diversity in cluster root morphology. Cluster roots form through synchronized development of densely packed determinate rootlets. They can comprise single clusters formed on parent axis, as found in members of the Proteaceae, such as *Hakeae* spp., *Leucadendron* spp., *Grevillea robusta*, and in the Fabaceae, such as white lupin (Lamont 1982, 2003; Skene *et al*. 1996; Lambers *et al*. 2006; Figure 6.1). However, some species from the Proteaceae, like *Banksia*

Table 6.1 Plant families having specialized compound (cluster or dauciform) root development and the prevalence of either nitrogen (N_2) fixing or mycorrhizal symbioses. (Adapted from: Dinkelaker *et al*. 1995; Skene 1998; Waters and Blevins 2000; Lamont 2003; Shane and Lambers 2005; Lambers *et al*. 2006.)

		Symbiosis		
Family	Root	Mycorrhizal	N_2 fixation	
			Rhizobial	Actinorrhizal
Fabaceae[a]	Cluster	–/+	+	–
Moraceae	Cluster	–	–	–
Betulaceae[a]	Cluster	–/+	–	+
Casuarinaceae[a]	Cluster	–/+	–	+
Eleagnaceae	Cluster	+	–	+
Myricaceae[a]	Cluster	–/+	–	+
Proteaceae	Cluster	–	–	–
Cucurbitaceae[a]	Cluster	+	–	–
Cyperaceae[a]	Dauciform	–/+	–	–

[a] Families noted as –/+ may be mycorrhizal but those species forming cluster roots are either non-mycorrhizal– or weakly mycorrhizal (+ = mycorrhizal/–/+ = weakly mycorrhizal).

grandis, form more complex compound clusters that form embedded mats in the soil profile (Lamont 1993, 2003; Adams and Pate 2002; Shane and Lambers 2005). Several features of dicot cluster root development and morphology are distinguished from that of typical dicot lateral roots. First, lateral roots are initiated usually in an alternating pattern from the pericycle of primary roots near the zone of metaxylem differentiation (Celenza *et al*. 1995; Charlton 1996), while cluster rootlets are initiated in waves along the axis of second and third order lateral roots (Skene and James 2000; Pate and Watt 2001; Lamont 2003). Second, typical lateral roots are initiated singularly opposite a protoxylem point, unlike cluster roots which are initiated in multiples opposite every protoxylem point within the wave of differentiation (Figures 6.1, 6.2). Third, cluster rootlets produce a superabundance of root hairs due to an apparent loss of regulation of trichoblast differentiation, while root hair development in typical roots is highly regulated and occurs from a discrete number of trichoblasts (Dolan 2001; Müller and Schmidt 2004). The length of cluster root segments varies from a few millimeters to 2–4 cm. Under P-deficient conditions cluster roots can comprise more than 70% of the plant root mass (Lamont 1982, 2003; Pate and Watt 2001; Lambers *et al*. 2006). The frequency of cluster roots in P-deficient soil and the accompanying increase in root hair density results in an increase in root surface area of greater than 100-fold (Lamont 2003; Vance *et al*. 2003; Shane and Lambers 2005). Another notable feature of cluster rootlets is that their growth is determinate, ceasing shortly after emergence, as contrasted to the indeterminate growth of lateral roots (Neumann and Martinoia 2002). This highly synchronous developmental pattern reflects that cluster root formation is an elegant, finely tuned process. Moreover, because root pericycle cells are arrested in the G2 phase of the cell cycle (Skene 1998; Skene and James 2000), cluster root initiation likely involves a hormone mediated concerted release of multiple pericycle cells from the G2 phase in a wave-like pattern along second-order lateral roots.

Root architectural changes, classified as cluster root types, also occur in the sedges (Cyperaceae) and the rushes (Restionaceae). Lamont (1974) characterized the cluster root morphology found in the Cyperaceae as dauciform due to the carrot-like developmental pattern along the root axis. As dauciform roots mature they develop large numbers of long (3–5 mm) root hairs (Figure 6.1; Shane and Lambers 2005; Shane *et al*. 2005). The development of dauciform roots in *Schoenus unispiculatus* was directly related to P availability, with their formation being suppressed as P availability increased (Shane *et al*. 2005). Recently Shane *et al*. (2006) have shown that dauciform roots of *S. unispiculatus* are structurally distinct from typical cluster roots, but functionally analogous to them. In the Restionaceae, the cluster root morphology has been characterized as capillaroid (Lamont 1982) due to the sponge-like, water-holding capacity of the root-soil aggregate. Capillaroid species have root clumps with exceptionally long root hairs (see Figure 6.3; Lambers *et al*. 2006).

Cluster root function

Similar to mycorrhizal association, cluster roots increase the root surface area and soil volume exploited for mining nutrients. In contrast to mycorrhizae, which grow over much of the entire root surface, the hairy and densely packed lateral rootlets in cluster root zones bind tightly to trapped soil particles and organic matter in localized soil volumes. Cluster root aggregates are most prominent in the upper layers of the soil profile where P is most abundant (Lamont 2003; Shane and Lambers 2005;

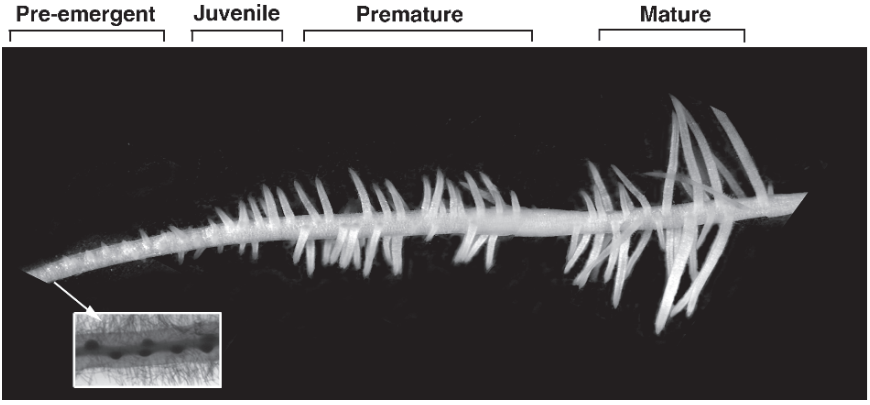

Fig. 6.3 Cluster root segment of P-deficient white lupin. Rootlet development proceeds from left to right. Pre-emergent zone contains rootlet confined to the root cortex and not yet emerged. Juvenile, premature, and mature zones contain emerged rootlets as described by Neumann and Martinoia (2002). Mature cluster roots have ceased elongating and have determinate development. Inset shows root hairs in pre-emergent zone. (Prepared by Dr. B. Bucciarelli.)

Lambers *et al*. 2006). The dense aggregation of cluster roots and soil facilitates the acquisition and uptake of nutrients, particularly P (Lamont 2003; Vance *et al*. 2003; Shane and Lambers 2005; Lambers *et al*. 2006). Enhanced capacity for nutrient acquisition and uptake by cluster roots occurs by effectively concentrating root exudates and plant nutrient uptake systems in localized patches of soil (Grierson and Attiwill 1989). Cluster roots exude organic acids (Gardner *et al*. 1983; Dinkelaker *et al*. 1995; Johnson *et al*. 1996a,b; Shane *et al*. 2006), protons (H^+; Dinkelaker *et al*. 1995; Neumann and Martinoia 2002; Yan *et al*. 2002; Shane *et al*. 2006), phenolics (Neumann *et al*. 1999; Weisskopf *et al*. 2006a,b), acid phosphatases (Dinkelaker *et al*. 1995; Gilbert *et al*. 1999; Miller *et al*. 2001), and iron chelate reductase (Dinkelaker *et al*. 1995; Neumann and Martinoia 2002). Cluster roots have enhanced expression of phosphate (Pi) transporters (Liu *et al*. 2001) and increased Pi uptake (Keerthisinghe *et al*. 1998; Neumann *et al*. 2000; Sousa *et al*. 2007). Exudation of carboxylates, H^+, phenolics, and acid phosphatases releases P bound to inorganic and organic particles making it available for rapid uptake by Pi transporters. Cluster root development accompanied by corresponding changes in cluster root metabolism and membrane uptake systems reflect an elegant, highly coordinated molecular response to P deficiency.

Although the development of cluster roots in Proteaceae and Cyperaceae has received growing attention, white lupin has served as the model for analysis of biochemical and molecular adaptations contributing to enhanced P acquisition and use by cluster roots (Dinkelaker *et al*. 1995; Watt and Evans 1999; Neumann and Martinoia 2002; Uhde-Stone *et al*. 2003a,b; Liu *et al*. 2005). As P deficiency occurs in white lupin, a cascade of changes in gene expression occurs resulting in synchronous development of: cluster roots having prolific root hair density; exudation of carboxylates, H^+, and enzymes; enhanced expression of membrane transporters; and apparent heightened sensitivity in roots to hormonal signals. Neumann *et al*. (2000) have staged cluster root development into four phases distinguished by rootlet emergence and biochemical response (Figure 6.3). In the juvenile and premature stages, cluster rootlets have emerged from the cortex and are actively elongating. At these stages rootlets are exuding malate and citrate in fairly equal amounts (300–700 nmol h^{-1} g^{-1} fw) accompanied by uniform rhizosphere pH (5–6) and little extrusion of protons. As the mature stage is attained, rootlet elongation stops, citrate exudation exceeds malate by four- to five-fold, copious amounts of acid phosphatase and phenolics are exuded accompanied by H^+ extrusion and a reduction in rhizosphere pH (Neumann and Martinoia 2002). Mature cluster root zones have increased transcript abundance for P transporters (Liu *et al*. 2001), acid phosphatase (Miller *et al*. 2001), sugar metabolism genes (Uhde-Stone *et al*. 2003a), and MATE-transporters (Uhde-Stone *et al*. 2005). It is noteworthy that total RNA concentration decreases as cluster root zones progress through development into the later phase of maturity, suggesting an increased turnover of nucleic acids to provide P for remobilization (Johnson *et al*. 1996a,b). The burst of exudation occurring in mature cluster root zones occurs over a three to four day period followed by the senescent stages of differentiation in which cluster roots turn brown and physically deteriorate (Watt and Evans 1999). Recent studies with cluster roots

of *Hakea*, Proteaceae (Shane *et al*. 2004; Shane and Lambers 2005) and with dauciform roots of *Schoenus*, Cyperaceae (Shane *et al*. 2006) show developmental stages and biochemical modifications analogous to those that occur in white lupin.

Phosphate acquisition

Phosphate acquisition from soil is enhanced in cluster root species (Gardner *et al*. 1982; Lamont 1982; Grierson 1992; Dinkelaker *et al*. 1995; Neumann *et al*. 2000; Keerthisinghe *et al*. 1998; Sousa *et al*. 2007). Enhanced acquisition of soil P occurs not only because of the biochemical and architectural changes that occur in cluster roots, which facilitate increased surface area for explorations and release of exudates to solubilize P, but also because Pi uptake is enhanced in cluster roots. Increased Pi uptake has been noted in cluster root zones of both white lupin (Keerthisinghe *et al*. 1998; Neumann *et al*. 2000) and *Hakea* (Sousa *et al*. 2007). The Pi concentration at which Pi uptake is half-maximal (Km) in cluster root species ranges from 1.0 μM for the high affinity uptake in low P soils to 40 μM under high P conditions. Sousa *et al*. (2007) showed a biphasic Pi uptake for *Hakea serica* indicating a high and low affinity system. Interestingly, Liu *et al*. (2001) isolated and characterized two Pi-transporter genes from white lupin cluster roots. The white lupin phosphate transporter 1 (*LaPT1*) was specifically expressed in cluster roots under P-deficiency and probably corresponds to a high affinity transporter. In contrast, *LaPT2* is expressed in all tissues in fairly high abundance and likely represents a low affinity transporter. We have noted the expression abundance of numerous transporter genes in P deficiency induced cluster roots of white lupin (Uhde-Stone *et al*. 2003b) ranging from Pi transporters to sulfate transporters, amino acid, and sugar transporters. The abundance of these transporters in white lupin cluster roots suggests the cluster root system is geared up for effective transport of many nutrients.

Modified carbon metabolism and partitioning is required for adaptation to P deficiency

Under normal growth and developmental 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 (Marschner *et al*. 1986; Kochian *et al*. 2004). 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 (Marschner *et al*. 1986; Hinsinger 2001; Ryan *et al*. 2001; Kochian *et al*. 2004). During P deficiency, roots show enhanced

accumulation of sugars, increased synthesis and exudation of carboxylates, and a dependence upon sugars or current phloem transport for P-stress induced gene expression in roots (Johnson *et al*. 1996a; Keerthisinghe *et al*. 1998; Watt and Evans 1999; Neumann and Martinoia 2002; Shane *et al*. 2004; Liu *et al*. 2005).

Convincing evidence exists for exudation of malate and citrate as a principal mechanism for relieving the edaphic stress of P deficiency. The release of carboxylates allows for the chelation of Al^{3+}, Fe^{3+}, and Ca^{2+} and the subsequent displacement of Pi from bound or precipitated forms (Gardner *et al*. 1982; Dinkelaker *et al*. 1995; Ryan *et al*. 2001; Vance *et al*. 2003; Shane and Lambers 2005) and may also make organic P more susceptible to acid phosphatase activity. Tricarboxylates, such as citrate, are more effective than dicarboxylates, such as malate, at displacing bound P due to their greater affinity for Fe^{3+}, Al^{3+}, and Ca^{2+} (Hinsinger 2001; Ryan *et al*. 2001; Kochian *et al*. 2004). While many plant species exude carboxylates under P-deficient conditions (Marschner *et al*. 1986; Ryan *et al*. 2001; Lambers *et al*. 2006), this trait achieves maximum effectiveness in cluster root species (Johnson *et al*. 1996b; Keerthisinghe *et al*. 1998; Neumann and Martinoia 2002; Kihara *et al*. 2003; Shane *et al*. 2004, 2006). When grown under P-deficient conditions, cluster roots exude 20- to 40-fold more citrate and malate than P-sufficient roots. Shane *et al*. (2004) reported that a significant accumulation of carboxylates (75 μmol g^{-1} fw) in *Hakea* cluster roots was coincident with the maximum rate of organic acid exudation. Likewise, Watt and Evans (1999) showed that organic acid exudation from mature lupin cluster roots peaked at 34 nmol min^{-1} g^{-1} fw, compared to near non-detectable levels in the early stages of their development.

Malate is frequently detected as the primary component of exudates in juvenile and premature cluster roots, while citrate predominates at peak exudation in mature cluster roots (Johnson *et al*. 1994, 1996b; Watt and Evans 1999; Neumann *et al*. 2000; Shane *et al*. 2004, 2006). Peak exudation has been referred to as the exudative burst. The amount of carbon exuded in citrate and malate can constitute from 10% to greater than 25% of the apparent current photosynthate of the plant (Johnson *et al*. 1994, 1996a; Neumann *et al*. 2000). Cluster roots and P-stressed roots in general are strong sinks for photosynthate (Johnson *et al*. 1996a; Watt and Evans 1999; Neumann *et al*. 2000; Shane *et al*. 2004; Hernández *et al*. 2007; Morcuende *et al*. 2007).

The striking change that occurs in the organic acid exudation of P-deficient cluster roots is invariably reflected in concurrent changes in RNA expression and activity of enzymes involved in C metabolism (Johnson *et al*. 1994; Neumann *et al*. 2000; Massonneau *et al*. 2001; Uhde-Stone *et al*. 2003a; Shane *et al*. 2004). Measurements of shoot and root CO_2 fixation with $^{14}CO_2$, accompanied by measurements of C partitioning and exudation, show that organic acids exuded from lupin cluster roots are derived from both photosynthetic CO_2 fixation and root dark (anapleurotic) CO_2 fixation (Johnson *et al*. 1994, 1996a,b). Some 60–65% of C exuded from roots is shoot derived while 35–40% is root derived. Exudation of carboxylates from roots is accompanied by an increase in the activities of sucrose synthase (SS), enzymes of glycolysis, and organic acids, particularly phospho*enol*pyruvate carboxylase (PEPC) and malate dehydrogenase (MDH; Johnson *et al*.

1994; Massonneau *et al*. 2001; Uhde-Stone *et al*. 2003a; Peñaloza *et al*. 2005; Figure 6.4). Similar changes in C metabolism have been noted for P-deficient roots of bean (Ciereszko *et al*. 1998; Hernández *et al*. 2007) and Arabidopsis (Morcuende *et al*. 2007).

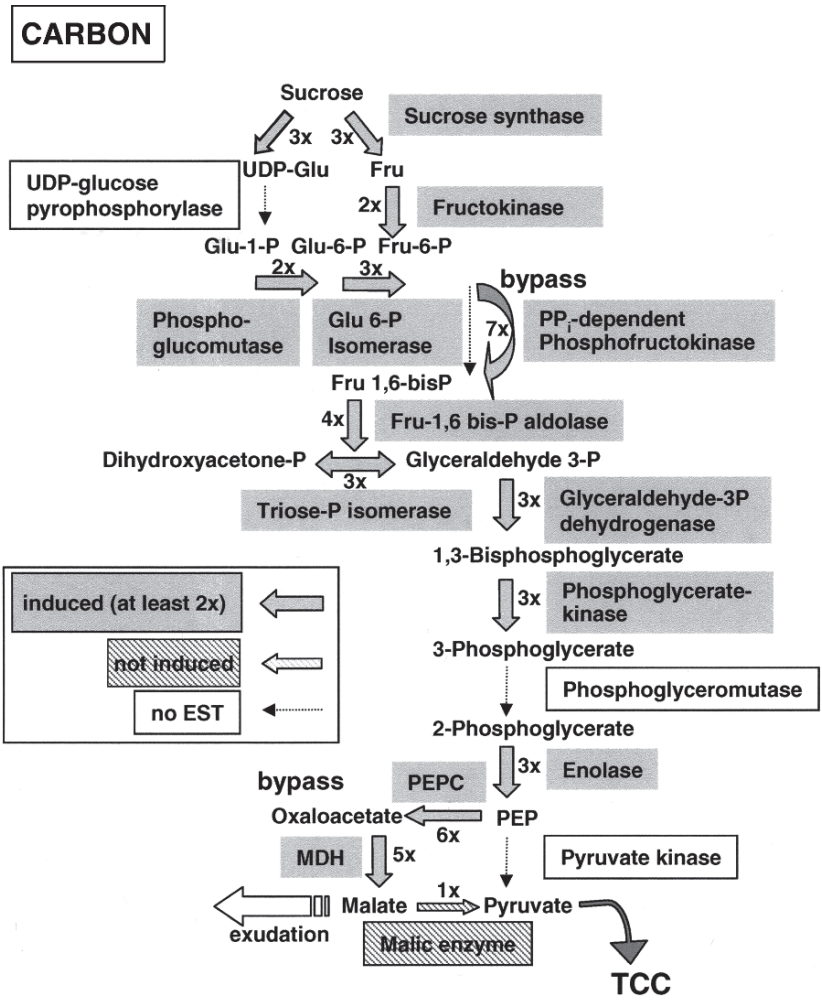

Fig. 6.4 Schematic representation of sucrose (carbon) metabolism through glycolysis with the formation of malate for exudation. ESTs with induced expression in P-deficient proteoid roots, compared to P-sufficient normal roots are represented in grey boxes. The average gene induction determined by two independent macroarrays is indicated at the corresponding arrows (e.g. 2x). Enzymes of the glycolytic pathway that were not found in the collection of 1,250 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 proteoid roots, compared to P-sufficient normal roots. (From Uhde-Stone *et al*. 2003a.)

Interestingly, even though cluster roots are strong sinks for C there is not a striking increase in respiration. Cluster root C metabolism and respiration appear to be relatively efficient due to adaptive changes that occur in response to low P. For example, P deficiency can limit the activity of pyruvate kinase (PK), an enzyme requiring Pi and ADP. The PEPC, MDH, NAD-malic enzyme pathway can bypass the PK entryway into the TCA cycle, thereby maintaining the flow of C while avoiding the use of ADP and generating Pi (Plaxton and Carswell 1999; Morcuende *et al*. 2007). Likewise, the reduction in cellular ADP and Pi in P deficient plants can result in reduced efficiency of respiration by inhibiting the cytochrome pathway of electron transport. Non-phosphorylating pathways that bypass energy-requiring steps, like the alternative oxidase (AOX) system, can maintain cellular metabolic integrity (Theodorou and Plaxton 1993; Day and Wiskich 1995; Vance *et al*. 2003). Recent studies show that AOX is enhanced in *Hakea* cluster roots (Shane *et al*. 2004), Arabidopsis (Morcuende *et al*. 2007), and bean (Hernández *et al*. 2007).

A series of elegant studies have now conclusively demonstrated a tight link between sugars and plant adaptation to P deficiency (Liu *et al*. 2005; Hernández *et al*. 2007; Karthikeyan *et al*. 2007; Morcuende *et al*. 2007; Müller *et al*. 2007). Sugars are required for the expression of many P deficiency induced genes in both roots and shoots. Liu *et al*. (2005) and Tesfaye *et al*. (2007) showed that the expression of a phosphate transporter (Pt), an acid phosphatase (APase), a multidrug toxin (MATE) protein, sucrose synthase (SS), hexokinase (HXK), and fructokinase (FK), in P deficiency induced cluster roots required sugar or phloem transport (Figure 6.5). Dark adaptation or blocking phloem transport reduced the expression of these genes to non-detectable levels in 24 hours, but re-exposure to light activated their transcription within a few hours. The interplay of sugars and P deficiency induced gene expression observed in white lupin has also been validated with Arabidopsis. Karthikeyan *et al*. (2007) and Müller *et al*. (2007) have shown that P deficiency induced gene expression and modified root architecture in Arabidopsis is tightly linked to sugar signaling, probably through HXK.

Hormonal regulation of cluster root development

Since cluster root development involves the synchronized initiation and growth of a large number of tertiary lateral roots in distinct wavelike patterns originating from primary and secondary lateral roots, it would not be surprising that the balance of hormones plays a role in this adaptive response to low P availability (Gilbert *et al*. 2000; Skene and James 2000). Many hormonally controlled developmental responses occurring in P-deficient Arabidopsis plants, which give rise to modified root architecture, appear also to be involved in modifying cluster root architecture. We have detected a number of plant hormone related ESTs during sequencing from cDNA libraries made to P-deficiency induced cluster roots (Table 6.2). As described later for Arabidopsis lateral roots, substantial support for the role of auxin in cluster root formation comes from observations showing: exogenous application of auxin

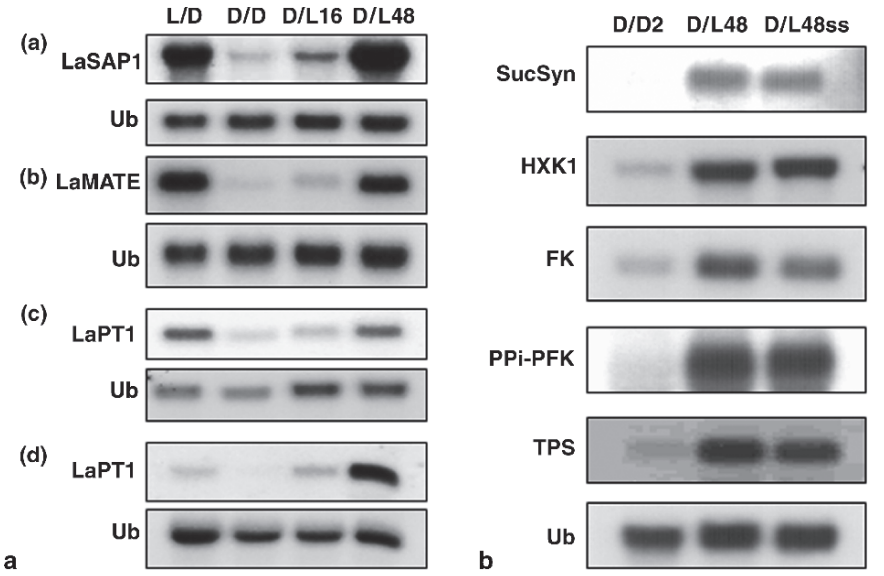

Fig. 6.5 RNA blot of lupin P-stressed induced genes that are co-regulated by products of photosynthesis. **a**. Total RNA from P-deficient proteoid roots and leaves were separated on agarose gels. Polyubiquitin probe (UB) was used as a control for loading and RNA quality. Light/dark regime: L/D 16/8 hour photoperiod; D/D dark-adapted for 24 hours prior to harvest; D/L18 or D/L48 plant transferred to continuous light for 16 or 48 hours after dark adaptation. Dark adaptation of P-deficient plants results in loss of RNAs encoding root expressed (a, b, c) LaSAP (lupin secreted acid phosphatase), LaMATE (lupin multidrug-toxin-efflux protein), and LaPT1 (lupin phosphate transporter). Dark adaptation of P-deficient plants results in loss of RNAs encoding leaf expressed (d) LaPT1. Upon returning plants to light mRNA abundance of P-deficient induced genes recovers (from Liu *et al*. 2005). **b**. Phosphorus deficiency and light affect expression of sugar-related genes in proteoid roots of white lupin. Total RNA isolated from proteoid roots of P-deficient white lupin plants at 14 days after emergence under different light regimes. Treatments: D/D2, P-deficient plants shaded in continuous dark for 48 hours; D/L48, dark-treated P-deficient plants re-exposed to continuous light for 48 hours; D/L48ss, one-half of the shoot of dark-grown plants re-exposed to light and the other one-half remaining in the dark. SucSyn, Suc synthase; HXK1, hexokinase; FK, fructokinase; PPi-PFK, PPi-dependent phosphofructokinase-1; TPS, trehalose-6-P synthase; Ub, polyubiquitin. Dark adaptation of P-deficient plants results in the loss of RNAs encoding genes of sugar metabolism. Upon returning P-deficient plants to light, P deficiency induced gene expression recovers. (From Tesfaye *et al*. 2007.)

stimulates cluster root formation in P-sufficient white lupin and *Protea* species (Gilbert *et al*. 2000; Skene and James 2000); auxin transport inhibitors block cluster root formation in P-deficient plants; and many genes involved in auxin synthesis and signaling are abundantly expressed in developing cluster roots of white lupin (Vance *et al*. 2003; Vance CP, Uhde-Stone C, Yamagishi M 2007; unpublished). These data clearly show a significant component of P-deficiency induced cluster root formation is due to auxin synthesis and transport. Currently, experiments are underway to assess auxin signaling in cluster roots through the transformation of cluster roots with the auxin reporter construct DR5-GUS (Ulmasov *et al*. 1997).

Table 6.2 Expressed sequence tags (ESTs) related to plant hormone balance isolated from phosphorus deficient white lupin cluster roots

EST Annotation	Number of ESTs[a]
Auxin influx/efflux	2
Auxin binding	3
Auxin response/repressed	10
IAA hydrolyase	3
Cytokinin oxidase	6
Methionine synthase	8
Aminocyclopropane carboxylic acid oxidase (ACC)	5
Giberrillin induced	14

[a]Number of ESTs detected in each category out of a total of 3,000 sequenced.

Although the role of ethylene in P-deficiency induced cluster root architecture is not clear, the fact that ethylene plays a role in root hair length, density, and development, as well as a role in lateral root emergence in P-deficient Arabidopsis (Ticconi and Abel 2004), suggests a similar role in cluster roots. As noted previously, cluster roots and dauciform roots have densely packed, unusually long root hairs. Studies with P deficiency-induced cluster roots of white lupin (Gilbert *et al*. 2000), *Casurina* (Zaid *et al*. 2003), and squash (Waters and Blevins 2000) have correlated ethylene production with cluster root formation. Gilbert *et al*. (2000) demonstrated a two- to three-fold increase in ethylene as cluster roots develop in P-deficient white lupin. Zaid *et al*. (2003) showed that Fe-deficiency stimulated cluster roots in *Casurina* and inhibition of ethylene synthesis reduced cluster root formation. Waters and Blevins (2000) noted a correlation between ethylene production, root iron reduction, and the formation of cluster roots in squash. We have found a number of genes involved in ethylene biosynthesis to be over-represented (Table 6.2) in our sequencing of ESTs derived from P-deficiency induced cluster root cDNA libraries, further suggesting a role for ethylene in cluster root architecture.

In their classical study of the physiology of cluster roots, Neumann *et al*. (2000) found that addition of cytokinins to lupin significantly reduced cluster root formation and cluster rootlet elongation. They also found elevated levels of cytokinin in four-week-old P-deficient white lupin roots, compared to P-sufficient roots. They postulated that auxin stimulates emergence of cluster rootlets in P-deficient plants, which results in increased production of cytokinin due to the numerous emerged root tips. In mature segments of P deficiency induced cluster roots we have found numerous ESTs with strong homology to cytokinin oxidase (Table 6.2), suggesting that cytokinins may be subject to degradation as cluster rootlets mature. 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 (Salama and Wareing 1979; Martín *et al*. 2000; Emery and Atkins 2002). Alternatively, *in planta* regulation of potentially large quantities of cytokinins, which could be released by the mass induction of cluster root meristems, may require cytokinin oxidase. As described later, cytokinins reduce lateral root initiation in Arabidopsis.

While strong correlative physiological and gene expression data suggest a critical role for auxins, cytokinins, and ethylene in P deficiency induced cluster root development, definitive genetic and biochemical experiments have yet to be performed. Salient questions to be addressed include: What is/are the internal signal(s) that initiate(s) the cascade of developmental biochemical and genetic changes resulting in cluster roots? How is determinacy in cluster roots regulated? Are reactive oxygen and programmed cell death part of the cluster root developmental phenomenon? Can gene knock-down and overexpression studies be harnessed to definitively answer questions regarding the role of growth hormones in cluster root development and function? Can the genetic control mechanism(s) for cluster root formation be identified and used to enhance P-uptake and use efficiency in other plant species? Recent advances in genetic transformation of root tissue via *Agrobacterium rhizogenes* (Boisson-Dernier *et al*. 2001; Limpens *et al*. 2004) facilitate high throughput experiments to evaluate gene function with either knock-down or overexpression approaches. Coupled with metabolomics and proteomics, new insights will be gained into the role of P deficiency in the development of complex root architecture.

ARABIDOPSIS

P deficiency mediates determinate growth of the primary root

Phosphorus availability has a marked effect on the root system architecture of Arabidopsis (Narang *et al*. 2000; Williamson *et al*. 2001; López-Bucio *et al*. 2002; Al-Ghazi *et al*. 2003; Chevalier *et al*. 2003; Müller and Schmidt 2004; Nacry *et al*. 2005). Growth under P-limiting conditions results in determinate growth of the primary root and redistribution of root growth from the primary root to lateral roots (Ticconi and Abel 2004). Reduced primary root growth under low P is accompanied by increased lateral root density, along with increased root hair length and number. Arabidopsis root biomass is concentrated near the soil surface, suggesting topsoil foraging by roots. Moreover, accessions with enhanced P acquisition also appear to have strengthened root penetration capacity (Narang *et al*. 2000).

Decreased primary root elongation during P deficiency is thought to involve localized sensing of P in the rhizosphere by root meristem and cap cells, which results in cessation of cell division in the primary root meristem (Sánchez-Calderón *et al*. 2005; Svistoonoff *et al*. 2007). Ticconi *et al*. (2004) identified an Arabidopsis mutant *pdr2* (Pi deficiency response) that has a disruption in P-sensing. This mutant shows a short-root phenotype that is specific for P deficiency. The short-root phenotype of *pdr2* is the result of inhibition of primary root cell division at P concentrations below 0.1 mM external P. Ticconi *et al*. (2004) proposed that *pdr2* monitors external environmental P and regulates primary root meristem activity to adjust root system architecture to maximize P acquisition. More recently, Svistoonoff *et al*.

(2007) identified an Arabidopsis gene that mediates the signaling between the root tip and contact with low P. The gene conditions reprogramming of plant root architecture. Previous studies by this group identified an Arabidopsis QTL designated low phosphate root (*LPR*) associated with arrest in primary root growth under low P (Reymond *et al*. 2006). Svistoonoff *et al*. (2007) then employed map-based cloning to identify the gene associated with the *LPR* QTL. They found that *LPR* encoded a multicopper oxidase (MCO) protein. Transcripts of the *LPR* MCO were most abundant in the root meristem and cap. *LPR* mutants lost the determinate primary root arrest phenotype under low P. They proposed that as the primary root tip came in contact with a low P area, MCO activity modifies either the amount or distribution of hormone-like compounds that signal determinacy in primary root growth and stimulates lateral root development.

Root hairs

The abundant development of lateral roots following cessation of primary root growth in low P environments is almost invariably accompanied by increased root hair density and length. Root-hairs are tubular-shaped cells specialized for nutrient uptake (Peterson and Farquhar 1996; Gahoonia and Nielsen 1998; Gilroy and 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; Dolan 2001). Root hairs can form as much as 77% of the root surface area of field crops (Peterson and Farquhar 1996). For plants lacking mycorrhizal associations, they are the primary site of nutrient uptake (Gahoonia and Nielsen 1998; Gilroy and Jones 2000; Jungk 2001). Root hair formation and growth is regulated largely by the supply of mineral nutrients, particularly P and NO_3^- (Bates and Lynch 1996, 2000; Gilroy and Jones 2000; Ma *et al*. 2001).

In Arabidopsis, trichoblasts are located over the junction of two underlying cortical cells. Trichoblasts can be distinguished by the late stages of embryogenesis. In recent years, root hair formation in Arabidopsis has become a model system for evaluating cell fate and hormonal interactions. Grierson *et al*. (2001) report that at least 40 genes in Arabidopsis affect root hair initiation and development. Five loci involved in root hair formation encode transparent testa glabra (*TIG*), glabra2 (*GL2*), constitutive triple response1 (*CTR1*), root hair defective6 (*RHD6*), and auxin resistant2 (*AXR2*; Masucci and Schiefelbein 1996). Analysis of these genes in Arabidopsis mutants demonstrated that a network of hormone interactions involving auxin and ethylene regulate root epidermal cell fate and root hair initiation (Tanimoto *et al*. 1995). Likewise, Parker *et al*. (2000) found eight genes whose loss-of-function mutants showed root hair defects. Although the function of these genes was not established, they were mapped to Arabidopsis chromosomes. More recently, Jones *et al*. (2006) showed that in addition to previously reported genes, some 606 novel genes had enhanced expression in Arabidopsis root hairs. They identified several gene families that appeared to be important in root hair

morphogenesis including cell wall synthesis enzymes, glycosylphosphatidylinositol (GPI) anchored proteins associated with lipid rafts, armadillo-repeat proteins, and leucine-rich receptor kinases. Importantly, a basic helix-loop-helix transcription factor has been shown to be the ancestral regulator of root hair development in Arabidopsis (Menand *et al*. 2007).

Phosphate availability regulates root hair elongation (Bates and Lynch 1996) and root hair density (Ma *et al*. 2001). The average length of root hairs on P-deficient plants was three-fold greater and root hair density was five-fold greater than in P-sufficient plants. Trichoblast files increased from 8 to 12 on P-deficient plants. Analysis of P acquisition in Arabidopsis wild type and root hair mutants, *rhd6* and *rhd2,* showed that wild type plants were more efficient than root hair mutants in obtaining P when plants were grown under low P conditions. However, there was no difference in growth when grown under high P conditions (Bates and Lynch 2000). Phosphorus deficiency altered radical cell pattern formation in roots by increasing cortical number and reduced epidermal cell elongation resulting in increased trichoblasts (Ma *et al*. 2001, 2003; Müller and Schmidt 2004). Modified root hair patterning was most evident in mutant genotypes having defects in first stage of epidermal cell differentiation, suggesting P deficiency signals are perceived very early in epidermal cell development. Modified root cell patterning in P-deficient plants is regulated by auxin-ethylene interactions (Dolan 2001; Stepanova *et al*. 2007).

Lateral roots

The prototype root architecture modification to occur in Arabidopsis in response to P deficiency is the promotion of lateral root development at the expense of primary root growth (Williamson *et al*. 2001; López-Bucio *et al*. 2002). Phosphorus deficiency appears to impair cell division in the primary root meristem, but stimulate elongation of initiated lateral root primordia and newly emerged lateral roots (Al-Ghazi *et al*. 2003; Malamy 2005; Nacry *et al*. 2005; Sánchez-Calderón *et al*. 2005). The root architecture changes induced by P deficiency are strikingly similar to those induced by either auxin addition or overproduction (Al-Ghazi *et al*. 2003; Malamy 2005; Nacry *et al*. 2005). Utilizing Arabidopsis mutants with lesions in auxin and ethylene signaling, a growing body of evidence indicates that P deficiency induced root architectural changes result from modifications in auxin and ethylene synthesis and/or signaling. The current working hypothesis is that P deficiency induces an increase in auxin synthesis and sensitivity in the primary root meristem, resulting in a modification or cessation of cell division. The modification in auxin signaling may result from P deficiency induced ethylene effects on auxin distribution (López-Bucio *et al*. 2002; Ma *et al*. 2003; Ortega-Martínez *et al*. 2007; Ruzicka *et al*. 2007; Swarup *et al*. 2007). Transcript profiling (Himanen *et al*. 2004; Laskowski *et al*. 2006) shows that more than 900 genes are differentially regulated in early lateral root initiation. Numerous genes related to auxin and ethylene signaling,

cell wall modification, and the cell cycle are rapidly induced as lateral root primordia are formed.

The plant hormone auxin (primarily indole-3-acetic acid [IAA]) has long been known to stimulate lateral root formation (Boerjan *et al*. 1995; Reed *et al*. 1998; Casimiro *et al*. 2001; Marchant *et al*. 2002). Nacry *et al*. (2005) showed that during P deficiency, IAA increases in the whole primary root and young lateral roots of Arabidopsis. Without IAA, only primary root growth was observed in Arabidopsis (Karthikeyan *et al*. 2007). Regardless of root P status, exogenous auxin application enhanced lateral root formation while suppressing primary root elongation in Arabidopsis (Al-Ghazi *et al*. 2003; Nacry *et al*. 2005; De Smet *et al*. 2007; Stepanova *et al*. 2007). Auxin transport inhibitors suppress lateral root formation in many species including Arabidopsis. As noted earlier, auxin has a striking effect on cluster root formation in white lupin and other cluster root species (Gilbert *et al*. 2000; Skene and James 2000).

Root architectural changes in response to P deficiency have been shown to be related to ethylene in Arabidopsis (López-Bucio *et al*. 2002; Ma *et al*. 2003). Low P conditions appear to enhance the roots' sensitivity to ethylene. Ethylene is known to be an effector of primary and lateral root cell elongation. Several recent reports document the interrelationship between auxin and ethylene in regulating root growth and development (Ruzicka *et al*. 2007; Stepanova *et al*. 2007; Swarup *et al*. 2007). Ethylene appears to stimulate auxin biosynthesis and increase auxin transport. Moreover, auxin must be present for ethylene inhibition of cell expansion. Ethylene may also interact with auxin to release lateral root meristems for growth. Thus, low P availability may alter root architecture by stimulating multicopper oxidase activity (Svistoonoff *et al*. 2007), which modulates the auxin-ethylene balance, resulting in determinate root growth and enhanced lateral root initiation.

Traditionally, cytokinins are thought to be negative regulators of root growth while having positive effects on shoot growth (Christianson and Warnick 1985; Werner *et al*. 2001, 2003; Aloni *et al*. 2006). Both N and P deficiency result in decreased cytokinin content (López-Bucio *et al*. 2003) accompanied by increased lateral root formation. Application of cytokinin inhibits root development and abolishes the auxin effect of increased lateral root formation. Cytokinin inhibition of primary root growth appears to occur through its effect on regulating root meristem size (Dello Ioio *et al*. 2007). Arabidopsis plants that overexpress cytokinin oxidase (CKX), the key enzyme regulating cytokinin degradation, have reduced cytokinin concentrations accompanied by increased lateral and adventitious root formation (Werner *et al*. 2003; Lohar *et al*. 2004). Li *et al*. (2006) have recently shown that cytokinins inhibit lateral root formation by blocking the pericycle founder cells transition at the G_2 phase. It is quite apparent that the modification of root architecture in P-deficient Arabidopsis is regulated by the subtle interaction of auxin, ethylene, and cytokinin. Localized changes in hormone transport, synthesis and cellular concentration shut down primary root growth, activate lateral root development, and increase trichoblast frequency giving rise to the root architecture favorable for topsoil foraging for P.

Phosphorus deficiency induced gene expression

Several studies of Arabidopsis (Hammond *et al*. 2003, 2004; Wu *et al*. 2003; Misson *et al*. 2005; Morcuende *et al*. 2007) and other species (Wang *et al*. 2001; Wasaki *et al*. 2003; Graham *et al*. 2006; Hernández *et al*. 2007) have now addressed whole genome changes in gene expression in response to P deficiency. Each study has noted that scores of genes respond with a two-fold change in expression. Hammond *et al*. (2004) noted that these changes can be classified as either early genes, which respond rapidly and frequently non-specifically to P deficiency or late genes, which lead to modifications in root architecture and metabolism. Invariably these studies have shown that early response genes include transporters particularly Pi transporters. However numerous other transporters also respond rapidly to P deficiency including aquaporins, sulfate transporters, sugar transporters, and amino acid transporters (Wang *et al*. 2001; Misson *et al*. 2005; Morcuende *et al*. 2007). Transcription factor genes also respond quickly to P deficiency, along with cell wall synthesis genes, PR genes, and acid phosphatases (Franco-Zorrilla *et al*. 2004). Genes induced in late responses include those encoding enzymes of glycolysis and the carboxylate cycle. Internal remobilization of P was affected by late response genes including: glucose-6P/Pi translocator, nucleotide pyrophosphatases, and glycerol-P-permeases (Misson *et al*. 2005). A large number of genes involved in phospholipid degradation and galacto- and sulfolipid synthesis also have enhanced expression after prolonged P deficiency.

Activation of these lipid-related genes probably reflects a shift in membrane biogenesis from incorporation of phospholipids to the use of galacto- and sulfo-lipids as membrane components (Essigmann *et al*. 1998; Misson *et al*. 2005). Morcuende *et al*. (2007) have completed an elegant study of Arabidopsis in which they followed the recovery of plants from P-stress over a 24-hour period. Plants subjected to P deficiency were supplemented with sufficient P to induce the recovery of genes suppressed under low P. They also evaluated metabolites and enzyme activities of several proteins. This analysis allowed for the identification of genes, proteins, and metabolites specifically involved in recovery from P deficiency. In addition to genes and pathways noted above, they found that phosphoenolpyruvate carboxylase (PEPC) and PEPC kinase were integrally involved in the shift in C metabolism that occurs during P deficiency. They also showed that C metabolism shifted toward starch synthesis under low P conditions and towards starch catabolism under high P conditions. Interestingly, Morcuende *et al*. (2007) found a striking increase in glycerophosphodiesterases (GPDEs) gene expression. GPDE genes have been recently shown to be important in root hair development (Jones *et al*. 2006) and the phenomenon of increased root hair length and density is a common response to P deficiency.

Whole genome studies have also led to the discovery of many transcription factors that appear to be important in response to P deficiency. Current evidence suggests that control of signaling P deficiency is complex, involving both positive and negative regulation of gene expression. Some of the transcription factors proven to be involved in P deficiency signal transduction include: MYBs, WRKYs, bHLH, Zn-finger proteins, and HD-Zip factors (Rubio *et al*. 2001; Hammond *et al*. 2004; Yi *et al*. 2005; Devaiah *et al*. 2007; Tesfaye *et al*. 2007).

SYNOPSIS

Phosphorus is second to N as the most limiting element for plant growth. Plants have evolved a number of effective strategies to acquire P and grow in P limited environments. Physiological, biochemical, and molecular studies of plant adaptations to P deficiency, which occur in non-mycorrhizal species, have provided striking new insights into how plants respond to P deficiency and which traits appear important in acquiring P. Because inexpensive P fertilizer is a limited resource and because many farmers of the world cannot afford P fertilizer (Steen 1997), it is imperative that plant biologists discover strategies and approaches that will enhance efficiency of P acquisition and use. The fact that we know modified root architecture, increased root hairs, and exuded carboxylates are key components in P acquisition offers targets to improve or modify in efforts toward increasing efficiency of P acquisition and use. A targeted approach is borne out by the recent identification of an Arabidopsis QTL gene affecting primary root extension and low P signal recognition as a multicopper oxidase (Svistoonoff *et al*. 2007). Utilizing a QTL approach for improving P acquisition in bean (Beebe *et al*. 2006) has resulted in beans with enhanced top-soil foraging, but the gene controlling this trait has not been cloned. With the wide array of genomic and bioinformatics research platforms now available, coupled to QTL and whole genome analysis, research into plant responses to P deficiency is moving toward an exciting phase, centered around signal transduction and regulation of root developmental plasticity and gene function, aimed at increasing efficiency of use. Sustainable cropping systems in the developed world and the need for efficient P acquisition in low P conditions of the developing world, require that plant research identify and exploit mechanisms to improve P use efficiency. Efforts that improve soil P availability to plants are urgently needed to maintain and increase economically and environmentally sustainable crop agriculture.

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