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Transcriptomics and hormonal regulation of cluster root development in phosphate-deficient white lupin

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1 Summary-Zusammenfassung

1.1 Summary

Among crops, white lupin (*Lupinus albus*) represents the extraordinary ability to acquire sparingly soluble soil phosphate (P_i) by formation of cluster roots (CRs), mediating intense exudation of phosphorus (P)-mobilising root exudates (citrate, phenolics, protons and acid phosphatase). It is widely used as a model plant for investigations of P acquisition by root-induced chemical modifications of the rhizosphere. During the last two decades, a large pool of information on CR function and physiology was obtained mainly by hypothesis-driven research. Based on these findings, this study was designed to get a more comprehensive picture of the metabolic changes during CR development using a transcriptome sequencing approach. The outcome of the transcriptome analysis was the basis for the formulation of research questions on the regulation of CR formation and function to be investigated more in detail:

Chapter I, focuses on transcriptome sequencing used for the first time for a systematic comparison of different stages in CR development. To get insights into the regulatory factors involved in CR formation, special emphasis was placed on hormone-related genes. Initiation of CR primordia in the pre-emergent (PE) zone was reflected by strongest expression of genes involved in transport and biosynthesis of auxins, brassinosteroids (BRs) and cytokinin receptors. Cluster root maturation, involving meristem degeneration and root hair proliferation was associated with strongly increased expression of ethylene-related transcripts and decreased expression of auxin- and BR-related genes. Also transcripts related with abscisic and jasmonic acids and cytokinin degradation were up-regulated in mature (MA) clusters.

The primary metabolism, highly expressed in juvenile (JU) clusters, underwent significant modifications during CR maturation with increased contribution of P_i-independent bypass reactions, promoting biosynthesis of organic acids. Citrate catabolism and respiration were down-regulated, triggering citrate accumulation in MA clusters. Up-regulation of phenylpropanoid pathways reflected accumulation of phenolics. Increased expression of transcripts encoding ALMT and MATE

transporters may be involved in the exudation of flavonoids and citrate, while up-regulation of transcripts encoding P_i transporters mediates subsequent uptake of mobilised P_i. Predominant expression of nucleotide degradation and secretory acid phosphatase in MA clusters coincides with P_i re-translocation and mobilisation of organic soil P. Up-regulation of the FIT transcription factor, usually mediating the expression of Fe deficiency responses (root hair proliferation, proton extrusion, Fe (III)-reduction, exudation of phenolics) can be observed also in MA clusters of P-deficient *Lupinus albus* even under Fe-sufficient conditions. This raises the question, whether FIT has a similar function in the regulation of P deficiency responses.

Chapter II, addresses the question whether sucrose acts as a shoot-borne signal for CR formation. External sucrose amendments to P-sufficient plants, at concentrations similar to those in PE root zones of P-deficient plants, induced CR formation comparable to P-deficient plants. Palatinose (25 mM), and combined application of glucose/fructose (both at 12.5 mM) failed to induce CR formation under P-sufficient conditions, demonstrating a specific signal function of sucrose and excluding osmotic and carbon source effects. However, CRs induced by sucrose were not functional with respect to citrate exudation, acid phosphatase and phosphoenolpyruvate carboxylase (PEPC) activities and expression of related genes (*LaMATE*, *LaSAP* and *LaPEPC*) quantified by RT-qPCR.

In Chapter III, the interactions of different phytohormones and sucrose on CR formation were investigated more in detail by an integrated approach of RT-qPCR, hormone translocation analyses and exogenous application of hormones or hormone antagonists. Shoot-to-root translocation of auxin was unaffected by P limitation, supporting the hypothesis that sucrose, rather than auxins, acts as major shoot-borne signal, triggering the induction of CR primordia. Ethylene may act as mediator of the sucrose signal, as indicated by strong inhibitory effects of the ethylene antagonist $CoCl_2$ on CR formation induced by sucrose or P limitation. As reported in other plant species, moderately increased production of ethylene and brassinosteroids (BRs) may induce biosynthesis and transport of root-borne auxins, indicated by increased expression of respective genes (*YUCCA*, *PIN1*, *AUX1*, *BR*, *ACC_oxidase*) in pre-emergent clusters. A role of BR in CR formation is further underlined by inhibitory effects of BR antagonists. The well-documented inhibition of root elongation by high doses of ethylene may be involved in the inhibition of lateral rootlets growth during

CR maturation, indicated by a massive increase of gene expression involved in ethylene production, associated with decline of transcripts with stimulatory effects (BR- and auxin-related genes). Based on these findings, models for the regulatory networks involved in CR formation (Chapter III) and function (Chapter I) have been developed.

1.2 Zusammenfassung

Lupinus albus (Weisslupine) ist eine Kulturpflanzenart mit außergewöhnlich stark ausgeprägter Fähigkeit zur Aneignung schwerlöslicher Bodenphosphate (P), was in diesem Fall durch die Ausbildung flaschenbürstenartiger Clusterwurzeln erreicht wird, welche die intensive Abgabe P-mobilisierender Wurzelabscheidungen (Citrat, Phenole, Protonen und saure Phosphatasen) vermitteln. Sie wird daher seit langem als Modellpflanze für Untersuchungen zur pflanzlichen P-Aneignung durch wurzelinduzierte Veränderungen der Rhizosphärenchemie genutzt. Während der vergangenen beiden Jahrzehnte wurden so durch überwiegend hypothesenorientierte Forschungsansätze bereits umfangreiche Informationen zur Funktion und Physiologie von Clusterwurzeln erarbeitet. Darauf basierend, wird in der vorliegenden Untersuchung nun versucht, über Transkriptom-Sequenzierung einen noch umfassenderen Überblick über die metabolischen Veränderungen während der Clusterwurzelentwicklung zu gewinnen. Auf dieser Basis wurden weiterführende Forschungsfragen zur Regulation und Funktion von Clusterwurzeln formuliert und detailierter untersucht:

Kapitel I beschreibt die Transkriptomanalyse, die hier zum ersten Mal für einen systematischen Vergleich verschiedener Stadien der Clusterwurzelentwicklung eingesetzt wurde. Um Einblicke in die regulatorischen Faktoren zu erhalten, die an der Bildung von Clusterwurzeln beteiligt sind, wurde besonderes Augenmerk auf Gene mit Bezug zum Phytohormonstoffwechsel gelegt. Die Induktion von Clusterwurzelprimordien in den subapikalen Seitenwurzelzonen, spiegelte sich in einer intensiven Expression von Genen wider, die am Transport und der Synthese von Auxinen, Brassinosteroiden (BR) und Cytokininrezeptoren beteiligt sind. Die weitere Entwicklung und „Reifung“ der Clusterwurzeln, die durch Meristemdegeneration und dichte Wurzelhaarbildung gekennzeichnet ist, war dagegen mit einer stark erhöhten Expression von Genen der Ethylenbiosynthese und

einer verminderten Expression der Auxin- und BR-Gene verbunden. Auch Transkripte des Abscisin-, und Jasmonsäurestoffwechsels, sowie des Cytokininabbaus waren verstärkt exprimiert.

Der Primärstoffwechsel mit besonders intensiver Exprimierung in jungen (JU) Clusterwurzeln zeigte während der weiteren Clusterwurzelentwicklung signifikante Modifikationen verbunden mit einer verstärkten Expression P_i-unabhängiger Ausweichreaktionen, die zur Biosynthese von organischen Säuren beitragen können. Dagegen war die Expression des Citratkatabolismus vermindert, was offensichtlich zur intrazellulären Akkumulation von Citrat beitrug. Die verstärkte Expression des Phenylpropanoidstoffwechsels ging mit einer erhöhten Akkumulation phenolischer Substanzen einher. Das vertärkte Auftreten von Transkripten für ALMT und MATE Transporter könnte die Abgabe von P-mobilisierenden Wurzelexudaten, wie Citrat und Flavonoiden widerspiegeln, während die verstärkte Expression von Transkripten für P_i-Transporter für die anschließende Aufnahme mobilisierter Phosphatanionen verantwortlich zu sein scheint. Verstärkte Expression des Nucleotidkatabolismus und sekretorischer, saurer Phosphatasen könnte im Zusammenhang mit internem P_i-Recycling und der Hydrolyse organischer Phosphatverbindungen im Boden stehen. Eine erhöhte Exprimierung des FIT Transkriptionsfaktors während der Clusterwurzelreifung, der normalerweise die koordinierte Induktion von Eisenmangelanpassungen vermittelt (Stimulierung des Wurzelhaarwachstums, Protonenabgabe, Esxudation phenolischer Sunstanzen, erhöhte Fe(III)-Reduktion), wirft die Frage auf, ob FIT möglicherweise ähnliche Funktionen bei der Regulation von P-Mangelanpassungen hat ?

Kapitel II widmet sich der Frage, ob Saccharose möglicherweise als sprossbürtiges Signal an der Bildung von Clusterwurzeln beteiligt ist. Bei gut mit P versorgten Pflanzen führte die externe Applikation von Saccharose im Konzentrationsbereich wie er in den subapikalen Wurzelzonen von P-Mangelpflanzen gemessen wurde, zu einer vergleichbaren Induktion von Clusterwurzeln wie P Mangel. Sowohl Palatinose (25 mM) als auch die kombinierte Gabe von Glucose und Fruktose (je 12.5 mM) waren dagegen nicht in der Lage, Clusterwurzelbildung zu induzieren, was eine spezifische Signalfunktion von Saccharose belegt und osmotische Effekte oder eine reine C-Quellenwirkung ausschließt. Allerdings waren im Unterschied zu P-Mangelpflanzen, die durch Saccharosegaben induzierten Clusterwurzeln inaktiv

im Hinblick auf Induktion der PEP-Carboxylaseaktivität, Angabe von Citrat und saurer Phosphatase und der Expression damit verbundener Gene (*LaMATE, LaSAP* und *LaPEPC*).

In Kapitel III wurden Interaktionen zwischen den an der Clusterwurzelentwicklung beteiligten Phytohormonen, in einem integrierten Ansatz aus RT-qPCR-Analyse, Hormontransportuntersuchungen, sowie der externen Applikation von Hormonen und Hormonantagonisten, genauer untersucht. Die Sproß/Wurzelverlagerung von Auxinen wurde durch den P-Ernährungsstatus nicht beeinflusst, was die Hypothese unterstützt, wonach eher Saccharose und nicht Auxin als primäres sprossbürtiges Signal bei der Induktion von Clusterwurzelprimordien wirkt.

Ethylen scheint an der weiteren Signaltransduktion im Wurzelgewebe beteiligt zu sein, was durch eine ausgeprägte Hemmung der Saccharose-, oder P-Mangel-induzierten Clusterwurzelbildung durch den Ethylenantagonisten $CoCl_2$ unterstrichen wird. Wie auch von anderen Pflanzenarten berichtet, kann eine moderat erhöhte Produktion von Ethylen und Brassinosteroiden die Biosynthese und den Transport wurzelbürtiger Auxine induzieren, was sich in einer erhöhten Expression der entsprechenden Gene (*YUCCA, PIN1, AUX1, BR, ACC oxidase*) in den subapikalen Seitenwurzelzonen widerspiegelt. Eine Beteiligung von Brassinosteroiden bei der Clusterwurzelbildung wird durch inhibitorische Effekte des Brassinosteroidantagonisten Brassinazol untertrichen.

Die gut beschriebene Hemmwirkung hoher Ethylenkonzentrationen auf das Wurzelwachstum steht möglicherweise auch im Zusammenhang mit der synchronen Wachstumshemmung der kurzen Seitenwurzeln im Clusterbereich, was durch die massive Erhöhung der Expression von Genen der Ethylenbiosynthese und einer zeitgleichen Expressionshemmung Wurzelwachstums-fördernder Gene der Auxin-, und Brassinosteroid synthese unterstrichen wird.

2 General introduction

2.1 Phosphorus: a looming global crisis

Phosphorus (P) belongs to the 17 essential elements required for plant growth and development (Marschner, 1995). It is required for energy metabolism, biosynthesis of nucleic acids, and also plays a key role in photosynthesis, respiration, and regulation of a number of enzymatic reactions (Raghothama, 1999). Plants take up phosphorus in its orthophosphate (P_i) forms ($H_2PO_4^-$ and HPO_4^{2-}). The plant available P_i in the soil solution usually occurs at very low concentration (0.1-10 µM; Hinsinger, 2001), although the total soil P content is frequently high. Crop yield of 30-40% of the world's arable land is limited by P deficiency (Runge-Metzger, 1995; Uexküll & Mutert, 1995). To maintain crop yield, application of phosphorus fertilizers is obligatory. However, only 20% or less of the applied phosphorus is removed by the first years' plant growth (Russell, 1973). Run-off of phosphorus is the primary factor leading to the eutrophication and hypoxia of the aquatic ecosystems (Runge-Metzger, 1995; Bumb & Baanante, 1996). Another problem which deserves attention is the prediction that inexpensive rock phosphate reserves are, by some estimates, going to be depleted in the next 50-100 years (Cordell *et al.*, 2009). However, the prognosis of global P reserves is currently under intensive discussion. Most recently, some authors argued that the world has over 300 years of rock phosphate reserves and over 1400 years of rock phosphate resources (Van Kauwenbergh *et al*., 2013). It should be emphasized that the estimation of rock phosphate reserves are subject to changes with updated information, discovery, economics, politics and new technology. Nevertheless, independent of the variable predictions, there is no doubt that rock phosphate is a limited natural resource and an indispensible mineral for fertilizer production and feeding the world.

2.2 Plant adaptations to phosphorus deficiency

Plants, naturally exposed to low phosphorus conditions, have evolved many adaptations to cope with this situation. Generally, there are two broad strategies for surviving in the low phosphorus environments: (1) those aiming at conservation of use; (2) those aiming at enhanced acquisition or uptake (Lajtha & Harrison, 1995;

Horst *et al.*, 2001; Vance, 2001; Vance *et al.*, 2003). Processes that conserve use of P include: decreased growth rate, increased internal P use efficiency, efficient P_i remobilisation from old tissues, modifications in carbon metabolism that bypass the P-requiring steps and the alternative respiration pathway (Schachtman *et al.*, 1998; Neumann *et al.*, 1999; Raghothama, 1999; Vance *et al.*, 2003; Plaxton & Tran, 2011). By comparison, processes that enhance P acquisition or uptake involve: accumulation and exudation of organic acid anions, production and secretion of phosphatases, modification of rhizosphere pH and redox potential (Neumann *et al.*, 1999; Neumann & Römheld, 2002), enhanced expression of P_i transporters (Raghothama, 1999), extended root growth with modified root architecture (Lynch & Brown, 2001), enhanced formation of fine roots and root hairs (Z. Ma *et al.*, 2001; Neumann & Römheld, 2002), symbiosis with mycorrhizae (Jakobsen *et al.*, 1992; Koide & Kabir, 2000; Burleigh *et al.*, 2002; Tibbett & Sanders, 2002; Read & Perez-Moreno, 2003) and formation of so-called "cluster roots" (CRs; Purnell, 1960; Skene, 1998).

2.3 Cluster roots and white lupin (*Lupinus albus* L.)

Cluster roots have been firstly noted by Engler (1894) and defined by Purnell (1960), given the name of "proteoid roots" since they were found to occur in members of the family Proteaceae. In many cases, members of this family are slow-growing sclerophyllous shrubs and trees, and a major component of the Mediterranean flora in Western Australia and South Africa (Neumann & Martinoia, 2002). The soils in these regions are among the most nutrient-impoverished soils in the world, particularly with phosphorus deficiency (Pate & Dell, 1984; McArthur, 1991; Specht & Specht, 2002). Apart from the Proteaceae family, proteoid roots were also found in a wide range of species and genera including members of the Betulaceae, Casuarinaceae, Cucurbitaceae, Cyperaceae, Eleagnaceae, Leguminosae, Moraceae, Myricaceae and Restionaceae, which are all adapted to infertile soils (Dinkelaker *et al.*, 1995; Skene, 1998). Therefore, the more general term of "cluster roots" has been introduced (Roelofs *et al.*, 2001).

Cluster roots are bottle-brush like rootlets, with limited growth and densely covered by root hairs, that arise from the pericycle opposite the protoxylem poles along the first-order of lateral roots (Fig. 1; Skene, 1998; Neumann & Martinoia, 2002). The

cluster root morphology differs between plant species, in that they may be simple, compound or complex clusters (Skene, 1998; Lambers *et al.*, 2006). Compound clusters, which tend to form dense root-mats in natural ecosystems, are formed by only a few genera within Proteaceae (Australian *Banksia* and *Dryandra* and South African *Orothamnus*) (Lamont, 1982). Simple clusters, which form numerous short, determinate rootlets, separated by unbranched regions from each other, are produced by most of the Australian and South African Proteacean genera and genera of other families such as *Lupinus albus* in the family Fabaceae (Lambers *et al.*, 2006).

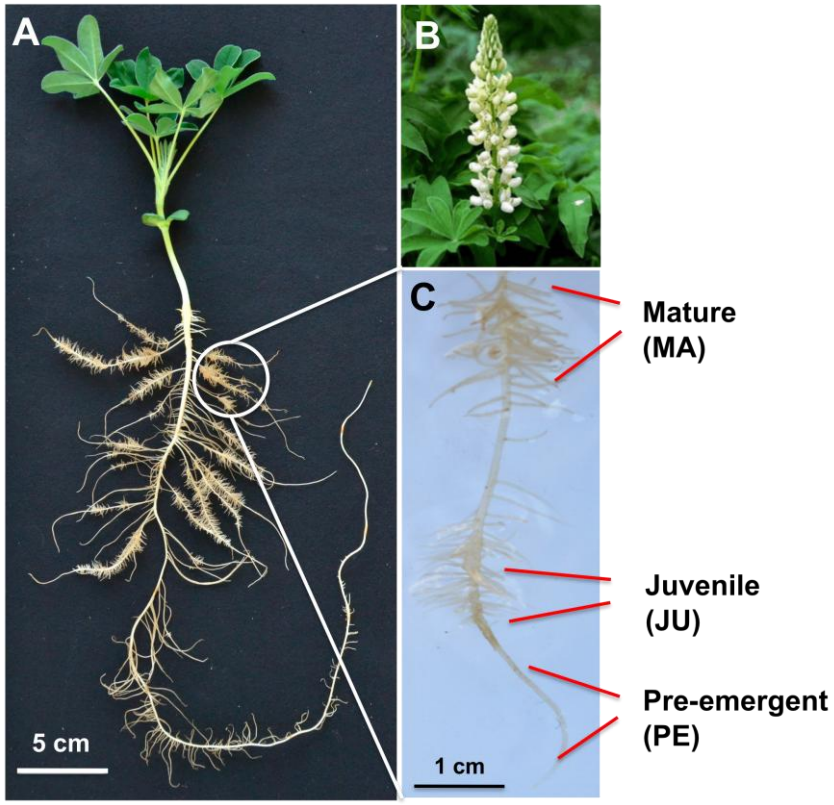

Fig. 1 (A) White lupin grown in hydroponic culture for 20 days after sowing (DAS) under P deficiency; (B) White lupin flowers; (C) Different developmental stages of cluster roots.

Among the plant species forming cluster roots, white lupin (*Lupinus albus*) is most thoroughly investigated and used as a model system to study plant adaptations for chemical P mobilisation by rhizosphere acidification, release of organic metal chelators (citrate, malate and phonolics), high activity of secretory acid phosphatase and expression of high-affinity P_i uptake (Figs. 1, 2; Neumann *et al.*, 2000; Neumann & Martinoia, 2002). It is an annual legume traditionally cultivated in the Mediterranean region and along the Nile valley (Huyghe, 1997), and produces

cluster roots within 4 to 5 weeks after germination particularly under P limitation (Kania, 2005) and also under Fe deficieny (Hagström *et al.*, 2001).

Cluster-rooted plant species are, in most cases, non-mycorrhizal (Skene, 1998), which might be an adaptation to the area of their distribution, where a short season of rainfall doesn't support the time-consuming development of association with arbuscular mycorrhizal (AM) fungi (Lamont, 1982; Gilbert *et al*., 1998). Broadly speaking, among the 18% of vascular plants that cannot form an AM symbiosis (Brundrett, 2009), there are two groups existing: one group is referred as Brassicaceae type, comprising those that typically occur in disturbed habitats, where soil phosphorus availability is high and competition with other plants is low; the other group is referred as Proteaceae type, comprising families that dominate on soils with extremely low phosphorus availability and have the root specialisations (e.g., cluster roots, dauciform roots) that allow them to mine soil P (Lambers & Teste, 2013). For the latter type of plants, chemical mobilisation of nutrients by root exudation of organic acid anions (mainly citrate and malate) released in huge amount, from individual clusters over a period of 1-3 days, might be an alternative strategy for nutrient acquisition instead of mycorrhizal associations (Skene, 1998; Neumann & Martinoia, 2002). The release of carboxylates by cluster roots of white lupin depends on the developmental stages (Fig. 2). These stages, from initiation to senescence, can be visually differentiated by the length of rootlets and color of the clusters and comprise: (1) the pre-emergent (PE) stage , as the stage for formation of cluster root primordia; (2) fast-growing, white colored juvenile (JU) clusters; (3) light brown, mature (MA) clusters with fully developed rootlets, the stage with maximum release of carboxylates; (4) senescent clusters with intensive browning (Kania, 2005; Figs. 1, 2).

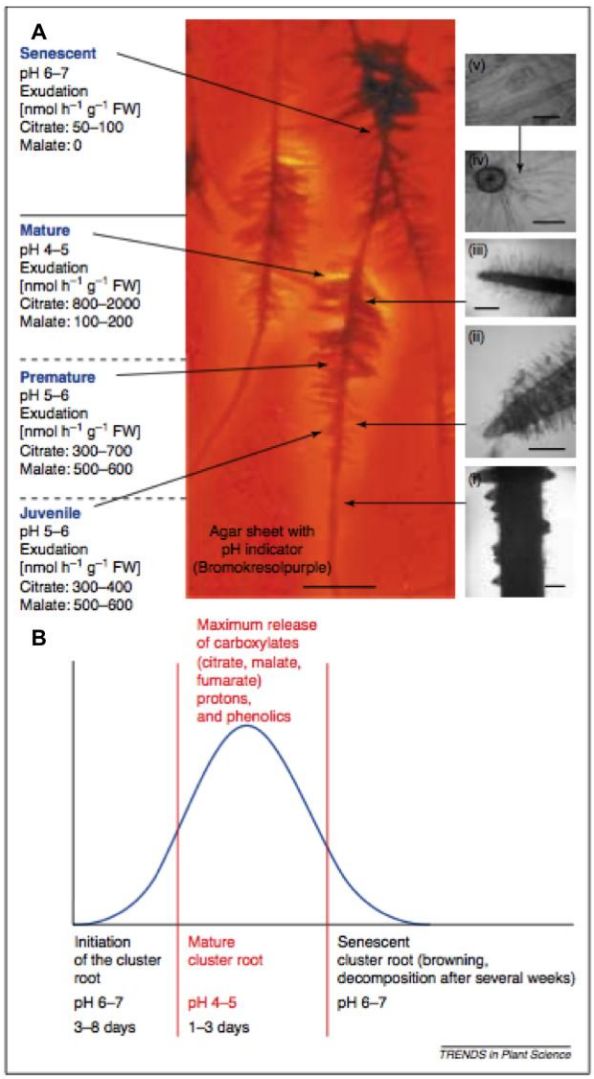

Fig. 2 Spatial and temporal variation in root morphology and root exudation during cluster root development. (A) Stages of cluster root development in *Lupinus albus*. Root-induced pH changes monitored by application of agar sheets with pH-indicator. Scale bar = 1 cm. (i) Juvenile: initial stage with emerging laterals. Scale bar = 500 µm. (ii) Juvenile: root hair development on a lateral rootlet, predominant exudation of malate. Scale bar = 260 µm. (iii) Mature: lateral rootlets without further growth activity, completely covered with root hairs; burst of citrate exudation and release of H^+. Scale bar = 250 µm. (iv) Transversal section of (iii) Scale bar = 260 µm. (v) Protozoa (*Vorticella*) living on the surface of root hairs in mature root clusters. Scale bar = 40 µm; (B) Temporal changes during cluster root development and root exudation in Proteaceae and *Lupinus albus* (from Neumann & Martinoia, 2002).

2.4 Induction and development of cluster roots

Cluster root formation is highly plastic and induced mainly by P deficiency, and in some species also by Fe deficiency (Neumann & Martinoia, 2002). Foliar P application to P-deficient plants can suppress formation of cluster roots (Marschner *et al.*, 1987; Shane *et al.*, 2003), suggesting a regulatory function of the internal P status (Dinkelaker *et al.*, 1995). However, cluster root formation seems to be stimulated also in nutrient-rich patches (Purnell, 1960; Skene, 1998), in upper soil layers, which are rich in organic matter (Lamont, 1973) and similarly in split-root compartments with local high P supply (Shen *et al.*, 2005), indicating an additional involvement of local signals (Neumann & Martinoia, 2002). Cluster root formation involves synchronised initiation and growth of a large number of rootlets along the first-order laterals in a distinct wave-like pattern. Thus it is not surprising that changes in hormonal balances are involved in this process (Gilbert *et al.*, 2000; Neumann *et al.*, 2000; Cheng *et al.*, 2011).

Auxin was thought to be implicated due to the stimulating effect on cluster root formation by exogenous application of IAA or NAA to P-sufficient plants (Gilbert *et al.*, 2000; Neumann *et al.*, 2000) and inhibitory effects by the application of auxin transport inhibitors (NPA or TIBA) to the P-deficient plants (Gilbert *et al.*, 2000). Recently, Meng *et al.* (2013) proposed that root-derived auxin and its redistribution along the first-order lateral roots may contribute to cluster root formation under P deficiency. Many genes related to auxin metabolism and signalling are highly expressed in developing cluster roots of white lupin (Uhde-Stone *et al.*, 2003; O'Rourke *et al.*, 2013; Chapters I, III). Additionally, Cheng *et al.* (2011) isolated an *IAA7/axr2* gene and transformed white lupin roots with an *IAA7/axr2*:GUS reporter, showing the *IAA7/axr2* reporter was highly active, and over a greater range, in P-deficient roots as compared with P-sufficient ones.

Neumann *et al.* (2000) have found a drastic inhibition of cluster root formation and of lateral rootlet elongation when cytokinins were applied to P-deficient white lupin plants. Also, elevated concentrations of cytokinins were detected, in roots of 4-week-old P-deficient white lupin as compared with P-sufficient plants. They postulated that auxin stimulates emergence of juvenile cluster rootlets in P-deficient plants, leading to increased production of cytokinins in the numerous emerged root tips. By contrast, a contig of five expressed sequence tags (ESTs) with homology to cytokinin oxidase (*CKX*) has been found in mature clusters induced by P deficiency

(Uhde-Stone *et al.*, 2003). Most recently, transcripts encoding CKX were also detected in P-deficient cluster roots (O'Rourke *et al.*, 2013), and particularly in P-deficient mature clusters (Chapter I) reflecting cytokinin degradation.

Also, elevated production of ethylene was detected in P-deficient roots of white lupin but the cluster root formation was not affected by application of ethylene inhibitors (Gilbert *et al.*, 2000). Moreover, enhanced expression of an EST with homology to 1-aminocyclopropane-1-carboxylate (ACC) oxidase, involved in biosynthesis of ethylene, was observed in P-deficient cluster roots as compared with P-sufficient normal roots of white lupin (Uhde-Stone *et al.*, 2003). Most recently, transcripts related to ethylene synthesis were also detected to be abundantly expressed in P-deficient clusters of white lupin (O'Rourke *et al.*, 2013), and particularly in mature clusters (Chapter I). Thus, it was speculated that ethylene may be involved in dense formation of root hairs (Neumann & Martinoia, 2002), but not in cluster root formation of white lupin. However, in other plant species like *Casuarina glauca*, cluster root formation under Fe deficiency was strongly inhibited by application of ethylene inhibitors (Zaid *et al.*, 2003), suggesting a direct role of ethylene in Fe deficiency-induced cluster root formation.

Recently, nitric oxide (NO) was demonstrated to be involved in the adaptive responses of white lupin to P limitation (Wang *et al.*, 2010). High levels of intracellular NO were detected in P-deficient cluster roots, particularly in cluster rootlet primordia before and during emergence and in mature root clusters. Application of the NO donor sodium nitroprusside (SNP) stimulated formation of cluster roots and lateral roots in P-deficient white lupin but not in P-sufficient plants. Based on the findings of Correa-Aragunde *et al.* (2006) in tomato (*Solanum lycopersicum*), NO seems to be required for lateral root initiation and primordia formation (Neumann, 2010; Cheng *et al.*, 2011) in the pericycle by modulating the expression of regulatory genes of the cell cycle, but not for the emergence of lateral roots, which additionally requires auxin (Neumann, 2010). Wang and coworkers (2010) further investigated enzymes putatively involved in NO generation in P-deficient cluster roots. Inhibitor studies and gene expression analyses suggested that NO synthase-like enzyme and xanthine dehydrogenase, but not nitrate reductase, are responsible for the production of NO in cluster roots. Xanthine dehydrogenase, as a key enzyme in purine catabolism, is highly expressed in mature clusters (Chapter I),

reflecting the massive *RNA* degradation (> 80%) during maturation of clusters for internal P remobilisation under condition of P starvation, with NO generation as a side product (Neumann *et al.*, 2000; Neumann, 2010).

Sucrose, derived from the photosynthesis, is the main form of mobile carbohydrates translocated from shoot to root. Under P deficiency, increased sucrose concentration was found in roots of different plant species, such as bean, soybean and *Arabidopsis* (Fredeen *et al.*, 1989; Ciereszko *et al.*, 1996; Ciereszko & Barbachowska, 2000; Hammond & White, 2008; Lei *et al.*, 2011). Chiou & Bush (1998) identified sucrose acts as a signal molecule involved in assimilate partitioning. Meanwhile, an increasing body of evidences supports the idea that sucrose derived from photosynthesis acts not only as carbon source but also as a systemic signal mediating plant responses to P limitation (Chapter II; Jain *et al.*, 2007; Karthikeyan *et al.*, 2007; Zhou *et al.*, 2008; Lei *et al.*, 2011). A sucrose signalling function in cluster root development has been proposed by Zhou *et al.* (2008).

2.5 Physiology of cluster roots

Under P deficiency, cluster root formation takes place over a time period of 4-5 weeks (Kania, 2005), and is characterised by distinct morphological and physiological changes. As mentioned above, the mature (MA) root clusters are the sites of morphological and physiological adaptations to mediate nutrient (particularly P) mobilisation in the rhizosphere (Neumann *et al.*, 2000). These modifications comprise development and proliferation of long, densely-spaced root hairs, secretion of P-mobilising root exudates, such as citrate, phenolics, phosphatases and protons (Fig. 3), induction of high-affinity P_i uptake systems and an increased Fe reductive capacity (Neumann & Martinoia, 2002; Chapter I). At the same time, the internal orthophosphate (P_i) status of MA clusters declines by more than 50% as compared with the JU clusters (Massonneau *et al.*, 2001), probably as a consequence of the well-documented P_i re-translocation from the old tissues (MA clusters) to the young-growing tissues (PE region and JU clusters) (Neumann *et al.*, 2000). Accordingly, the metabolic pathways requiring P are inhibited and replaced by the P-independent bypass reactions (Plaxton & Tran, 2011) to support P_i-recycling in MA clusters (Chapter I).

Among the carboxylates secreted by CRs, citrate is the most efficient carboxylate anion to mobilise sparingly available soil P, followed by oxolate. Malate, secreted mainly in juvenile clusters, has a much lower mobilising effect (Jones, 1998). Accumulation of 50-90 µmol of citric acid per g soil has been reported in the rhizosphere soil of cluster roots in white lupin (Dinkelaker *et al.*, 1989; Gerke *et al.*, 1994; Li *et al.*, 1997), which is sufficient for the desorption of phosphorus from sparingly soluble Ca-, Al- and Fe-phosphates and from P-adsorbing Fe/Al humic acid complexes, mainly by mechanisms of ligand exchange, and dissolution of P sorption sites in the soil matrix (Gardner *et al.*, 1983; Gerke *et al.*, 1994; Neumann *et al.*, 2000). Mobilisation of soil organic P fractions is related to secretion of acid phosphatase (APase) (Neumann *et al.*, 1999). However, in many soils the availability of organic P is limited mainly due to the low solubility of organic P forms such as Ca/Mg-, and Fe/Al-phytates (representing the major proportion of soil organic P) and the limited mobility of the root-borne phosphohydrolases (APase, phytase) (Neumann *et al.*, 2000). In this context, the release of carboxylates may help to mobilise also the sparingly soluble organic P esters and thereby increase their availability for enzymatic hydrolysis by phosphohydrolases adsorbed on the root surface (Neumann & Römheld, 2007; Fig. 3). Apart from P mobilisation, an increased reduction of Mn(IV)-oxide and Fe(III) was also detected in the rhizosphere of CRs, probably due to the increased activity of membrane-bound reductase and excretion of large amounts of reducing compounds such as phenolics (mainly isoflavoniods) (Neumann *et al.*, 2000; Neumann & Martinoia, 2002).

The carboxylates secreted into the rhizosphere are a preferential carbon source for rhizosphere bacteria and fungi. Accordingly, white lupin roots have evolved mechanisms to reduce the microbial growth in the rhizosphere of active CRs, which include rapid and strong acidification in the rhizosphere of mature clusters to reduce bacterial populations, excretion of toxic phenolics (maily isoflavonoids) and antifungal cell wall-degrading enzymes (chitinase and glucanase) at the stage preceding the citrate excretion (Weisskopf *et al.*, 2006). Moreover, carboxylate exudation is a well-programmed process, which occurs as strong pulses within a short period of time (1-3 days) into the restricted volume of rhizosphere soil (Neumann & Martinoia, 2002). These complex strategies have obviously been

evolved for protection of carboxylates against microbial degradation (Weisskopf *et al.*, 2006), making it more efficient for mobilisation of P.

The finding that senescent clusters accumulating high intracellular concentration of citric acid without citrate exudation suggests that citrate exudation may be mediated by a specific tightly-controlled transport mechanism located in plasma membrane (Neumann *et al.*, 2000). Exposing P-deficient cluster roots of white lupin to anion channel inhibitors (ethacrynic acid and anthracene-9-carboxylic acid) decreases citrate exudation by 50%, indicating that citrate exudation may be mediated by anion channels (Neumann *et al.*, 2000). Moreover, multidrug and toxic compound extrusion (MATE) transporters have been implicated in the root exudation of citrate and flavonoids (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007; Gottardi *et al.*, 2013), while malate efflux seems to be mediated by the aluminum-activated malate transporters (ALMT) (Sasaki *et al.*, 2004). Up to 23% of the total plant dry weight is lost as root exudation of carboxylates (Dinkelaker *et al.*, 1989). Plants may have evolved mechanisms to control the carboxylates exudation. Surprisingly, a WRKY domain-containing transcription factor WRKY46 was reported to function as transcriptional repressor of *ALMT1* in *Arabidopsis*, which regulates aluminum-induced malate secretion (Ding *et al.*, 2013). Similarly, in the absence of Al, the putative Al-binding protein AlBP might function as a negative regulator of citrate flux through *SbMATE*. Upon Al exposure, AlBP binds Al, causing a conformational change in AlBP and allowing citrate to flow out of root cells through *SbMATE* (Liu & Kochian, unpublished data). Root exudation of organic acids (e.g., citrate and malate) that mobilise the sparingly available P and chelate toxic Al^{3+} in the rhizosphere have been identified as an effective mechanism to cope with P deficiency and Al toxicity (J. F. Ma *et al.*, 2001; Ryan *et al.*, 2001; Kochian *et al.*, 2004). The question is whether malate and citrate exudation under P deficiency is also regulated in the similar manner?

For mineralisation of organic P forms in soils, root secretory acid phosphatase (APase) activity increases dramatically in cluster roots of P-deficient white lupin (Ozawa *et al.*, 1995; Gilbert *et al.*, 1999; Neumann *et al.*, 1999; Miller *et al.*, 2001; Wasaki *et al.*, 2003), particularly in mature and senescent clusters (Neumann *et al.*, 1999). The mobilisation of organic P forms, prior to enzymatic hydrolysis via APase,

seems to be another function of massive citrate exudation in CRs (Neumann & Römheld, 2007).

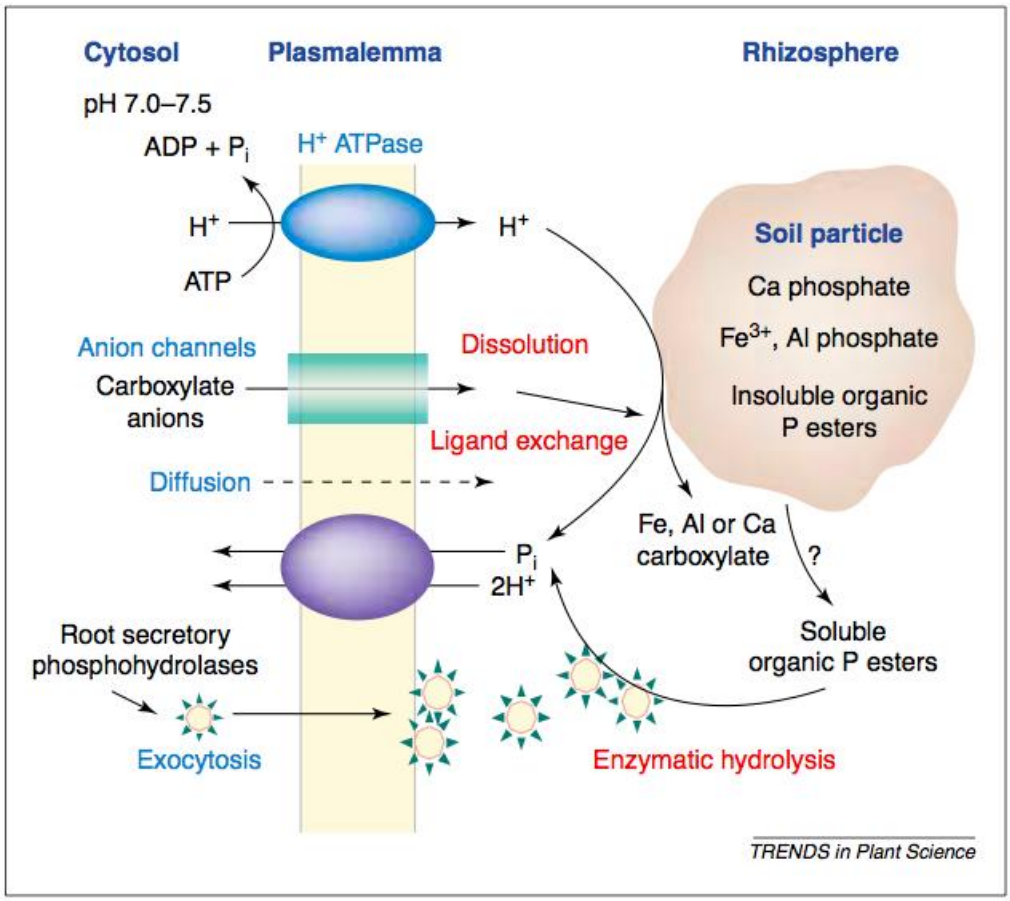

Fig. 3 Model for root-induced chemical phosphate mobilisation in the rhizosphere by exudation of carboxylates, protons and root secretory phosphohydrolases (from Neumann & Martinoia, 2002).

2.6 Physiological adaptations of cluster roots in P-deficient white lupin

2.6.1 Alternative glycolytic pathways

Since many enzymes of the glycolytic pathway depend on P_i or adenylates as co-substrates, these metablic processes might be impaired under severe P limitation (Vance *et al.*, 2003). However, in this situation, many plants, in general, show remarkable flexibility by utilisation of P_i-independent bypass reactions (Duff *et al.*, 1989; Palma *et al.*, 2000; Vance *et al.*, 2003; Plaxton & Podestá, 2006; Plaxton & Tran, 2011). While ATP and ADP-levels decline under P starvation (Ashihara *et al.*, 1988; Duff *et al.*, 1989), pyrophosphate (PP_i) concentration remains as an alternative

P pool (Duff *et al.*, 1989; Dancer *et al.*, 1990; Plaxton & Tran, 2011). The well-documented PP_i-dependent glycolytic bypass reactions include: (1) cleavage of sucrose by the sucrose synthase pathway and active transport of protons mediated by a PP_i-dependent H^+ pump in the tonoplast (Plaxton & Carswell, 1999); (2) PP_i-dependent phosphofructokinase (PFK) bypassing ATP-dependent PFK (Theodorou *et al.*, 1992; Theodorou & Plaxton, 1996; Plaxton & Carswell, 1999; Plaxton & Tran, 2011). In addition, other alternative glycolytic pathways comprise nonphosphorylating NADP-dependent glyceraldehyde-3P dehydrogenase (NADP-G3PDH) bypassing P_i-dependent NAD-G3PDH and phosphoglycerate kinase (Duff *et al.*, 1989; Theodorou *et al.*, 1992; Plaxton & Carswell, 1999; Vance *et al.*, 2003; Plaxton & Tran, 2011) and combined activities of PEP carboxylase, MDH and NAD-malic enzyme (Theodorou & Plaxton, 1993; Vance *et al.*, 2003; Plaxton & Tran, 2011), when the activity of pyruvate kinase (PK) is severely inhibited by P limitation. All these alternative reactions can also be found in cluster roots of P-deficient white lupin (Chapter I; Neumann *et al.*, 1999, 2000; Massonneau *et al.*, 2001; Uhde-Stone *et al.*, 2003; Vance *et al.*, 2003; O'Rourke *et al.*, 2013). Accordingly, these bypass reactions maintain the carbon flow from glycolysis into TCA cycle under P limitation, and additionally the PEPC-mediated non-photosynthetic CO_2 fixation provides a substantial proportion of carbon (> 30%) to carboxylate production in cluster roots (Johnson *et al.*, 1994; Johnson *et al.*, 1996a, b).

2.6.2 Inhibited citrate turnover in TCA cycle

In contrast to increased production of organic acids, decreased activity of aconitase, catalysing the turnover of citrate to isocitrate, was reported by Neumann *et al.* (1999) and Kihara *et al.* (2003) in mature clusters of white lupin. Aconitase is rapidly inactivated by H_2O_2 (Verniquet *et al.*, 1991), which can be produced by inhibited respiration (Minagawa *et al.*, 1992; Purvis & Shewfelt, 1993). Also NO, produced in connection with P_i recycling by nucleotide degradation in mature clusters, is a potent inhibitor of aconitase (Neumann *et al*., 2010; Wang *et al*., 2010; Gupta *et al.*, 2012). Moreover, Kihara *et al.* (2003) reported inhibited activity of NADP-isocitrate dehydrogenase (IDH), catalyzing the turnover of isocitrate to 2-oxoglutarate (2-OG), in mature clusters of white lupin under P deficiency. A reduced NO_3^- uptake and

assimilation is a common feature of P-deficient plants (Le Bot *et al.*, 1990; Rufty *et al.*, 1990; Pilbeam *et al.*, 1993; Gniazdowska *et al.*, 1999; Neumann *et al.*, 2000) and accordingly the demand for 2-OG, which is an important acceptor for amino N in N assimilation (Lancien *et al.*, 1999), is also reduced. The reduced N assimilation and a lower demand for 2-OG may in turn affect the citrate turnover which provides the precursor for 2-OG (Neumann *et al.*, 2000), such as IDH activity. Thus, the huge amount of citrate accumulation and exudation in mature clusters of white lupin under P deficiency might be a consequence of both increased production (via PEPC) and decreased turnover of citrate in TCA cycle (Chapter I; Neumann *et al.*, 2000).

2.6.3 Reduced respiration with the exception of alternative oxidase (AOX)

In cluster roots of P-deficient white lupin, reduced mitochondrial respiration has been reported (Johnson *et al.*, 1994; Neumann *et al.*, 1999; Massonneau *et al.*, 2001; Kania *et al.*, 2003) potentially caused by shortage of ADP and P_i as substrates of the cytochrome (Cyt) electron transport pathway, and more generally, by impairment of mitochondrial function due to limited biosynthesis of proteins (Bingham & Farrar, 1988; Williams & Farrar, 1990; Bingham & Stevenson, 1993; Wanke *et al.*, 1998). The imbalanced carbon metabolism and electron transport can cause an overproduction of reactive oxygen species (ROS) in the respiration chain and of the oxidised product H_2O_2 (Purvis & Shewfelt, 1993; Wagner & Krab, 1995). Consequently, the TCA cycle would be inhibited by the shortage of oxidised pyridine nucleotides (Lee, 1979) and the overproduction of H_2O_2 that might inhibit the aconitase activity (Verniquet *et al.*, 1991) for citrate turnover (Lance & Rustin, 1984; Neumann *et al.*, 2000).

However, as known so far, plants and some fungi and protists additionally exhibit an alternative respiration pathway, the so-called alternative oxidase (AOX) (Day & Wiskich, 1995). This cyanide-resistance oxidase catalyses the oxidation of ubiquinone and reduces the oxygen to water in a single four-electron step without conserving energy by bypassing the energy-conserving steps in the normal Cyt pathway (Day *et al.*, 1996; Siedow & Umbach, 2000) as a mechanism to counteract the overproduction of free radicals.

2.6.4 RNA degradation for P_i recycling

Cluster root development from the juvenile to senescent stage in white lupin under P deficiency is associated with a 90% loss of total *RNA* (Neumann *et al.*, 2000; Massonneau *et al.*, 2001), reflecting P_i recycling which is subsequently retranslocated to pre-emergent and juvenile cluster roots with a high energy demand due to intensive growth activity (Neumann & Martinoia, 2002).

2.7 Research questions and hypotheses

Based on the large pool of information on CR physiology obtained mainly by hypothesis-driven approaches, this study was designed to get a more comprehensive picture of the metabolic changes during CR development starting with a transcriptome sequencing approach. The outcome of the transcriptome analysis provided the basis for the formulation of research questions to be investigated more in detail:

(1) Which genes/transcripts are contributing to cluster root formation and function of white lupin (*Lupinus albus*) under P deficiency?

(2) Does sucrose act as a signal controlling cluster root formation?

(3) Which hormonal interactions are involved in cluster root formation?

The first research question is addressed in an article entitled "The regulatory network of cluster root function and development in phosphate-deficient white lupin (*Lupinus albus* L.) identified by transcriptome sequencing".

The article entitled "A re-assessment of sucrose signalling involved in cluster root formation and function in phosphate-deficient white lupin (*Lupinus albus* L.)" addresses the second research question using an axenic hydroponic culture system to characterise sucrose-mediated signalling events involved in formation and function of cluster roots.

The third research question is addressed in an article entitled "Hormonal interactions during cluster root development in phosphate-deficient white lupin (*Lupinus albus* L.)", using a combined approach of RT-qPCR based analysis of hormone-related genes, hormone transport studies and external application of hormones and hormone antagonists.

3 Chapter I - The regulatory network of cluster root function and development in phosphate-deficient white lupin (*Lupinus albus* L.) identified by transcriptome sequencing

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3.1 Abstract

Lupinus albus serves as model plant for root-induced mobilisation of sparingly soluble soil phosphates via the formation of cluster roots (CRs) that mediate secretion of protons, citrate, phenolics and acid phosphatases (APases). This study employed next generation sequencing to investigate the molecular mechanisms behind these complex adaptive responses at the transcriptome level. We compared different stages of CR development, including pre-emergent (PE), juvenile (JU) and the mature (MA) stages. The results confirmed that the primary metabolism underwent significant modifications during CR maturation, promoting the biosynthesis of organic acids, as had been deduced from physiological studies. Citrate catabolism was down-regulated, associated with citrate accumulation in MA clusters. Up-regulation of the phenylpropanoid pathway reflected the accumulation of phenolics. Expression of genes encoding ALMT and MATE transporters correlated with the exudation of citrate and flavonoids. The expression of transcripts related to nucleotide degradation and APases in MA clusters coincided with the re-mobilisation and hydrolysis of organic phosphate resources. Most interestingly, expression analysis of hormone-related genes suggested a central role of ethylene during CR maturation. This was associated with the up-regulation of the iron (Fe) deficiency regulated network that mediates ethylene-induced expression of Fe deficiency responses in other species. Finally, transcripts related to abscisic and

jasmonic acids were up-regulated in MA clusters, while auxin- and brassiosteroid-related genes and cytokinin receptors were most strongly expressed during CR initiation. Key regulations proposed by the *RNA*-seq data were confirmed by RT-qPCR and some physiological analyses. A model for the gene network regulating cluster root development and function is presented.

3.2 Introduction

Phosphorus (P), as one of the 17 essential elements required for plant growth and development, belongs to the most limiting plant nutrients in arable lands (Marschner, 1995). Plant P uptake occurs exclusively in its orthophosphate (P_i) forms ($H_2PO_4^-$ and HPO_4^{2-}) dissolved in the soil solution. Although the total amount of soil P usually exceeds the plant requirements by several orders of magnitude, it is mainly fixed in sparingly available organic and inorganic P forms. The diffusion-based delivery from the soil solid phase is frequently much too slow to match the plant's demand for optimal growth and therefore requires additional adaptations for acquisition of nutrients with limited solubility. These adaptations can be grouped into strategies towards: (1) enhanced spatial acquisition of the available fraction of sparingly soluble nutrients by increasing the root surface involved in nutrient absorption and (2) increasing nutrient solubility by root-induced chemical changes in the rhizosphere (Neumann & Römheld, 2002).

While 80% of land plants are able to establish symbiotic associations with arbuscular mycorrhizal fungi to improve mainly spatial P_i acquisition (Koide & Kabir, 2000; Smith *et al.*, 2000; Burleigh *et al.*, 2002; Tibbett & Sanders, 2002), efficient strategies to increase the solubility of sparingly available soil P sources are much less abundant. Particularly in various non-mycorrhizal plant species, the development of so-called "cluster roots" (CRs), formed by closely-spaced lateral rootlets with limited growth, has been identified as one of the most efficient adaptations for acquisition of sparingly soluble mineral nutrients (mainly P) by chemical modifications of the rhizosphere, comprising release of organic metal chelators (citrate, malate, phenolics), protons and secretory phosphohydrolases (Neumann & Martinoia, 2002; Vance *et al.*, 2003; Shane & Lambers, 2005; Neumann & Römheld, 2007; Lambers *et al.*, 2011). Cluster roots are a characteristic feature in many species of the Proteaceae and other plant families, adapted to highly

weathered soils in Australia and South Africa (Neumann & Martinoia, 2002). However, the most detailed knowledge on mechanisms determining CR function is based on investigations of white lupin (*Lupinus albus* L.), as one of the few highly P-efficient cluster-rooted crops.

Due to the limited P availability, crop yield on 30-40% of the world's arable lands relies on high inputs of soluble P_i fertilizers (Runge-Metzger, 1995; Uexküll & Mutert, 1995), which are mainly produced from mined rock phosphate, as a non-renewable resource (Cordell *et al.*, 2009). In face of this situation, research efforts towards more sustainable use of P_i fertilizers have attracted increased attention in the recent past, including attempts to understand natural plant adaptations to P limitation, as a potential source of approaches to improve P efficiency of crops by optimised agronomic management strategies, and genetic or biotechnological modifications (Vance *et al.*, 2003; Cheng *et al.*, 2011).

During the last decade, enormous progress has been made in the investigation of the metabolic adaptations, determining the function of CR, mainly based on physiological measurements and molecular characterisation of the underlying metabolic processes (Neumann & Martinoia, 2002; Vance *et al.*, 2003; Shane & Lambers, 2005; Neumann & Römheld, 2007; Lambers *et al.*, 2011). The use of genomic studies could provide a far more comprehensive picture but up to date, traditional genomic studies were mainly focussing on well-characterised model systems, such as *Arabidopsis*, *Medicago truncatula* and maize (*Zea mays*) (O'Rourke *et al.*, 2013). However, even without availability of whole genome data, novel techniques based on next generation sequencing provide the opportunity to conduct transcriptome studies also in other plant species. By using the Illumina platform, the expression pattern of transcripts in a specific tissue, under certain abiotic/biotic conditions, is detectable. This approach has already been proven as a successful tool for investigations on *Lupinus albus*: O'Rourke *et al.* (2013) presented the first *Lupinus albus* gene index (LAGI 1.0) of P_i-deficient and -sufficient plants, using *RNA*-seq analysis (Illumina GA-IIx Platform). A total of 2128 transcripts differently expressed in response to P_i deficiency were identified (O'Rourke *et al.*, 2013). Coupled with physiological experiments addressing hormonal effects on cluster root formation, new insights into biochemical and molecular changes induced by P limitation were provided. These experiments

mainly focussed on leaves, roots and CRs of P_i-deficient and -sufficient plants. However, individual root clusters of P_i-deficient white lupin undergo distinct developmental changes, starting with the induction of CR primordia at 2-3 cm behind the root tip of first-order laterals (pre-emergent stage), followed by outgrowth of juvenile clusters, finally reaching the mature stage when the second-order lateral rootlets exhibit no more growth activity. Chemical rhizosphere modifications for nutrient mobilisation are mainly restricted to the mature stage (Fig. 1A; Neumann *et al.*, 1999, 2000). Cluster roots are continuously formed throughout the whole growth period and the development of each individual root cluster proceeds within a time period of 7-8 days (Neumann & Martinoia, 2002). Consequently, the root systems of P_i-deficient white lupin comprise clusters in all developmental stages (Fig. 1B). To get more detailed insights into the characteristic changes of gene expression during CR development, we used the *RNA*-seq analysis of pre-emergent (PE), juvenile (JU) and mature (MA) CR stages (Fig. 1A). In combination with previous physiological data, this approach identified novel key components of the regulatory network underlying the development, metabolic processes and function of CR.

3.3 Material and methods

3.3.1 Plant culture

Six seedlings of white lupin (*Lupinus albus* L. cv. Feodora) pre-germinated on filter paper were transferred into pots containing 2.5 L of aerated nutrient solution with 2 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe (III)-EDTA, 100 µM KH_2PO_4 at pH 5.6-5.8. Phosphate was omitted in the -P treatments. Replacement of nutrient solution was conducted every four days. Plant culture was performed with three biological replicates in a growth chamber with a light period of 16 h, light intensity of 200 mmol m^{-2} s^{-1}, 24/18 ℃ day/night temperature and a relative humidity of 60%. At 20 days after sowing (DAS), the root parts were harvested and separated into: pre-emergent root segments of first-order laterals without root tips (PE), juvenile CRs that had not reached their final length (JU), and mature CRs (MA) located just basal to JU clusters (Fig. 1A) of P-deficient plants. Mature clusters were separated from senescent clusters with dark brown

coloration. Root material was immediately frozen in liquid nitrogen and stored at -80 ℃ for further analysis.

3.3.2 *RNA* extraction, *cDNA* library preparation and sequencing

Samples from three biological replicates were pooled into one, for each stage of CR development. Total *RNA* was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNase-Free DNase Kit (Qiagen) was used to remove potential contamination from genomic *DNA* according to manufacturer's instructions. Reverse transcription and sequencing with the Illumina HiSeq 2000 technology were performed by the sequencing company (GATC Biotech AG, Konstanz, Germany). Mapping against public available *Lupinus albus* transcript database LAGI 1.0 (O'Rourke *et al.*, 2013) was done with Rsubread (Liao *et al.*, 2013). The output SAM-file contained 59.2% (PE), 58.8% (JU) and 57.5% (MA) successfully mapped reads. After normalization using Bioconductor, fold changes and relative read numbers were determined by DESeq (Version 1.8.3) (Anders & Huber, 2010). The procedure followed the vignette (Analyzing *RNA*-Seq data with the DESeq package; working without any replicates), the input were raw count tables.

Sequences were annotated with Mercator (http://mapman.gabipd.org/web/guest/mercator) and classified according to MapMan (http://mapman.gabipd.org/web/guest/mapman) functional plant categories as previously described (Straub *et al.*, 2013a). Metabolic pathways were visualized with MapMan 3.5.1R2 and its build-in module PageMan, showing DESeq's fold change values. Significance was determined by Wilcoxon Rank Sum test (PageMan Z-score below -1.96 or above 1.96, MapMan probability below 0.05).

3.3.3 Quantitative real-time PCR for confirmation of *RNA*-seq data

In a second experiment, total *RNA* of CR in different developmental stages was isolated with the innuPREP Plant *RNA* kit (Analytik Jena AG, Jena, Germany) using three independent biological replicates. Contaminating genomic *DNA* was removed with a RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. The complementary *DNA* (*cDNA*) was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Gene-specific primers were designed using Primer3Plus. Quantitative real-time PCR was performed as previously described (Straub *et al.*,

2013b). Primer sequences of selected reference genes (*PP2AA3*, *UBC*) and other genes can be found in Appendix Suppl. Table 1.

3.3.4 Statistical analysis

Quantitative real-time PCR data are shown as means ± SE. One-way ANOVA with least significant difference (LSD) test at $P < 0.05$ was used to test the differences. The software used was SAS for Windows Version 9.4 (SAS Institute Inc, Cary, NC, USA).

Sequencing data from this article can be accessed at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number: E-MTAB-2253.

3.4 Results

3.4.1 *RNA*-seq analysis and validation of *RNA*-seq expression profiles by quantitative real-time PCR (RT-qPCR)

Three complementary *DNA* (*cDNA*) libraries were constructed from pooled samples of PE, JU and MA developmental stages of CR, harvested at 20 days after sowing (DAS) (Fig. 1A). In total, 146 738 732 Illumina reads were generated from the three *cDNA* libraries. Mapping against the publicly available *Lupinus albus* transcript database (LAGI 1.0) yielded 103 147 unique sequences. A large number of housekeeping genes with low, intermediate and higher expression levels were expressed similarly across all samples, validating the *mRNA* quality, extraction and normalization procedure.

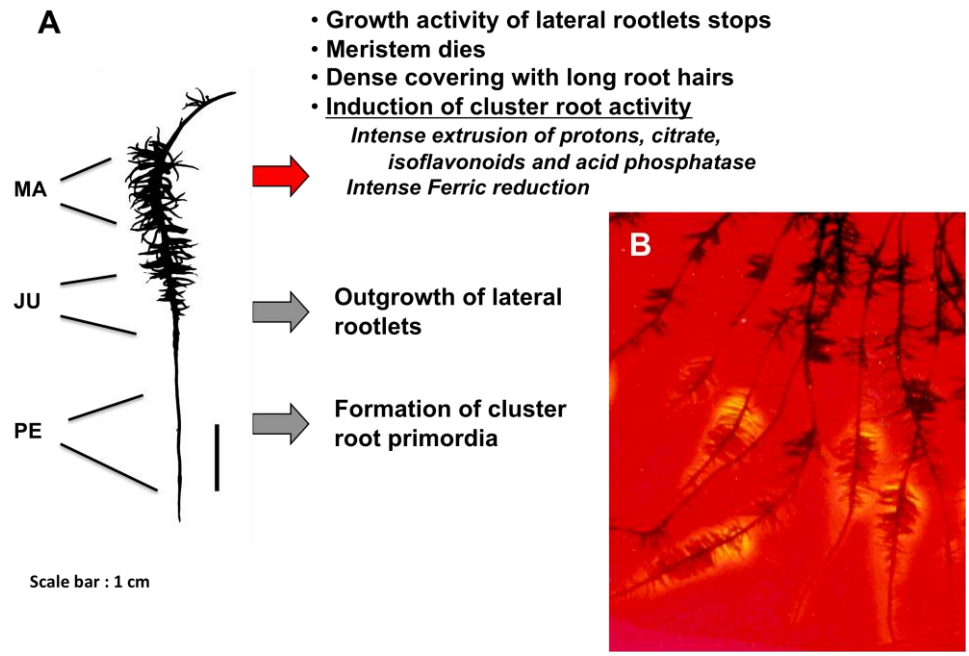

Fig. 1 (A) Developmental stages of cluster roots in white lupin (*Lupinus albus* L.) grown for 20 days after sowing (DAS) under P deficiency. PE, pre-emergent clusters without root tips; JU, juvenile cluster root; MA, mature cluster root; (B) Root system with cluster roots in different stages of development and activity (proton extrusion visualized with pH indicator, modified after Neumann *et al*., 2000).

The differential expression of key genes in different gene categories was validated in independent experiments with quantitative real-time PCR (RT-qPCR). These analyses included genes involved in hormonal metabolism (Fig. 2A, B), root carbon metabolism (Fig. 2C), transporters and enzymes for nutrient uptake and root exudation (Fig. 2C, D), as well as various transcription factors regulating root growth responses and root exudation (Fig. 2C, D). Sub-apical root zones (2-3 cm) of phosphate-supplied plants (+P_AP) and –P (-P_PE) plants, as well as JU (-P_JU) and MA (-P_MA) clusters were included into this analysis. In total, 23 differentially regulated transcripts from the *RNA*-seq analyses were evaluated by RT-qPCR, with most of them (20) showing qualitatively the same behavior when analyzed with both methods, yielding a highly consistent data set as compared with the *RNA*-seq analysis (Table 1 and Fig. 2).

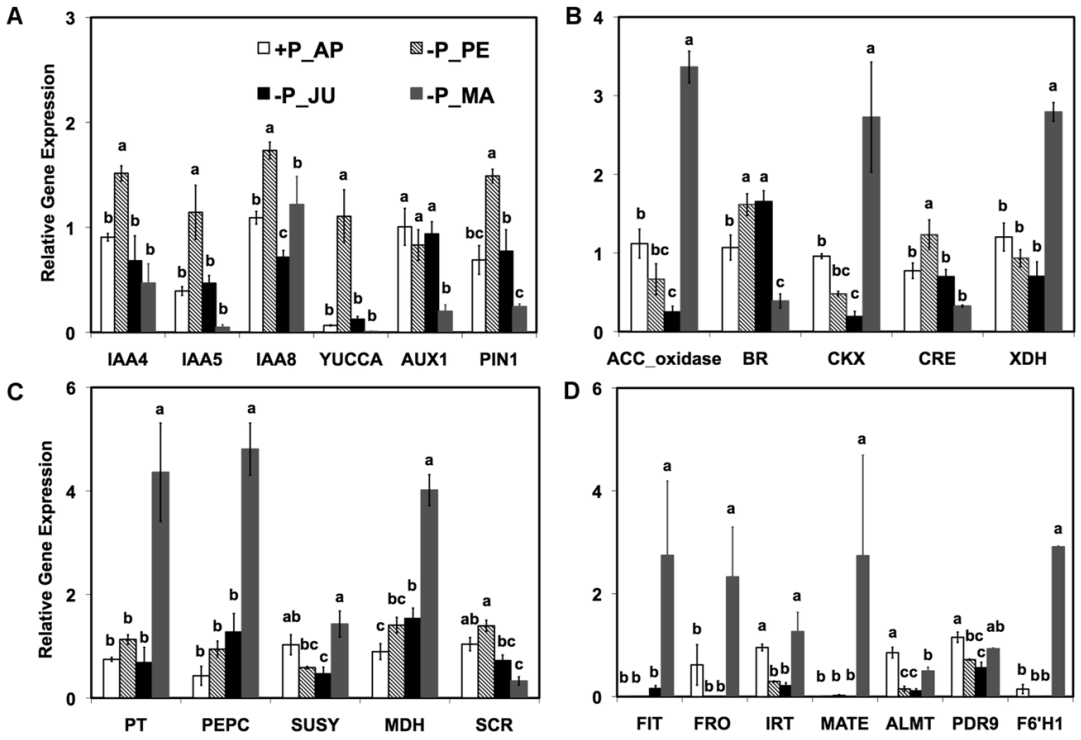

Fig. 2 RT-qPCR evaluation of transcripts involved in hormones and root carbon metabolism, phosphate and ion transporters and transcription factors. (A) Expression pattern of auxin-related transcripts; (B) Expression pattern of transcripts involved in metabolism of ethylene (ACC_oxidase), brassinosteroids (enzyme in BR biosynthesis pathway), cytokinin (CKX and CRE) and nitric oxide (XDH); (C) Expression pattern of transcripts involved in phosphate uptake (PT), biosynthesis of organic acids (PEPC), SUSY, MDH and the scarecrow (SCR) transcription factor; (D) Expression pattern of transcripts involved in iron acquisition, FIT, FRO2, IRT1, organic compounds biosynthesis or extrusion, MATE, ALMT, PDR9 and F6'H1. The expression level is indicated relative to the reference genes. The values are means ± SE (n = 2-3). Different lowercase letters denote significant differences ($P < 0.05$). +P_AP, sub-apical first-order lateral root zone (without root tips) of P-sufficient plants; −P_PE, pre-emergent clusters (without root tips) of P-deficient plants; −P_JU, juvenile clusters of P-deficient plants; −P_MA, mature clusters of P-deficient plants; *YUCCA*, encoding a flavin monooxygenase–like enzyme, catalyzing tryptophan-dependent auxin biosynthesis; *AUX*, auxin influx carrier; *PIN*, auxin efflux carrier; *ACC*, 1-aminocyclopropane-1-carboxylate; *BR*, involved in the conversion of the early brassinosteroid precursor 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis pathway; *CKX*, cytokinin oxidase; *CRE*, cytokinin receptor; *XDH*, encoding xanthine dehydrogenase in purine metabolism; *PT*, phosphate transporter; *PEPC*, phosphoenolpyruvate carboxylase;

SUSY, sucrose synthase; *MDH*, malate dehydrogenase; *FIT*, FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR; *FRO*, FERRIC REDUCTION OXIDASE; *IRT*, IRON-REGULATED TRANSPORTER; *MATE*, multidrug and toxic compound extrusion; *ALMT*, aluminum-activated malate transporter; *PDR9*, PLEIOTROPIC DRUG RESISTANCE9; *F6'H*, Fe(II)- and 2-oxoglutarate-dependent dioxygenase (2OGD) family.

3.4.2 Differentially expressed transcripts potentially involved in cluster root development

Lateral root development involves the phytohormone auxin as a key player, together with cytokinins, ethylene, brassinosteroids (BRs), and abscisic acid (ABA) (Fukaki & Tasaka, 2009). As CRs are a specialised type of lateral roots, these phytohormones are likely also crucial for CR development and function.

The *YUCCA* pathway was proposed as a common indole-3-acetic acid (IAA) biosynthesis pathway (Zhao *et al.*, 2001) in higher plants. Indole-3-acetic acid is then transported from source to sink tissues via polar auxin transport, which requires influx (*AUXs*) and efflux (*PINs*) carriers (Marchant *et al.*, 2002; Benková *et al.*, 2003; Blilou *et al.*, 2005).

Table 1 *RNA*-seq expression profile for different developmental stages of cluster roots under P deficiency (selected). Pairwise comparisons of different developmental stages of cluster roots (JU versus PE, MA versus JU and MA versus PE) are calculated. Expression ratios, as log2 fold change, of each comparison are shown. Negative values indicate lower expression in JU compared with PE, lower expression in MA compared with JU, and lower expression in MA compared with PE, respectively. Expression ratios of 0 indicate no difference. *YUCCA*, key enzyme for tryptophan-dependent auxin biosynthesis; *AUX*, auxin influx carrier; *PIN*, auxin efflux carrier; *ACC*, 1-aminocyclopropane-1-carboxylate; *SAM*, S-Adenosyl-Methionine; *BR*, involved in the conversion of the early brassinosteroid precursor 24-methylenecholesterol to campesterol; *CKX*, cytokinin oxidase; *CRE*, cytokinin receptor; *XDH*, xanthine dehydrogenase in purine metabolism; *PT*, phosphate transporter; *SAP*, secretory acid phosphatase; *PEPC*, phosphoenolpyruvate carboxylase; *SUSY*, sucrose synthase; *MDH*, malate dehydrogenase; *SPX, SYG1/Pho8/XPR1; GPX-PDE*, glycerophosphodiester phosphodiesterase; *PHO2*, phosphate 2; *AOX*, alternative oxidase; *SCR*, SCARECREW; *FIT*, FER-LIKE IRON DEFICIENCY INDUCED

TRANSCRIPTION FACTOR; *FRO*, FERRIC REDUCTION OXIDASE; *IRT*, IRON-REGULATED TRANSPORTER; *MATE*, multidrug and toxic compound extrusion; *ALMT*, aluminum-activated malate transporter; *PDR9*, PLEIOTROPIC DRUG RESISTANCE9; *F6'H*, Fe(II)- and 2-oxoglutarate-dependent dioxygenase (2OGD) family; *F3'H*, flavonoid 3'-hydroxylase.

Sequence identifier	Expression ratio (log2)			Annotation
	JU/PE	MA/JU	MA/PE	
LAGI01_44916	-2	0	-2	*IAA4*
LAGI01_23355	-1	-3	-3	*IAA5*
LAGI01_23949	-1	0	-1	*IAA8*
LAGI01_22491	-3	-3	-6	*YUCCA*
LAGI01_20170	-2	0	-2	*AUX1*
LAGI01_18436	-1	-1	-2	*PIN1*
LAGI01_38922	0	2	2	*ACC oxidase*
LAGI01_19375	0	0	1	*ACC synthase*
LAGI01_32571	-1	2	1	*SAM synthase*
LAGI01_12936	2	-3	-1	*BR synthesis*
LAGI01_30270	-1	3	2	*CKX*
LAGI01_4624	-1	-1	-2	*CRE*
LAGI01_2030	1	1	2	*XDH*
LAGI01_49868	1	2	3	*PT*
LAGI01_21085	0	3	3	*SAP*
LAGI01_11832	1	0	1	*PEPC*
LAGI01_91	1	0	1	*SUSY*
LAGI01_41467	1	-1	0	*MDH*
LAGI01_37377	1	2	2	*Formamidase*
LAGI01_24729	2	0	2	*Nodulin*
LAGI01_46560	-1	2	2	*SPX3*
LAGI01_36851	0	1	1	*GPX-PDE1*
LAGI01_32637	0	1	1	*GPX-PDE2*
LAGI01_2742	-1	1	0	*PHO2*
LAGI01_1568	0	1	1	*AOX*
LAGI01_12303	-1	-2	-3	*SCR*
LAGI01_31302	1	2	3	*FIT*
LAGI01_14166	1	5	6	*FRO*
LAGI01_44181	0	1	1	*IRT*
LAGI01_21605	1	6	7	*MATE*
LAGI01_25811	-1	2	0	*ALMT*
LAGI01_11575	1	1	2	*PDR9*
LAGI01_46101	1	5	5	*F6'H1*
LAGI01_30448	1	1	2	*F3'H1*

The *RNA*-seq and RT-qPCR data revealed a massive differential expression of the auxin network in the different root zones. The strongest expression of auxin-related and auxin-responsive genes (*IAA 4/5, IAA 8, YUCCA, AUX1* and *PIN1*) was found in the PE zone, the region for induction and development of CR primordia. The

expression of auxin-related transcripts declined consistently in the JU stage and was decreased further in MA regions (Table 1 and Fig. 2A).

Cytokinins have already been implicated in CR formation of white lupin and showed a complex antagonistic interplay with auxin (Neumann *et al.*, 2000). Transcripts encoding cytokinin oxidase (CKX), an enzyme that catalyzes the irreversible degradation of cytokinins, were up-regulated 8-fold in MA as compared with JU clusters. By contrast, the transcript encoding the cytokinin receptor (CRE) was down-regulated 2-fold in MA as compared with the JU clusters (Table 1). These expression patterns were again confirmed by RT-qPCR (Fig. 2B).

Ethylene is another phytohormone that was postulated to be involved in the root development and root hair formation of CR in white lupin (Gilbert *et al.*, 1997; Watt & Evans, 1999b). In the *RNA*-seq data, as well as in the RT-qPCR, transcripts encoding enzymes involved in ethylene biosynthesis, 1-aminocyclopropane 1-carboxylic acid (ACC) oxidase and ACC synthase, were most highly expressed in MA clusters. Consistently, *RNA*-seq analysis further revealed also the highest expression of a transcript related to formamidase in MA clusters, an enzyme providing precursors for ethylene biosynthesis (Table 1). The expression of a transcript encoding an enzyme in the brassinosteroid (BR) biosynthesis pathway, involved in the conversion of the early brassinosteroid precursor 24-methylenecholesterol to campesterol, was inversely related with transcripts of ethylene biosynthetic enzymes. The BR biosynthesis pathway was up-regulated in JU as compared with the PE stage but dramatically down-regulated in MA clusters (Table 1).

Additionally, *RNA*-seq analysis also revealed the up-regulation of transcripts involved in abscisic acid (ABA) and jasmonic acid (JA) metabolism in MA clusters (Fig. 3N and Appendix Suppl. Fig. S5).

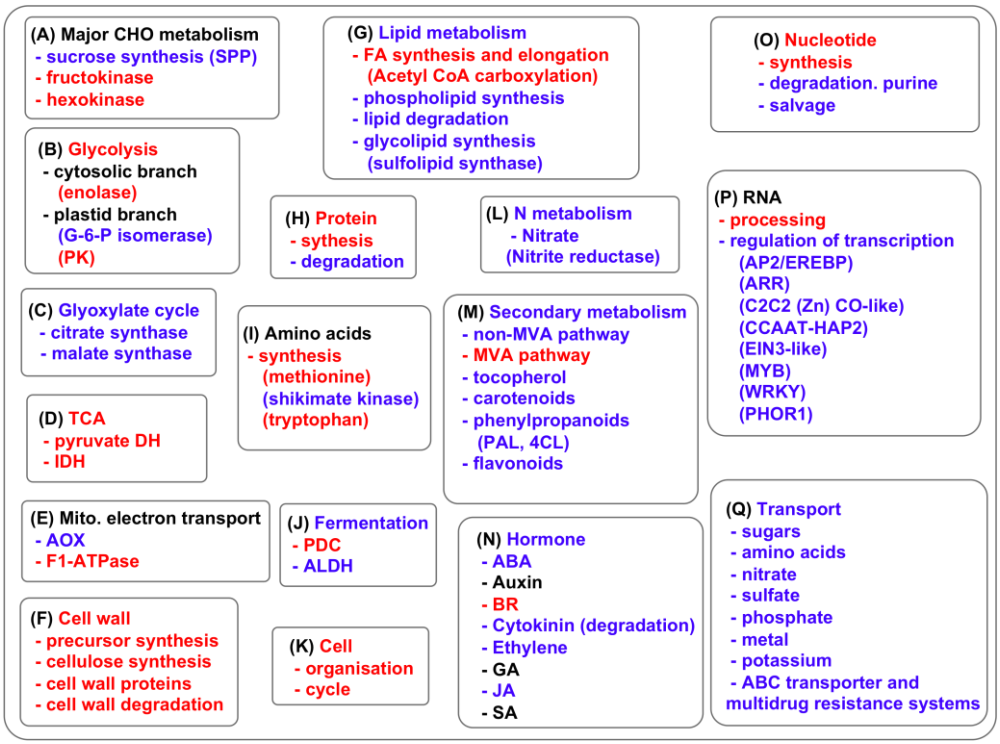

Fig. 3 General transcriptional differences from different metabolic categories. Mature clusters (MA) compared with juvenile clusters (JU). Blue color indicates significant up-regulation in MA as compared with JU, while red indicates significant down-regulation (Z-score < 1.96). Black shows no significant difference. The coloring is according to the Z-scores of the Wilcoxon test. A Z-score of ± 1.96 represents a P-value of 0.05. The figure was generated according to PageMan. The metabolic categories are related to: (A) Major CHO metabolism; (B) Glycolysis; (C) Glycoxylate cycle; (D) TCA; (E) Mitochondrial electron transport; (F) Cell wall; (G) Lipid metabolism; (H) Proteins; (I) Amino acids; (J) Fermentation; (K) Cell cycle and organization; (L) N metabolism; (M) Secondary metabolism; (N) Hormones; (O) Nucleotides; (P) RNA; (Q) Transport. CHO, carbohydrate; SPP, suc-phosphatase; G-6-P, glucose-6-phosphate; PK, pyruvate kinase; TCA, tricarboxylic acid cycle; pyruvate DH, pyruvate dehydrogenase; IDH, isocitrate dehydrogenase; AOX, alternative oxidase; FA, fatty acid; PDC, pyruvate decarboxylase; ALDH, aldehyde dehydrogenase; MVA, mevalonate; PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate-CoA ligase; ABA, abscisic acid; BR, brassinosteroid; GA, gibberellic acid; JA, jasmonic acid; SA, salicylic acid.

The key hormone-related gene expression pattern under P-sufficient conditions (+P) was then compared with that in PE, JU and MA stages of CRs in P-deficient plants (Fig. 2A, B) by RT-qPCR. As expected from the fact that CRs are not formed under

P-sufficient conditions, the expression pattern of auxin-, cytokinin-, BR- and ethylene-related genes revealed by RT-qPCR did not match the different developmental stages of the CRs in -P plants. As a consequence, a temporally and spatially distinct hormonal network of gene expression appeared to be associated with each developmental stage of CR formation.

3.4.3 Differentially expressed transcripts involved in cluster root function

In functional CRs of P-deficient white lupin, large amounts of organic acids (mainly malate and citrate), protons, phenolics and acid phosphatases (APases) that are involved in P mobilisation, are released into the rhizosphere (Dinkelaker *et al.*, 1995, 1997; Watt & Evans, 1999b; Neumann *et al.*, 2000; Massonneau *et al.*, 2001; Neumann & Martinoia, 2002). Malate is the dominant organic acid accumulating and released mainly in apical root segments and JU clusters, whereas citrate is dominant in MA clusters and released in pulses within 1-3 days (Neumann *et al.*, 1999). The *RNA*-seq data nicely validated these physiological data (Fig. 3).

The transcript encoding malate dehydrogenase (MDH), the enzyme reversibly catalyzing the oxidation of malate to oxaloacetate, was highly expressed in JU clusters, and declined during CR maturation (Table 1). By contrast, the gene related to phosphoenolpyruvate carboxylase (*PEPC*) was up-regulated in MA clusters (Fig. 2C). At the same time, transcripts of the metabolic pathways involved in citrate consumption, such as tricarboxylic acid (TCA) cycle enzymes (particularly isocitrate dehydrogenase, Figs. 3D, 4B) and cytochrome dependent respiration (Fig. 3E) were down-regulated. This transcriptional regulation pattern is consistent with preferential accumulation of citrate in MA clusters, and was confirmed by *in situ* formazan staining of dehydrogenase activities in the TCA cycle (Fig. 5).

Phenolics (mainly isoflavonoids) are also released in large amounts during CR development (Neumann *et al.*, 1999; Weisskopf *et al.*, 2006). These compounds are supposed to be involved in P mobilisation and protection of citrate against microbial degradation (Neumann *et al.*, 1999; Weisskopf *et al.*, 2006; Tomasi *et al.*, 2008). Accordingly, *RNA*-seq data revealed up-regulation of transcripts encoding flavonoid 3'-hydroxylase (F3'H1), as well as a Fe(II)- and 2-oxoglutarate-dependent dioxygenase family gene (*F6'H1*), both involved in the biosynthesis of flavonoids (Table 1, Fig. 2D). Accordingly, also the biosynthetic sequences providing flavonoid

precursors, such as the phenylpropanoid metabolism was found to be up-regulated in MA clusters (Fig. 3M).

The release of root secretory acid phosphatase (APase) in P-deficient white lupin is discussed as a crucial mechanism to acquire organic P forms in soils (Ozawa *et al.*, 1995; Gilbert *et al.*, 1999; Neumann *et al.*, 1999; Miller *et al.*, 2001; Wasaki *et al.*, 2005). In accordance with earlier reports (Massonneau *et al.*, 2001; Wasaki *et al.*, 2005), the *RNA*-seq data confirmed an intense (8-fold) up-regulation of the transcript encoding APase in MA clusters (Table 1).

The release of P-mobilising root exudates as well as the subsequent uptake of mobilised P_i requires the expression of transport proteins. Multidrug and toxic compound extrusion (MATE) transporters have been implicated in the root exudation of citrate and flavonoids (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007; Gottardi *et al*., 2013), while malate efflux seems to be mediated by the aluminum-activated malate transporters (ALMTs) (Sasaki *et al.*, 2004). Accordingly, *MATE* and *ALMT* transcripts were found to be most strongly expressed in MA clusters (Table 1, Fig. 2D). The same held true for transcripts encoding phosphate transporters (PTs, Table 1, Fig. 2C), which were clearly most abundant in the MA regions, in accordance with their P_i transport function in the region of P_i mobilisation.

Interestingly, the *RNA*-seq analysis and the RT-qPCR revealed the preferential expression of the iron uptake regulatory network in MA clusters, including the *FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR* (*FIT*, Table 1, Fig. 2D). This transcription factor is commonly crucial for the coordinated regulation of Fe deficiency responses of dicotyledonous plant species and non-graminaceous monocots (Ling *et al.*, 2002; Colangelo & Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005; Bauer *et al.*, 2007). Moreover, despite the fact that the lupin plants in above experiments were grown under Fe-sufficient conditions, MA root clusters also showed a concerted up-regulation of key genes of the iron deficiency network, such as the *FERRIC REDUCTION OXIDASE2* (*FRO2*) (Robinson *et al.*, 1999) and the *IRON-REGULATED TRANSPORTER 1* (*IRT1*) (Vert *et al.*, 2002, Table 1, Fig. 2D).

In contrast to the observed up-regulation of many genes involved in organic acid metabolism, biosynthesis of phenolics, the release of P-mobilising root exudates and

P_i uptake (Table 1, Fig. 2C, D), MA root clusters were characterised by a collective down-regulation of large parts of the primary metabolism, including the repressed synthesis of cell wall compounds (Fig. 3F), proteins (Fig. 3H), amino acids (Fig. 3I) and nucleotides (Fig. 3O). By contrast, typical metabolic sequences involved in P_i recycling, such as nucleotide degradation (Fig. 3O), replacement of phospholipids by sulfolipids (Fig. 3G) were consistently up-regulated. Furthermore, strongly P_i-dependent metabolic pathways for carbohydrate catabolism (glycolysis, cytochrome-dependent respiration) were consequently replaced by more P_i-independent metabolic bypass reactions (Plaxton & Tran, 2011), such as sucrose synthase (*SUSY*, Fig. 2C), UDP glucose phosphorylase, PEP carboxylase (*PEPC*, Fig. 2C) and alternative oxidase (*AOX*, Fig. 3E).

3.5 Discussion

The development of functional root clusters usually takes place over a time period of 7-8 days (Neumann & Martinoia, 2002) and is characterised by distinct morphological and physiological changes (Fig. 1). Cluster root formation starts in the pre-emergent (PE) stage from the pericycle, 2-3 cm behind the tip of first-order laterals. Cluster root primordia are initiated in rows opposite the protoxylem poles (Neumann & Martinoia, 2002). Outgrowth of juvenile (JU) clusters occurs during 3-4 days and maturation proceeds until the lateral rootlets have reached their final length of 3-5 mm. This process is associated with meristem inactivation (Watt & Evans, 1999b). The mature (MA) root clusters are the site of morphological and physiological alterations to mediate nutrient (particularly P) mobilisation in the rhizosphere (Fig. 1, Neumann *et al.*, 2000). These modifications comprise development and proliferation of long, densely-spaced root hairs, secretion of P-mobilising root exudates, such as citrate, phenolics, phosphatases and protons, induction of high-affinity P_i uptake systems and an increased Fe reductive capacity (Neumann & Martinoia, 2002). At the same time, the internal P_i status of MA root clusters drastically declines by more than 50% as compared with JU clusters (Massonneau *et al.*, 2001), probably as a consequence of the well-documented P_i re-translocation from older tissues to young, actively-growing organs in P-deficient plants. This is associated with a massive decline in total *RNA* concentrations of MA clusters by approximately 90% (Massonneau *et al.*, 2001), suggesting that also *RNA*

degradation contributes to P recycling. Thus, the metabolism of MA clusters seems to be highly specialized to the functions related with P mobilisation, while other metabolic activities are largely down-regulated. Indeed, *RNA*-seq analysis confirmed this hypothesis, demonstrating a down-regulation of many transcripts involved in the primary metabolism, such as the biosynthesis of cell wall components (Fig. 3F), fatty acids (Fig. 3G), proteins (Fig. 3H), amino acids (Fig. 3I) and nucleotides (Fig. 3O) in MA clusters as compared with the JU stage. By contrast, metabolic pathways involved in P_i recycling and P_i-independent metabolic bypass reactions (Plaxton & Tran, 2011), including the biosynthesis of exudate compounds, such as organic acids (particularly citrate), phenolics, secretory acid phosphatase, were all up-regulated in MA clusters as compared with JU ones (Table 1, Figs. 2, 3). This also applied for transporters potentially involved in root exudation, P_i uptake and re-translocation. The activity of MA root clusters lasts for approximately 1-3 days, until the nutrients in the explored soil volume are extracted. Thereafter, the senescent CRs of soil-grown plants undergo complete decomposition within 2-3 weeks (Neumann & Martinoia, 2002).

3.5.1 Hormonal control of cluster root development

Cluster roots are specialised lateral roots and therefore, a similar hormonal regulation as in lateral root formation must be expected. Auxin plays a key a role in the regulation of lateral root formation, starting with priming of founder cells in the pericycle, followed by induction, development and outgrowth of lateral root primordia, which involves *PIN*-mediated transport, gradient formation and signalling of shoot- and root-borne auxin (Chang *et al.*, 2013). Accordingly, RT-qPCR analysis revealed the highest expression of various genes involved in auxin biosynthesis and transport (*YUCCA*, *AUX1*, *PIN1*) in the PE stage, followed by a continuous decline during outgrowth and CR maturation (Fig. 2A). This is in line with previous reports on the expression of auxin-related genes in CRs (Meng *et al.*, 2013). A key role of auxins was also suggested by stimulation of CR formation after external auxin application even in P-sufficient plants (Gilbert *et al.*, 2000; Neumann *et al.*, 2000; Skene & James, 2000; Hocking & Jeffery, 2004), while the application of auxin transport antagonists exerts inhibitory effects (Gilbert *et al.*, 2000).

An interesting new finding was the observation that similar to auxins, the expression of a transcript involved in conversion of the early brassinosteroid (BR) precursor 24-methylenecholesterol to campesterol in the BR biosynthesis pathway was high in PE and JU clusters, but declined dramatically during CR maturation. Lateral root formation can be promoted by BRs via the stimulation of acropetal auxin transport (Fukaki & Tasaka, 2009). Outgrowth of lateral root primordia seems to be particularly dependent on acropetal transport of shoot-borne auxin (Chang *et al.*, 2013) and accordingly, the highest expression of BR-related genes was found in actively-growing JU clusters (Table 1, Fig. 2B).

Low levels of ethylene promote lateral root initiation by stimulation of auxin biosynthesis, while high ethylene concentrations exert inhibitory effects on lateral root initiation (Fukaki & Tasaka, 2009) and also on root elongation (Torrey, 1976). Consequently, the expression of transcripts encoding 1-aminocyclopropane-1-carboxylate (ACC) oxidase, S-adenosylmethionine (SAM) synthase and formamidase, as enzymes mediating ethylene biosynthesis, was low in PE and JU clusters, but a massive up-regulation was indicated by *RNA*-seq and RT-qPCR data (Table 1, Fig. 2B) in MA clusters, which exhibit no more growth activity. Up-regulation of genes involved in ethylene biosynthesis in CRs of white lupin is highly consistent with the data set of O'Rourke *et al.* (2013), although that study did not differentiate between the different developmental stages of CRs. Increased ethylene production seems to be a general characteristic of P-deficient tissues (Lynch & Brown, 1997) and accordingly, Gilbert *et al.* (2000) reported increased production of ethylene also in root systems with CRs of P-deficient white lupin. This observation is in line with the lower P status of MA root clusters (Neumann *et al.*, 1999; Massonneau *et al.*, 2001) as massive P_i re-translocation occurs from MA to JU and PE stages, which have a higher P requirement. Although Gilbert *et al.* (2000) did not find effects of ethylene inhibitors on CR formation in *Lupinus albus*, in other plant species (*Casuarina glauca*), cluster root development was clearly stimulated by the external application of ethylene precursors (ACC) and inhibited by various ethylene antagonists (Zaid *et al.*, 2003). Moreover, ethylene has also been implicated in the promotion of root hair development (Jung & McCouch, 2013) and accordingly, formation of long and densely-spaced root hairs covering the lateral rootlets is

characteristic for CR maturation and is associated with increased ethylene production (Neumann & Martinoia, 2002).

Cytokinins exert inhibitory effects on lateral root formation by affecting *PIN*-mediated auxin transport (Fukaki & Tasaka, 2009). In accordance, Neumann *et al.* (2000) demonstrated the inhibition of CR formation and elongation of lateral rootlets by external application of the synthetic cytokinin kinetin. Moreover, cytokinin-induced inhibition of auxin transport seems to be also involved in the formation of the auxin gradient that is required for the induction of lateral root primordia (Jung & McCouch, 2013). This may explain the preferential expression of cytokinin receptor (*CRE*) genes particularly in the PE stage of CR development (Table 1, Fig. 2B). Neumann *et al.* (2000) also reported elevated cytokinin concentrations in the root tissue of P-deficient white lupin, compared with P-sufficient control plants. As cytokinins are preferentially produced in growing root tips (Aloni *et al.*, 2006), it was speculated that auxin-induced formation of JU cluster rootlets in P-deficient plants results in the increased production of cytokinins due to the large number of root tips (Neumann *et al.*, 2000). The enhanced accumulation of cytokinins in the JU clusters may in turn contribute to the inhibition of rootlet elongation during CR maturation. Although inhibition of lateral root formation by high cytokinin levels is mainly discussed in the context of primordia induction (Jung & McCouch, 2013), inhibitory effects on lateral root elongation are also documented, possibly mediated by stimulation of ethylene biosynthesis (Skoog & Miller, 1957; Cary *et al.*, 1995). The increased expression of transcripts encoding cytokinin oxidase (CKX) in MA root clusters (Table 1, Fig. 2B), similarly reported by Uhde-Stone *et al*. (2003), may indicate that cytokinin degradation occurs as a response to elevated cytokinin production by lateral rootlets in the JU stage. However, interactions of cytokinins and auxins are also required for development and maintenance of meristems (Su *et al.*, 2011). In contrast to the PE stage, high expression of transcripts related to cytokinin oxidase (CKX), mediating cytokinin degradation, and the lack of cytokinin receptor (*CRE*) transcripts (Fig. 2B) may reflect cytokinin deficiency in the root tips of MA root clusters, finally leading to meristem inactivation, repression of their further elongation and formation of the functional clusters (Watt & Evans, 1999b).

Apart from the hormones mentioned above, the transcriptome studies on CR development also demonstrated distinct changes in transcript levels related to other hormones, such as abscisic and jasmonic acids (up-regulated in MA clusters, Fig. 3N, Appendix Suppl. Fig. S5), suggesting a putative involvement in the regulatory network of CR development, which requires further investigations. The same holds true for nitric oxide (NO) related gene expression and the potential role of NO signalling in CR induction (Wang *et al.*, 2010; Meng *et al.*, 2012).

3.5.2 Metabolic changes during cluster root development

In accordance with the lower P status of MA clusters and the postulated re-translocation of P_i to actively-growing JU tissues, *RNA*-seq analysis revealed an up-regulation of metabolic pathways related to P_i recycling: this was indicated by up-regulation of transcripts involved in P_i transport, degradation of nucleotides and synthesis of sulfolipids (Table 1, Figs. 2C, 3O, G). The latter replace phospholipids in biomembranes under P-deficient conditions (Plaxton & Tran, 2011). The massive 90% decline in total *RNA* contents during CR maturation (Massonneau *et al.*, 2001) is consistent with the transcriptome data.

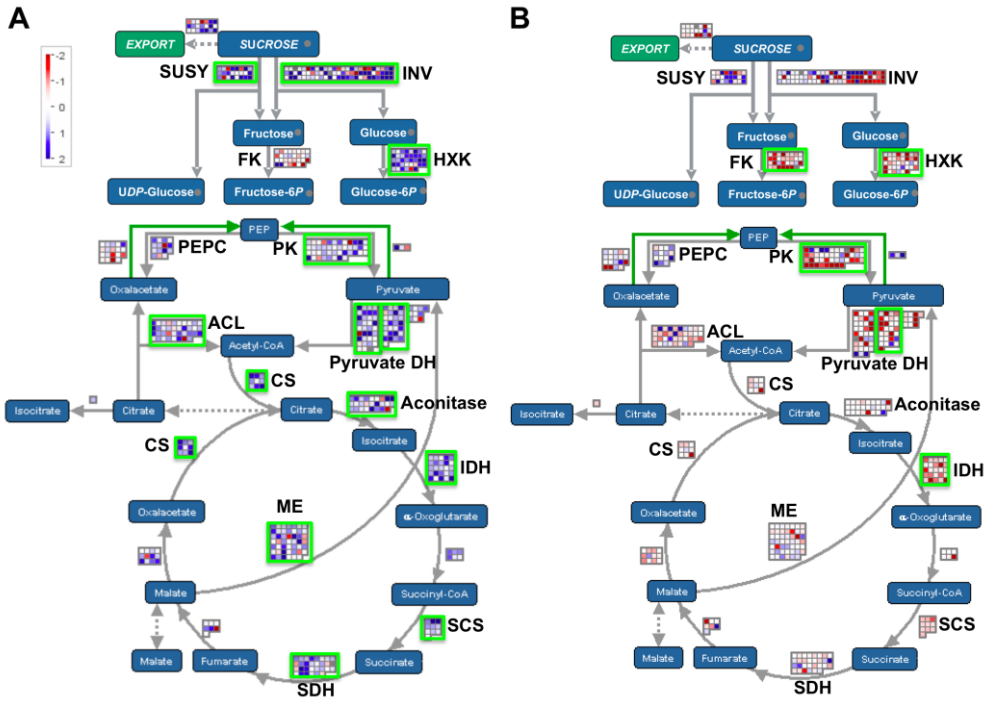

Fig. 4 Glycolysis and TCA cycle classified by the MapMan software. Differences in transcriptional expression levels between JU and PE clusters (A) and MA and JU clusters (B). In (A), blue represents higher gene expression in JU as compared with PE clusters

while red represents lower gene expression; in (B), blue represents higher gene expression in MA as compared with JU clusters, red represents lower gene expression. PE, pre-emergent clusters without root tips; JU, juvenile cluster root; MA, mature cluster root. Green squares indicate significant differences at $P < 0.05$ by Wilcoxon Rank Sum test. SUSY, sucrose synthase; INV, invertase; FK, fructokinase; HXK, hexokinase; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; ACL, ATP citrate lyase; Pyruvate DH, pyruvate dehydrogenase; CS, citrate synthase; IDH, isocitrate dehydrogenase; SCS, succinyl-CoA ligase; SDH, succinate dehydrogenase; ME, malic enzyme.

Furthermore, the down-regulation of highly P_i-dependent glycolytic pathways for carbohydrate catabolism (hexokinase, fructokinase, pyruvate kinase, Figs. 3A, B, 4B) and their replacement by more P_i-independent bypass reactions (SUSY, PEPC, Fig. 2C), as well as down-regulation of cytochrome-dependent respiration and the induction of AOX (Fig. 3E), typical for P-deficient tissues (Plaxton & Tran, 2011), were all confirmed by our transcriptome data and physiological measurements (Neumann *et al*., 2000; Massonneau *et al*., 2001; Kania *et al.*, 2003).

Metabolic pathways involved in biosynthesis of organic acids, such as the glyoxylate cycle (malate synthase, citrate synthase) and the PEP carboxylase-dependent carbohydrate catabolism were up-regulated in MA clusters (Figs. 3C, 2C), thereby mediating the biosynthesis of the carboxylates released as root exudates for P mobilisation (Neumann *et al.*, 2000). However, CR maturation is typically characterised by a shift from malate, as major carboxylate accumulating in the JU stage, to almost exclusive and extremely high citrate accumulation in MA clusters (Neumann *et al.*, 2000; Neumann & Martinoia, 2002). This is of particular interest, since in many soils citrate is much more efficient in P mobilisation as compared with malate (Neumann & Römheld, 2007). Based on physiological measurements revealing a down-regulation of citrate-degrading enzymes in the TCA cycle (Fig. 5, Neumann *et al.*, 2000) and reduced respiration (Neumann *et al.*, 1999) in MA root clusters, it was postulated that citrate accumulation is a consequence of both increased biosynthesis of organic acids and reduced activity of metabolic pathways involved in citrate consumption (Neumann & Martinoia, 2002).

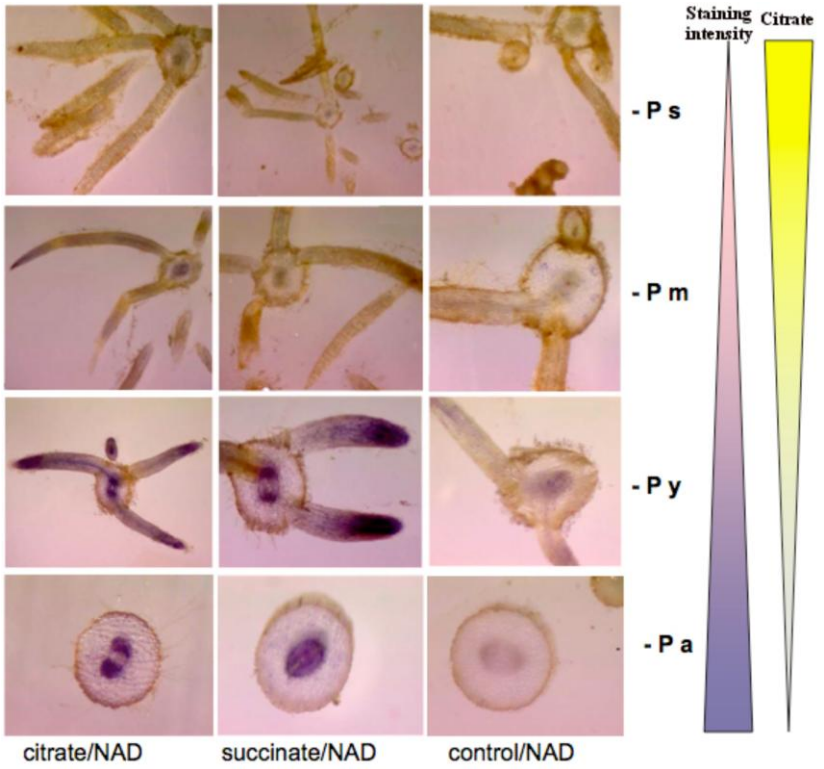

Fig. 5 Histological formazan staining of TCA cycle dehydrogenase activities (after Seligman & Rutenburg, 1951) in different root zones of P-deficient white lupin using citrate and succinate as substrates as related to citrate accumulation in the root tissue. –P a, 10 mm apical root zone of lateral root of P-deficient plants; -P y, juvenile cluster roots; -P m, mature cluster roots; -P s, senescent cluster roots; NAD, nicotinamide adenine dinucleotide (from Kania, 2005).

This hypothesis was nicely confirmed by the current *RNA*-seq data, demonstrating a significant down-regulation of transcripts for the TCA cycle enzyme isocitrate dehydrogenase and of transcripts related to cytochrome-dependent respiration (Figs. 4B, 3E). Furthermore, the increased accumulation and root exudation of phenolics (particularly isoflavonoids) during CR maturation, with putative functions in P_i mobilisation (Tomasi *et al*., 2008) and the protection of nutrient-mobilising root exudates against microbial degradation (Weisskopf *et al*. 2006), was reflected in the increased expression of transcripts related with the phenylpropanoid metabolism and biosynthesis of flavonoids (Table 1, Figs. 3M, 2D). In many plant species, root exudation of citrate and phenolics is mediated by MATE transporters (Neumann & Römheld, 2007). The relevant transcripts were found to be up-regulated during CR maturation (Table 1, Figs. 2D, 3Q, Massonneau *et al.*, 2001; Uhde-Stone *et al.*, 2003;

O'Rourke *et al.*, 2013). Accordingly, recent investigations by Gottardi *et al*. (2013) using an RNA interference (RNAi) gene-silencing approach, confirmed the role of a MATE transporter in isoflavonoid (genistein) exudation from CR of white lupin. However, for citrate exudation, inhibitor studies and patch clamp experiments (Neumann *et al.*, 1999; Zhang *et al.*, 2004) are more compatible with the involvement of an anion channel. The RT-qPCR data demonstrated the up-regulation of a candidate gene with homology to *ALMT* at the MA stage, which was also highly expressed in roots of P-sufficient plants (Fig. 2D). Therefore, the mechanism of citrate exudation in MA clusters and the putative involvement of *MATE* transporters and/or anion channels remain to be elucidated.

Interestingly, P deficiency induced changes during CR maturation, comprising proliferation of root hairs, increased exudation of phenolics, citrate and protons, and an increased Fe reductive capacity (Neumann & Martinoia, 2002), strongly resemble the Fe deficiency response of so-called Strategy I plants (Neumann & Römheld, 2007). In Fe-deficient dicots and non-graminaceous monocots, the coordinated Strategy I response is mediated via ethylene-induced activation of the *FIT* transcription factor (Lingam *et al.*, 2011). Surprisingly, *RNA*-seq and RT-qPCR analysis identified that MA root clusters of P-deficient white lupin show increased expression of genes involved in ethylene biosynthesis (Table 1, Fig. 2B) and increased expression of the *FIT* transcription factor (Table 1, Fig. 2D) even with sufficient Fe supply. The up-regulation of *FIT* transcripts in CRs has been reported also by O'Rourke *et al.* (2013). Moreover, an increased expression of transcripts encoding ferric reductase (FRO2) and the Fe^{2+} transporter (IRT1) as integral parts of the *FIT*-mediated Strategy I Fe deficiency response was characteristic for CR maturation under P-deficient conditions (Table 1, Fig. 2D). An artificial, latent Fe deficiency due to insufficient Fe supply via the nutrient solution could be excluded by plant analysis which revealed shoot Fe concentrations of 80-100 mg kg^{-1} dry matter, considered as optimal for lupin culture (Brennan *et al*., 2008). This raises the question whether the relation of CR activity under P limitation and the Strategy I Fe deficiency response share at least partially the same signalling pathways and whether this regulation pattern is specific for CR maturation or may also control similar P deficiency induced alterations in rhizosphere chemistry observed in other plant species (Neumann & Römheld, 2007). The up-regulation of a transcript

encoding ferric reductase (FRO2) in MA clusters is in line with the observation of increased Fe reductive capacity at the CR surface (Neumann & Martinoia, 2002). *Lupinus albus*, as many other lupin species, is naturally adapted to moderately acidic soils (Gladstones, 1970) where Fe deficiency is usually not a severe problem. Although intense expression of Fe deficiency responses, such as release of Fe-mobilising root exudates (citrate, phenolics) may contribute to some extent also to P acquisition from iron phosphates (Tomasi *et al.*, 2008), an up-regulation of adaptations for Fe acquisition under these conditions would bear the risk of excessive Fe uptake and Fe toxicity. Post-translational down-regulation of Fe uptake could represent a possibility to overcome this problem. Splitting of soluble Fe(III) complexes with citrate and phenolics via increased expression of ferric reductase (*FRO2*) observed at the root surface of MA clusters may be a strategy to liberate these exudate compounds to make them available again for P mobilisation. Accordingly, Dinkelaker *et al.* (1989) reported intense accumulation of Fe in the rhizosphere of CRs, while P was extracted and depleted. Furthermore, Manganese (Mn) uptake across roots partially captures the pathway for Fe(II) and a shoot accumulation of Mn correlates well with CR formation (Neumann & Martinoia, 2002).

Another metabolite involved in the regulation of Strategy I Fe deficiency responses is NO, which promotes *FIT* expression via the stimulation of ethylene biosynthesis (García *et al.*, 2011). Interestingly, intense NO production has been also reported in MA root clusters of white lupin (Wang *et al.*, 2010). This NO is synthesised most probably via xanthine dehydrogenase, which was up-regulated at the transcript level in MA root clusters (Table 1, Fig. 2B) (Wang *et al.*, 2010). Xanthine dehydrogenase is involved in nucleotide degradation, which is triggered by the low P_i status in MA clusters (Neumann, 2010). Thus, it may be speculated that increased NO production is a direct consequence of nucleotide degradation for P_i remobilisation during CR maturation, which then in turn induces ethylene production (García *et al.*, 2011). By triggering the observed Strategy I responses via the *FIT* transcription factor, as well as inhibition of lateral rootlet elongation and induction of root hair formation, ethylene controls metabolic and morphological features of the MA clusters at the same time. Moreover, inhibitory effects of NO, on the activity of aconitase and on mitochondrial respiration, as well as stimulation of the alternative oxidase pathway

have been documented (Ederli *et al.*, 2006; Hayat *et al.*, 2009). All these metabolic events are characteristic for MA root clusters (Fig. 3E) (Neumann & Martinoia, 2002) and have been implicated in the intracellular accumulation of citrate prior to the pulse of exudation (Neumann, 2010). Accordingly, application of the NO donor sodium nitroprusside to juvenile and mature root clusters stimulated citrate efflux, whereas the NO scavenger (c-PTIO) had inhibitory effects (Wang *et al*., 2010). Moreover, NO seems to be also required for the induction of lateral root primordia in the pericycle, by modulating the expression of regulatory genes of the cell cycle (Correa-Aragunde *et al.*, 2006). Accordingly, NO accumulation was also detected in PE root clusters (Wang *et al*., 2010) and discussed as a common signal mediating P deficiency and Fe deficiency responses in white lupin probably acting via the scarecrow (*SCR*) transcription factor (Meng *et al.*, 2012), which was predominantly expressed in PE clusters (Fig. 2C).

The presented transcriptome sequencing analysis of different stages in CR development of *Lupinus albus* confirmed many earlier physiological data on metabolic changes associated with CR function and some primary investigations on gene expression during CR formation. Moreover, it provided fascinating novel insights in the complex regulatory transcriptional network that determines the initiation, maturation and function of cluster roots. The resulting network was summarised in a hypothetic model that is presented in Fig. 6.

Experiments with foliar P application and split-root studies have demonstrated that shoot signals are also important factors triggering the formation of CRs (Marschner *et al.*, 1987; Shane *et al.*, 2003; Shen *et al.*, 2005). Auxin, and more recently, also increased shoot to root translocation of sucrose under P-deficient conditions, have been identified involving in cluster root formation (Liu *et al.*, 2005; Zhou *et al.*, 2008). The declining P-nutritional status triggers P_i re-translocation and re-mobilisation from older tissues (older leaves, basal root zones and MA root clusters) to young actively-growing organs (young leaves, root tips, PE and JU clusters). Within the P_i remobilisation process, *RNA* degradation is associated with increased NO production (via xanthine dehydrogenase). Nitric oxide stimulates the biosynthesis of ethylene, triggering the proliferation of root hairs and inhibiting lateral rootlet elongation. If ethylene, as an easily diffusible gaseous signal molecule,

also inhibits the elongation of first-order laterals, this explains the dense spacing of second-order laterals forming the root clusters.

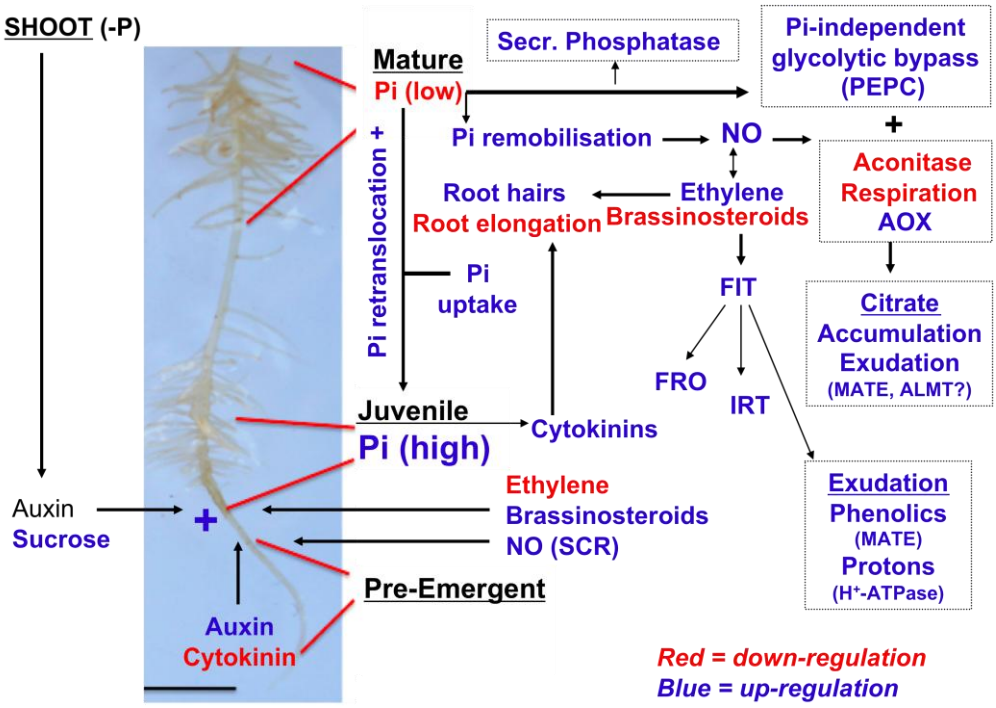

Fig. 6 Schematic representation of the regulatory network involved in cluster root formation and function including phytohormones and other factors (see main text for detailed description). Blue color indicates up-regulation, while red color indicates down-regulation. Scale bar: 1 cm.

Increased ethylene production also triggers increased ferric reductase activity at the root surface, the exudation of protons and phenolics in a coordinated response, which is mediated by the *FIT* transcription factor. The low P status in MA clusters induces the increased expression of high-affinity P_i uptake systems, secretory acid phosphatases and the induction of P_i-independent metabolic bypass reactions, promoting the biosynthesis of organic acids. The increased levels of NO also inhibit TCA cycle enzymes (aconitase) and respiration, leading to a reduced turnover of citrate and to citrate accumulation in the CR tissue. After reaching a threshold concentration of approximately 30 µmol g^{-1} root fresh weight (Neumann *et al.*, 2000; Aloni *et al.*, 2006), citrate is released via MATE transporters and/or the identified anion channel candidates.

In PE and JU clusters, an improved P-nutritional status due to P_i re-translocation enables the formation and outgrowth of new lateral rootlets. These are regulated by

synergistic interactions of auxin, cytokinins, brassinosteroids, NO and low levels of ethylene. Numerous root tips in JU clusters contribute to the increased biosynthesis and basipetal transport of cytokinins, which exerts inhibitory effects on lateral rootlet elongation during CR maturation, possibly also by increasing ethylene biosynthesis.

3.6 Outlook

The proposed scheme, based on gene expression and physiological data, may be used as a starting point for more detailed investigations of the signalling pathways and the regulatory network determining CR function and development, as a model system to understand the mechanisms of efficient nutrient acquisition in the rhizosphere.

One of the most interesting aspects of CR formation is the question whether development of CR requires unique regulators, exclusively present only in cluster-rooted plant species. The fact that root clustering can also be observed in many other plant species under specific growth conditions, such as localized nutrient supply (Jing *et al.*, 2010), exogenous hormone applications (Hinchee & Rost, 1992; Kaska *et al.*, 1999), inoculation with phytohormone-producing soil microorganisms (Kaska *et al.*, 1999; Sukumar *et al.*, 2013), points to the possibility that differences in the quantitative expression of general regulatory factors for root development may be the major determinants of CR morphology and CR function. This may indicate that by a favourable combination of common gene expression and hormonal signalling, many crop roots may form the highly complex CRs. This hypothesis is consistent with the current study, where the identified network of developmental, metabolic and hormonal signals links common plant strategies for root morphological and functional responses with highly specific cluster root development and function in P-deficient white lupin.

3.7 Appendix

3.7.1 Authors' contributions

ZW carried out data analysis and most of the lab work, DS and HY helped with the transcriptome sequencing data analysis and RT-qPCR validation, AK performed the

histological formazan staining of TCA cycle dehydrogenase activities, ZW, GN and UL designed the study, ZW wrote the manuscript with final corrections provided by GN, UL and JS. All authors read and approved the final manuscript.

3.7.2 Acknowledgements

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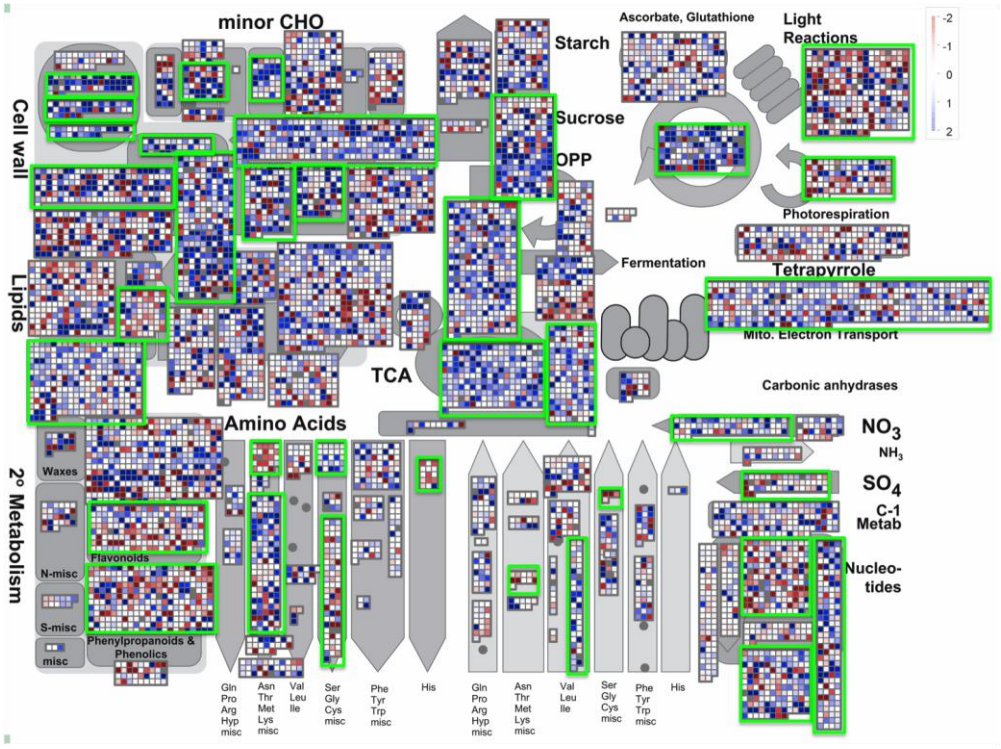
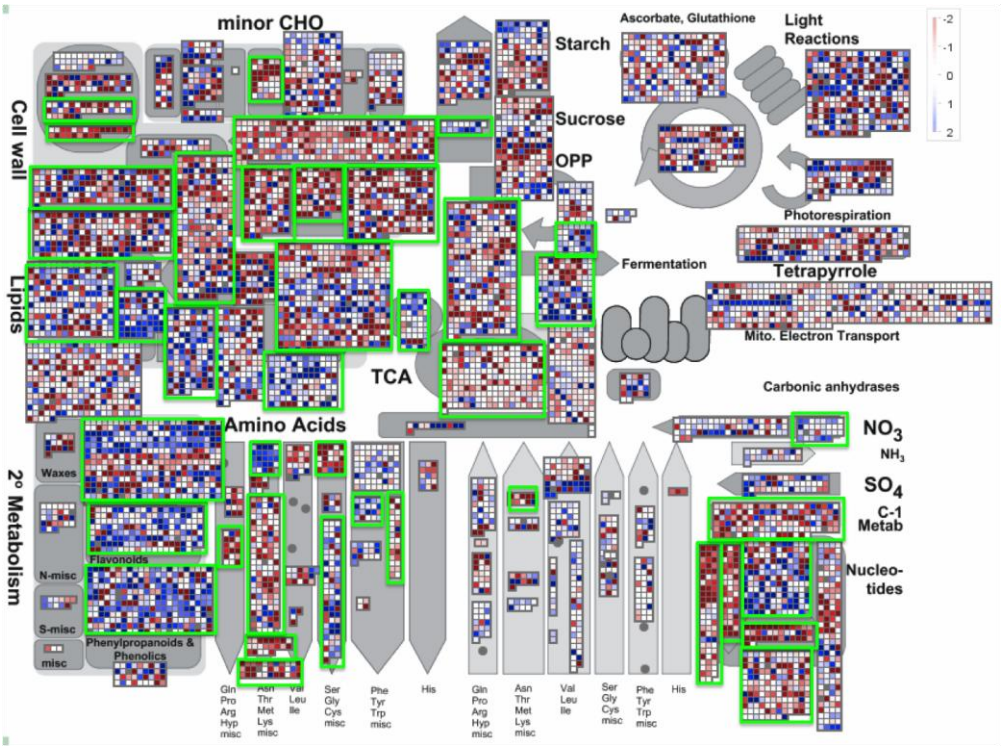
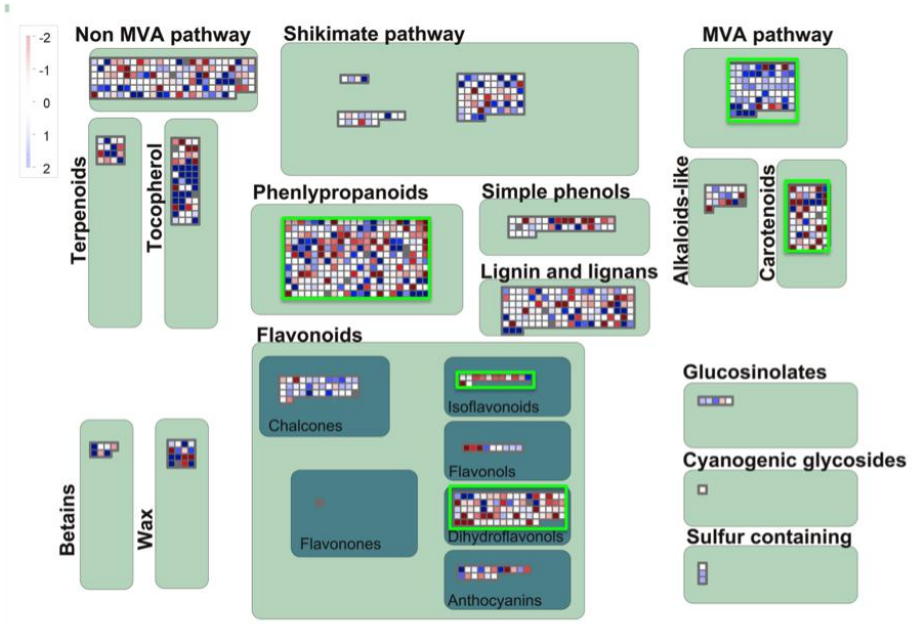
3.7.3 Supplemental materials



Suppl. Fig. S1 Transcriptome overview of juvenile clusters (JU) as compared with pre-emergent ones without root tip (PE). Higher expression in JU as compared with PE was indicated by blue color and lower expression was in red color. Green squares indicate significant regulation at $P < 0.05$ by the Wilcoxon Rank Sum test, template from MapMan.

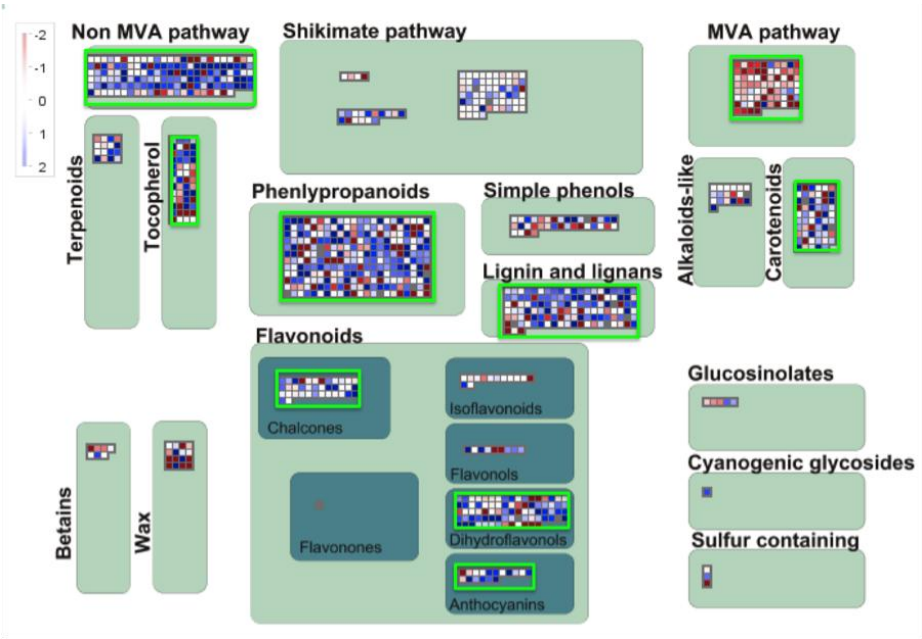


Suppl. Fig. S2 Transcriptome overview of mature clusters (MA) as compared with juvenile ones (JU). Higher expression in MA as compared with JU was indicated by blue color and lower expression was in red color. Green squares indicate significant regulation at $P < 0.05$ by the Wilcoxon Rank Sum test, template from MapMan.

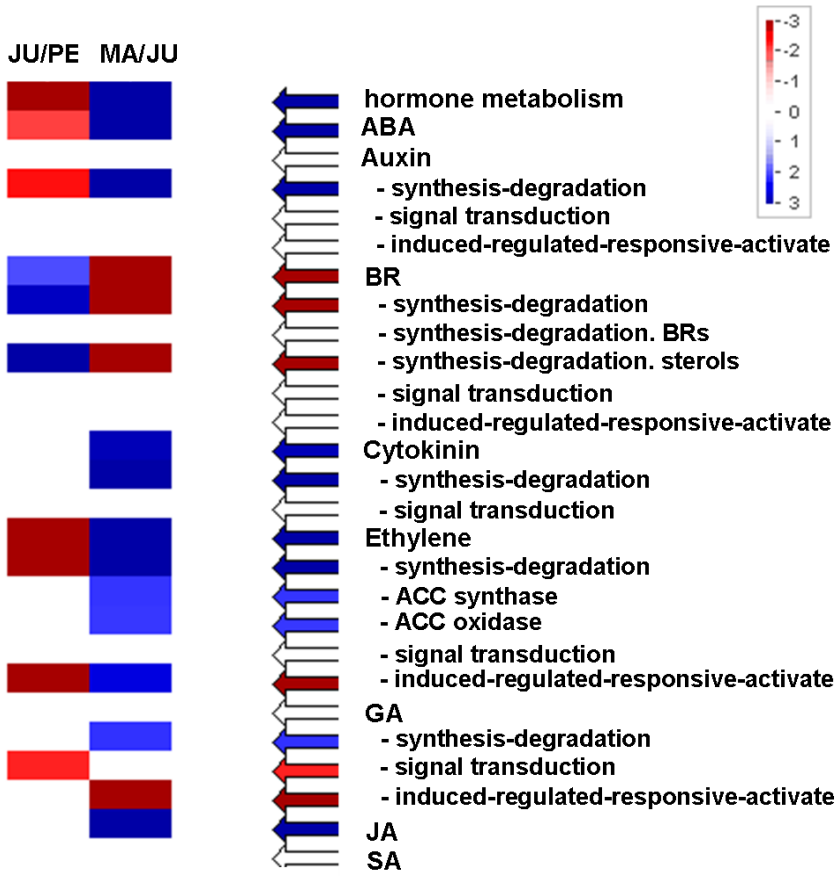


Suppl. Fig. S3 Secondary metabolism of juvenile clusters (JU) as compared with pre-emergent ones without root tip (PE). Higher expression in JU as compared with PE was indicated by blue color and lower expression was in red color. Green squares indicate

significant regulation at P < 0.05 by the Wilcoxon Rank Sum test, template from MapMan.



Suppl. Fig. S4 Secondary metabolism of mature clusters (MA) as compared with juvenile ones (JU). Higher expression in MA as compared with JU was indicated by blue color and lower expression was in red color. Green squares indicate significant regulation at P < 0.05 by the Wilcoxon Rank Sum test, template from MapMan.



Suppl. Fig. S5 Differential expression profiles of categories related to hormones. The comparisons are juvenile clusters (JU) as compared with pre-emergent without root tip (PE), mature clusters (MA) as compared with juvenile clusters (JU). Blue color indicates significant up-regulation of JU as compared with PE, MA as compared with JU, respectively, while red indicates significant down-regulation. White color shows no significant difference (Z-score < 1.96). The coloring is according to the Z-scores of Wilcoxon test. Z-score of ± 1.96 represents a P-value of 0.05. The plot was generated using PageMan. ABA, abscisic acid; BR, brassinosteroid; GA, gibberellic acid; JA, jasmonic acid; SA, salicylic acid.

Suppl. Table 1 Primer pairs used for Quantitative real-time PCR.

Annotation	Sequence	
	Forward	Reverse
PP2AA3	TGCATGCATAGAGCACCAAG	CATTGTTGAGCTTGCTGAGG
UBC	TCGGCGGATCCTGATATTAC	CTGGAACAGAAAAGGCAAGC
IAA4	CCACAATTGCAGCACATCTC	GTTCTTCAACGGTTGTGTGG
IAA5	GATGATAAGCCAGCGGAAAC	ACCACCATGCCATAAGAAGC
IAA8	CCTGGCTTTCCATCAACTTC	TGTGCAGCTAATGGTGCTTC
YUCCA	TAGCTTCAATGTGGCAGCAC	ATGGCATAAGAGGGAGTTGG
AUX1	ATGCTGAGGGCAATGTTAGG	CACTTTTGGTGGACATGCTG
PIN1	CAAAGTGTTTGGGAGGGTTG	GGAGCAACTTGAGCAAAAGG
ACC_oxidase	TTGGGTAGGCTTGGTTTGTC	CTACAACCCAGCCAATGATG
BR	AATGGCGAGCACGCTTATAG	TCCCTCAAAAGATGGTCTCG
CKX	GGCACAACCTTCAGATTTGG	CCCAAATTGGCCTAATCCTC
CRE	ACTTCACATCGGCTCCAAAC	AGGGAAAGACATGCCTGATG
XDH	CTTTCAAGGCGAAGGATCTG	TCACGTGCCTTCACAAAGTC
PT	AGCATACTCGGACATGATGG	TTGCCTCTGGCCTATCTTTC
PEPC	TAGAGATGCACAGCACACGAC	AGAATCACGCAATCGGAGTC
SUSY	ATCATCTCCTGTGCCCTTTG	ATTAAAGGTGGCAGCACTGG
MDH	TCCAGTTGCTGAGGTCAATG	TTCTGTGCCTCCATCTTGTG
SCR	CAACCAATGGACAGCAACAG	TGCCTCATAAGGTTGGGAAC
FIT	TGTCACCTTCACACTGCAAG	TATCACCCAAGCCCCATTAG
FRO	TGCTTTGGGTTGCCTCTATC	ATGCCAAGAGGGCCTTTTAC
IRT	CGGCGCACCTAAAAGTAATC	CGAAGCACTTGATCACAACG
MATE	CTTCTGCATCTTGCACCATC	ATGCACAGTGCCATTCTCTG
ALMT	GATGGAGTTGGTGGAAATGC	TAGAAACCCTGCAAGGGAAG
PDR9	CTCCCTCTTGGATCTTTTGC	GTGGGGTTTTTGGCTTTCTC
F6'H1	TCACAGGCACACCATGATTC	TCAATGGACCTGAGCATGAG

4 Chapter II - A re-assessment of sucrose signalling involved in cluster root formation and function in phosphate-deficient white lupin (*Lupinus albus* L.)

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4.1 Abstract

Apart from substrate functions, a signalling role of sucrose in root growth regulation is well established. This raised the question whether sucrose signals might also be involved in formation of cluster roots (CRs) under phosphate (P_i) limitation, mediating exudation of phosphorus (P)-mobilizing root exudates, e.g., in *Lupinus albus* and members of the Proteaceae. Earlier studies demonstrated that CR formation in *Lupinus albus* was mimicked to some extent by external application of high sucrose concentrations (25 mM) in the presence of extremely high P supply (1-10 mM), usually suppressing CR formation. In this study, we re-addressed this question using an axenic hydroponic culture system with normal P supply (0.1 mM) and a range of sucrose applications (0.25-25 mM). The 2.5 mM sucrose concentration was comparable with internal sucrose levels in the zone of CR initiation in first-order laterals of P-deficient plants (3.4 mM) and induced the same CR morphology. Similar to earlier studies, high sucrose concentrations (25 mM) resulted in root thickening and inhibition of root elongation, associated with a ten-fold increase of the internal sucrose level. The sucrose analogue palatinose, and a combination of glucose/fructose failed to stimulate CR formation under P-sufficient conditions, demonstrating a signal function of sucrose and excluding osmotic or carbon source effects. In contrast to earlier findings, sucrose was able to induce CR formation, but had no effect on CR functioning with respect to citrate exudation, *in-vitro* activity and expression of genes encoding phosphoenolpyruvate carboxylase,

secretory acid phosphatase and MATE transporters, mediating P-mobilizing functions of CRs.

4.2 Introduction

Apart from mycorrhizal associations and biological nitrogen fixation, formation of cluster roots (CRs) is regarded as one of the major adaptations for mineral nutrient acquisition in higher plants (Skene, 1998). Cluster roots are bottlebrush-like clusters of lateral rootlets with limited growth, densely covered with root hairs and formed in response to phosphate (P_i) or iron (Fe) limitation (Skene, 1998; Hagström *et al*., 2001; Neumann & Martinoia, 2002). The rootlets with a length of 5-10 mm emerge from the pericycle opposite the protoxylem poles along the first-order lateral roots in members of the Proteaceae, Casuarinaceae and some other plant species adapted to soils with low P_i availability (Purnell, 1960; Dinkelaker *et al*., 1989; Skene, 1998; Lambers *et al*., 2012). Due to the largely increased surface area, these specialized root structures are able to mobilise sparingly soluble soil P, but also other nutrients, such as Fe, Mn, Zn and Mo, via the concentrated release of organic chelators (citrate, malate, phenolics), ectoenzymes (acid phosphatases), protons and reductive changes in the rhizosphere (Gardner *et al*., 1983; Dinkelaker *et al.*, 1997; Gilbert *et al.*, 1999).

Among crops, white lupin (*Lupinus albus*) is one of the few plants that can form CR under P and Fe deficiency (Hagström *et al*., 2001). In the recent past, this plant species was used as an illuminating model to investigate the plant adaptations for chemical mobilisation of sparingly available soil P sources (Skene, 2000; Neumann & Martinoia, 2002). However, only limited knowledge exists concerning the regulatory processes determining the distinct patterns of CR formation. Foliar P application to P-deficient plants can suppress formation of CR (Marschner *et al.*, 1987; Shane *et al.*, 2003), suggesting a regulatory function of the internal P status (Dinkelaker *et al.*, 1995). However, cluster root formation seems to be stimulated also in nutrient-rich patches (Purnell, 1960; Skene, 1998), in soil layers rich in organic matter (Li *et al.*, 2010) and similarly in split-root compartments with local high P supply (Shen *et al*., 2005), indicating an additional involvement of external signals (Neumann & Martinoia, 2002). Expression studies of related genes and external application of hormones and hormone antagonists suggest an interplay of hormonal factors, such as auxin, cytokinin, ethylene, nitric oxide (NO), giberillic

acid and brassinosteroids (BRs) (Gilbert *et al.*, 2000; Neumann *et al.*, 2000; Wang *et al.*, 2010; Meng *et al.*, 2012, 2013; O'Rourke *et al.*, 2013; Chapter I) on CR formation, exploiting the modules of normal lateral root development (Fukaki & Tasaka, 2009).

Of course, cluster root development and function are both strongly dependent also on shoot carbon supply, as demonstrated by shading and stem girdling experiments (Liu *et al.*, 2005). Accordingly, Dinkelaker *et al*. (1989) have shown that a carbon fraction comprising about 23% of the total plant dry weight in P-deficient white lupin can be released as citrate to mediate P mobilisation in the rhizosphere of mature root clusters. Only 30% out of this carbon fraction were assimilated directly in the root tissue by anaplerotic CO_2 fixation via phosphoenolpyruvate (PEP)-carboxylase (Johnson *et al.*, 1996a). However, apart from the function as carbon source, the translocated sucrose to the roots can also act as signal molecule for plant growth and development (Chiou & Bush, 1998) and seems to be also involved in root growth responses to P limitation (Jain *et al.*, 2007; Karthikeyan *et al.*, 2007; Hammond & White, 2008, 2011). Therefore, a potential role of sucrose signalling in the formation and function of CR has been discussed by Liu *et al*. (2005) and Zhou *et al*. (2008), suggesting a function in the expression of P starvation-induced genes, such as high-affinity P transporters (*LaPT1*), PEP-carboxylase (*LaPEPC3*) and secretory acid phosphatase (*LaSAP*), as well as in cluster root development. Unfortunately, these studies on the sucrose effects have been conducted either with very young lupin seedlings that lack cluster roots (Liu *et al.*, 2005) with extremely high P concentration (1-10 mM) or under very artificial culture conditions, where lupin plants were grown over four weeks in sterile agarose media (Zhou *et al.*, 2008). However, root development in agar over extended time periods is frequently biased by the depletion of nutrients and particularly by the slow diffusion of oxygen (Barrett-Lennard & Dracup, 1988), which is known to induce ethylene accumulation. Ethylene is an important hormonal factor involved in lateral root formation with concentration-dependent stimulatory or inhibitory effects (Fukaki & Tasaka, 2009). Moreover, experiments addressing sucrose effects on cluster root function were so far considering only the transcriptional level, but did not investigate the related root physiological responses.

In order to minimize the risk of artifacts arising from the culture conditions, the present study revisited the potential role of sucrose signalling in CR function and development, using a sterile hydroponic culture system with aerated nutrient solution, which has been similarly employed in many earlier studies on cluster root physiology of *Lupinus albus* (Keerthisinghe *et al.*, 1998; Neumann *et al.*, 1999; Massonneau *et al.*, 2001; Kania *et al.*, 2003). To avoid rapid microbial contamination induced by sucrose amendments to the growth media, plant culture was conducted under axenic conditions (Fig. 1) according to von Wirén *et al*. (1995).

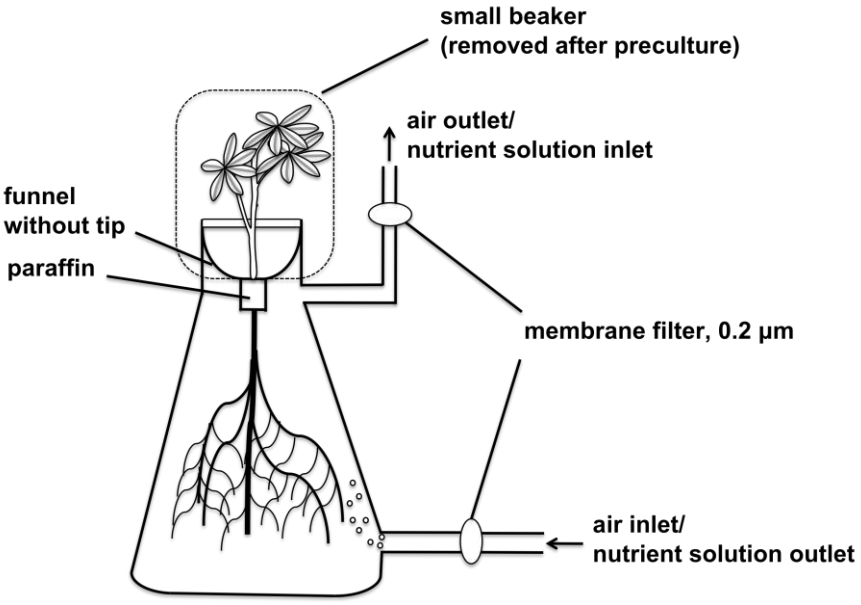

Fig. 1 Hydroponic system for plant culture under axenic conditions (modified after von Wirén *et al.*, 1995; for description of the details see the main text).

4.3 Materials and methods

4.3.1 Plant culture

Seeds of white lupin (*Lupinus albus* L. cv Feodora) were surface sterilized using 95% (v/v) ethanol for 10 min, washed 3 times with sterilized distilled water, followed by incubation in 10% (w/w) NaClO for 7 min and 6 times washing with sterile distilled water. Seeds were germinated on 1% (w/v) agar plates for four days in darkness, and then transferred to bottles containing 600 ml sterilized nutrient solution with 2 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe(III)-EDTA, with (+P) or without (-P) 100 µM KH_2PO_4 at

pH 5.6-5.8 and with (+Suc) or without (-Suc) sucrose (0.25-25 mM). In some experiments, palatinose (25 mM) or glucose (12.5 mM) plus fructose (12.5 mM) were added instead of sucrose.

For sterile hydroponic culture, all bottles, nutrient solutions and other instruments were autoclaved before use. Sucrose, glucose, fructose and palatinose were added by sterile filtration using 0.2 μm membrane filters (FP 30/0.2 CA-S, Whatman GmbH, Dassel, Germany). Also inlet and outlet tubes for aeration were equipped with 0.2 μm sterile membrane filters. During pre-culture stage (10 days after sowing, DAS), plant shoots were covered with small beakers (Fig. 1). Thereafter, the beakers were removed and bottles were sealed with sterilized paraffin at the entry point of the shoots. Plants were kept in a growth chamber with a light period of 16 h, and 24/18 ℃ day/night temperature, light intensity of 200 μmol m^{-2} s^{-1}, and relative humidity of 60%. Replacement of sterile nutrient solution was carried out every 5-7 days in a clean bench using the inlet and outlet aeration tubes after removal of the sterile filters.

Sterility of the growth media was checked by plating tests on 1% (w/v) agar media containing 100 μM KH_2PO_4 and 25 mM sucrose. Aliquots of the nutrient solutions (15 ml) were frozen at -20 ℃ for further analysis of exudate compound (citrate) and the pH was determined.

4.3.2 Root analysis

At final harvest, roots were scanned using Epson STD 4800 scanner and analysis was conducted using the WinRHIZO program (Regent Instruments Inc., Quebec, Canada).

4.3.3 Analysis of citrate in root exudates

Citrate is the major organic acid in root exudates of P-deficient *Lupinus albus* (Neumann *et al.*, 1999). Sub-samples of the root exudates were used for measuring the citrate concentration using a commercial enzymatic assay (R-Biopharm AG, Darmstadt, Germany) as described by Dinkelaker *et al.* (1989) via citrate lyase-mediated conversion of citrate to oxaloacetate. In presence of the enzymes L-malate dehydrogenase (L-MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and

L-lactate by oxidation of the reduced nicotinamide-adenine dinucleotide (NADH). The amount of NADH oxidized in these reactions is stoichiometric to the amount of citrate. NADH oxidation is monitored spectrophotometrically at 340 nm.

4.3.4 Enzyme activities

4.3.4.1 PEP-carboxylase

Determination of *in-vitro* activities of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was carried out according to Neumann & Römheld (1999). Frozen samples of the root tissue were homogenized in 1 ml cold Tris-HCl buffer (25 mM, pH 8.0, with 0.5 M sucrose and 2 mM DTT) per 200 mg root fresh weight, together with a small proportion of insoluble polyvinylpolypyrolidone (PVPP) for adsorption of phenolics. Insoluble material was removed by centrifugation (4 °C, 10 min at 20 000 g). Activity of PEPC in the supernatant was determined spectrophotometrically, monitoring the oxidation of NADH at 340 nm for 3 min in a malate dehydrogenase (EC 1.1.1.37) coupled reaction. Lactate dehydrogenase (EC 1.1.1.27) was included into the assay medium to account for decarboxylation of oxaloacetate during the test.

4.3.4.2 Acid phosphatase

For determination of secretory acid phosphatase activity, 2 cm excised apical root segments without root tips obtained from first-order laterals (for +P -Suc) and of mature CR (for -P -Suc, -P +Suc and +P +Suc treatments) were washed twice with distilled water for 5 min to remove contents of wounded cells. Thereafter, 0.5 ml distilled water + 0.4 ml Na-acetate buffer (0.2 M, pH 5.2) + 0.1 ml p-nitrophenyl phosphate (NPP, 0.15 M) were added. After 1 min incubation at 25-30 °C, 0.8 ml of the reaction media were removed and mixed with 0.4 ml of 0.5 M NaOH to terminate the reaction. The absorption of the dephosphorylation product p-nitrophenol (PNP) was determined spectrophotometrically at 405 nm. Thereafter, the root segments were used for root dry weight (DW) determination.

4.3.5 Determination of reducing sugars

Determination of reducing sugars and sucrose was carried out with minor modifications according to Blakeney & Mutton (1980). Shortly, frozen root tissues mixed with approximately 10 mg of activated charcoal were homogenized in 1 ml 70% (v/v) ethanol per 50 mg root fresh weight using mortar and pestle. After

homogenization, insoluble material was removed by centrifugation (5 min at 16 000 g). The supernatant was divided into two sub-samples: one aliquot (25 µl) with addition of 25 µl invertase solution [10 mg Yeast Invertase (Grade VII, Sigma I-4504) in 50 ml H_2O and 50 ml 0.2 M Na-acetate buffer pH 4.8], 100 µl distilled water and 100 µl Na-acetate buffer (0.2 M, pH 4.8), while the other aliquot (25 µl) was mixed with 100 µl distilled water and 100 µl Na-acetate buffer (0.2 M, pH 4.8) only. After 2 h incubation at room temperature, 1.25 ml color reagent (hydroxybenzoic acid-hydrazide 5g L^{-1} in a solution of 14.7 g trisodiumcitrate $\cdot 2H_2O$, 1.47 g $CaCl_2 \cdot 2H_2O$ and 20 g NaOH per liter) was added to both sample aliquots and boiled for 4 min. Just prior to the boiling step, the second aliquot also received 25 µl invertase solution. After cooling, absorption was measured spectophotometrically at 430 nm and quantification was performed by comparison with glucose standards. The sucrose content was calculated from the difference between the sub-samples with and without sucrose hydrolysis by the invertase treatment.

4.3.6 Quantitative real-time PCR

Total *RNA* was isolated with the innuPREP Plant RNA kit (Analytik Jena AG, Germany). Contaminating genomic *DNA* was removed with a RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. The *cDNA* was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Gene-specific primers were designed using Primer3Plus. Quantitative real-time PCR was performed as previously described (Straub *et al.*, 2013b). The primer pairs used are: *LaUbiquitin*-F 5'-TTGCGGACTACAACATCCAG-3' and *LaUbiquitin*-R 5'-GGC CTTCACGTTATCAATGG-3'; *LaMATE*-F 5'-CTTCTGCATCTTGCACCATC-3' and *LaMATE*-R 5'-ATGCACAGTGCCATTCTCTG-3'; *LaSAP*-F 5'-TGGCCT TACCAAAAGCTGAG-3' and *LaSAP*-R 5'-GCCTTTTTCCACTCGTTACC-3'; *LaPEPC*-F 5'-TAGAGATGCACAGCACACGAC-3' and *LaPEPC*-R 5'-AGAATC ACGCAATCGGAGTC-3'.

4.3.7 Statistics

Data are shown as means ± standard error (SE). One-way ANOVA with the least significant difference (LSD) test was used to test the differences. The software used was SAS for Windows Version 9.4 (SAS Institute Inc, Cary, NC, USA).

4.4 Results

4.4.1 Sucrose effects on cluster root formation

To examine the effect of sucrose application on CR formation, white lupin seedlings were gown for 23 days after sowing (DAS) in sterile hydroponic culture with or without amendments of 25 mM sucrose (Suc) and phosphate (P_i). Only a small number of CRs appeared in the +P and -Suc control treatment (10 clusters per plant), while CR formation was significantly increased in the treatments without P supply. However, a similar increase of CRs (up to 40 clusters per plant) was induced also in P-sufficient plants amended with 25 mM sucrose in the growth medium (Figs. 2, 3A).

Fig. 2 Root morphology of white lupin as affected by phosphate and sucrose application. Plants were grown in sterile hydroponic culture with or without 100 μM KH_2PO_4 (P) and 25 mM Sucrose (Suc) for 23 days after sowing (DAS). From left to right: +P –Suc, -P -Suc, -P +Suc, +P +Suc. Scale bar: 5 cm.

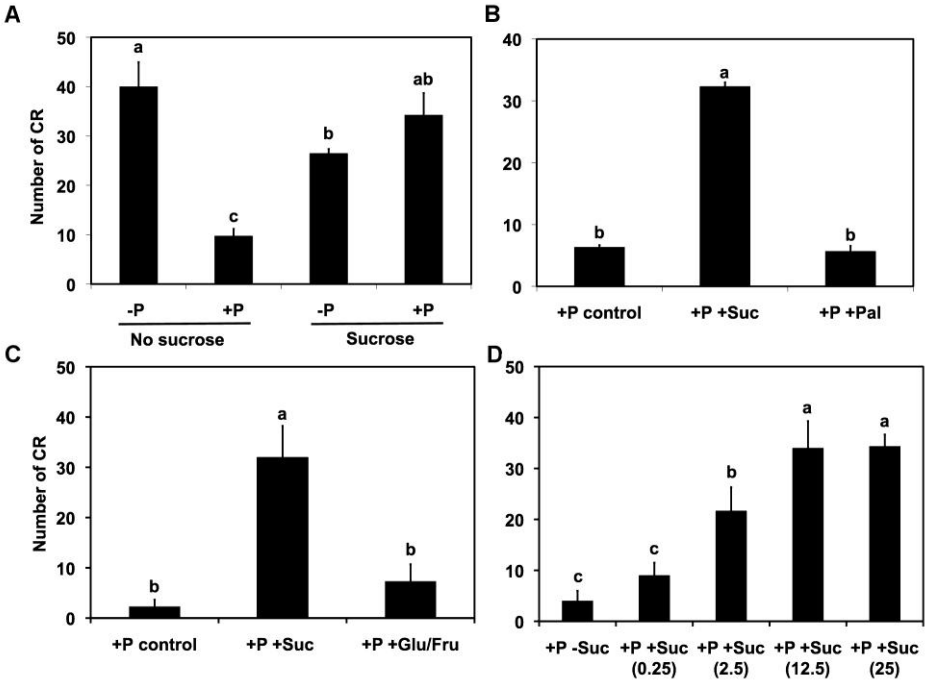

Fig. 3 Effect of sucrose (Suc), palatinose (Pal) and glucose plus fructose (Glu/Fru) application on cluster root (CR) formation of white lupin. Plants were grown in sterile hydroponic culture containing: (A) 0 (-P) or 100 μM (+P) KH_2PO_4, supplemented with or without 25 mM sucrose (Suc) for 23 days after sowing (DAS); (B) 25 mM sucrose (Suc) and 25 mM palatinose (Pal) for 26 DAS; (C) 25 mM sucrose (Suc) and 12.5 mM glucose plus 12.5 mM fructose (Glu/Fru) for 22 DAS; (D) Different levels of sucrose (0, 0.25, 2.5, 12.5 and 25 mM) for 23 DAS. Means ± SE of three to four biological replicates are presented. Different letters denote significant differences ($P < 0.05$).

Sucrose supply increased not only the number of CRs in P-sufficient plants, but was also associated with changes in the root morphology, leading to the formation of shorter and thicker roots (Figs. 2, 4F).

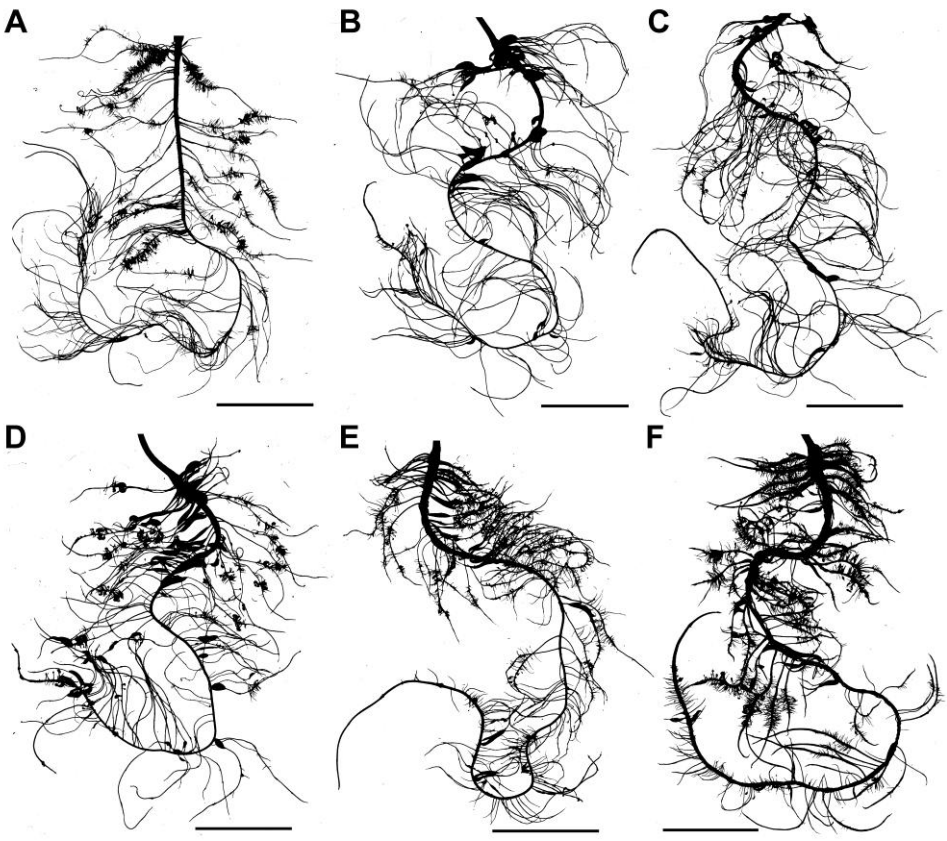

Fig. 4 Root morphology of white lupin as affected by different levels of sucrose application. Plants were grown in sterile hydroponic culture without phosphate (P) and sucrose (Suc) supply or with supplement of 100 μM KH_2PO_4, together with 0, 0.25, 2.5, 12.5, 25 mM sucrose (Suc) for 23 days after sowing (DAS). (A) -P –Suc; (B) +P –Suc; (C) +P +Suc (0.25 mM); (D) +P +Suc (2.5 mM); (E) +P +Suc (12.5 mM); (F) +P +Suc (25 mM). Scale bar: 5 cm.

Increased shoot to root translocation of assimilates and an increased accumulation of sucrose in the root tissue under P limitation is a well-described phenomenon (Marschner, 1995; Hammond & White, 2008; Lei *et al.*, 2011). To investigate whether external sucrose application mimics this effect in P-sufficient plants, we determined the sucrose concentrations in the pre-emergent stage of CR development, localized in the 2-3 cm sub-apical root segments of the first-order laterals. On a fresh weight basis, P-deficient plants accumulated a root tissue concentration of approximately 3.5 mM sucrose, which was increased by a factor of 10 (35 mM) in P-sufficient plants with external sucrose supply of 25 mM (Table 1).

Table 1 Tissue concentration of sucrose at different developmental stages of first-order lateral roots of white lupin (2-3 cm sub-apical segments for +P -Suc, 2-3 cm sub-apical pre-

emergence CR and juvenile CR for -P -Suc and +P +Suc). Plants were grown in sterile hydroponic culture without or with supplement of 100 μM KH_2PO_4, supplemented with or without 25 mM sucrose (Suc) for 22 days after sowing (DAS). At each stage, means ± SE of three biological replicates are presented. Different letters denote significant differences ($P < 0.05$). FW: fresh weight.

Root zone	Sucrose concentration (mmol kg^{-1} root FW)
+P -Suc sub-apical	1.8 ± 0.4 b
-P -Suc sub-apical pre-emergent	3.4 ± 1.1 b
-P -Suc juvenile	3.0 ± 0.6 b
+P +Suc sub-apical pre-emergent	35.1 ± 0.7 a
+P +Suc juvenile	30.2 ± 0.8 a

Based on these data, the effect of sucrose application on CR formation of P-sufficient plants was tested also over a range of lower concentrations to meet the physiological level detected in P-deficient plants. Cluster root formation continuously increased with increasing sucrose concentrations up to 12.5 mM (Fig. 3D). Decreasing the initially supplied sucrose concentration of 25 mM in the growth medium by a factor of 10 to 2.5 mM, resulted in cluster root formation and root morphology very similar to P-deficient plants (Fig. 4A, D) and root thickening or inhibition of lateral root elongation was no longer detectable (Fig. 4A, D, F).

To test the specificity of the sucrose effect on CR formation, we also applied the sucrose analog palatinose at 25 mM, which is not metabolized by higher plants (Sinha *et al.*, 2002). Palatinose was not able to stimulate CR development in P-sufficient plants (Fig. 3B). In order to decide whether sucrose was acting as a signal compound for CR formation or simply as a carbon source, a mixture of 12.5 mM glucose and 12.5 M fructose was supplied to +P plants as a carbon source, which can then be easily metabolized (Wind *et al.*, 2010). However, only 25 mM sucrose, but not the mixture of glucose and fructose, was able to induce CR formation in +P plants (Fig. 3C).

4.4.2 Sucrose effects on cluster root activity

To test whether CRs, induced by sucrose amendments to P-sufficient plants, exhibit also the typical root-induced alterations in rhizosphere chemistry (Neumann & Martinoia, 2002), we investigated the exudation of citrate and acid phosphatase as well as the proton extrusion. In complementary analyses, the expression of genes encoding secretory acid phosphatase (*LaSAP*), PEP carboxylase (*LaPEPC*) as a key enzyme for biosynthesis of organic acids and MATE transporters (*LaMATE*) potentially mediating citrate exudation was measured. Root extrusion of protons was measured by the acidification of the growth medium.

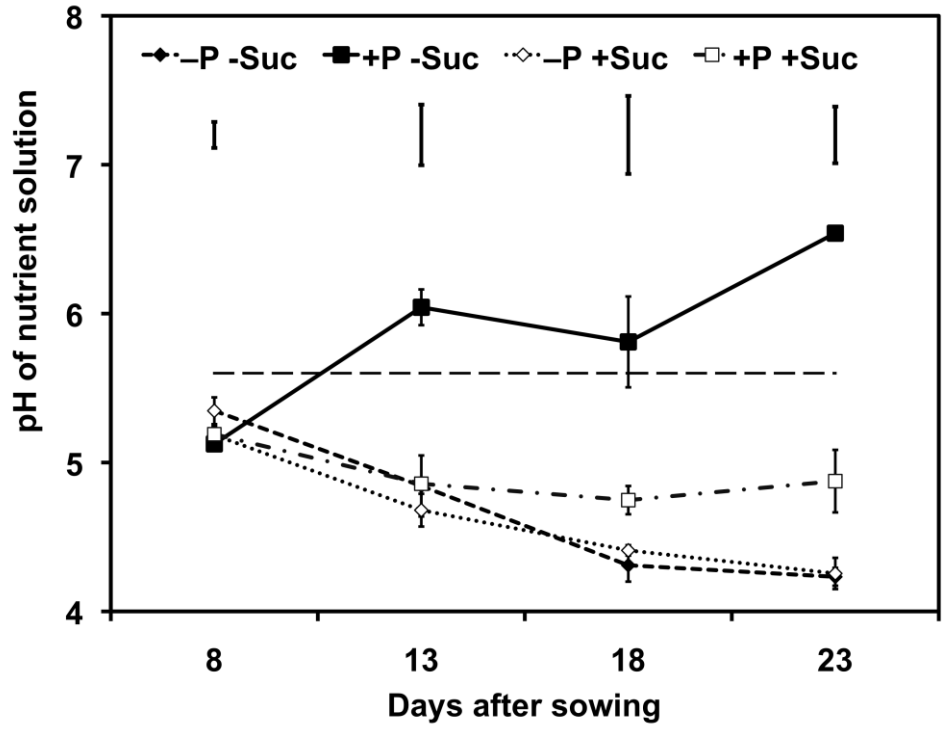

Fig. 5 Root-induced proton release of white lupin measured as the acidification of growth media before refreshing the nutrient solution. Plants were grown in sterile hydroponic culture containing 0 (-P) or 100 µM (+P) KH_2PO_4, supplemented with or without 25 mM sucrose (Suc) for 23 days after sowing (DAS). The horizontal dashed line indicates the initial pH of the nutrient solution (5.6). Means ± SE of four biological replicates are presented. Vertical bars denote $LSD_{0.05}$ values.

Starting from the initial pH of 5.6, a decrease to pH 4.2 was quantified in P-deficient plants. By contrast, only a moderate acidification to pH 4.8 was measured in P-

sufficient plants supplied with sucrose, while +P plants without sucrose amendment increased the medium pH to approximately 6.8 (Fig. 5).

A massive increased root exudation of citrate, which is induced simultaneously with proton extrusion (Neumann *et al.*, 2000), was detected in P-deficient plants (Fig. 6A). Accordingly, a gene encoding MATE transporters, which has been implicated in root exudation of citrate and phenolic compounds in other plant species (Neumann & Römheld, 2007) were transcriptionally up-regulated (Fig. 6B). However, neither an increased citrate exudation, nor up-regulation of *LaMATE* gene was detectable in root clusters induced by sucrose treatments under P-sufficient condition (Fig. 6).

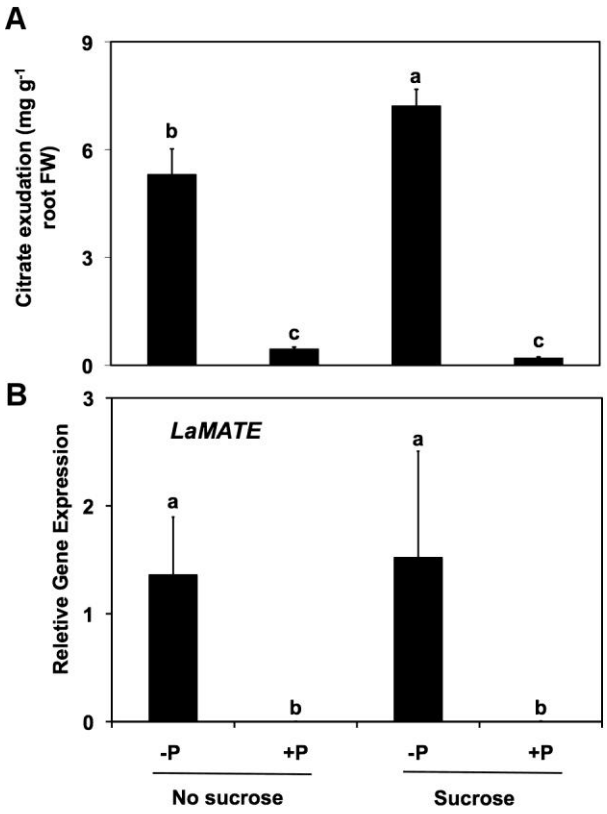

Fig. 6 Cumulative exudation of citrate within 5 days (A) and expression of the *LaMATE* gene (B), encoding MATE transporter potentially mediating citrate exudation, of white lupin grown with and without phosphate (P) and sucrose (Suc) application. Plants were grown in sterile hydroponic culture containing 0 (-P) or 100 µM (+P) KH_2PO_4, supplemented with or without 25 mM sucrose (Suc) for 23 days after sowing (DAS). For (A), Means ± SE of four biological replicates are presented, different letters denote significant differences ($P < 0.05$); For (B), 2 cm excised apical root segments without root

tips obtained from first-order laterals were harvested for +P –Suc treatment, and mature cluster roots were harvested for -P –Suc, -P +Suc, +P +Suc treatments. Means ± SE of two to three biological replicates are presented. Gene expression was normalized to the *LaUbiquitin* expression levels of each sample.

In accordance with the increased root exudation of citrate, the *in-vitro* activity of PEP carboxylase, a key enzyme for the biosynthesis of organic acids, and the expression of the *LaPEPC* gene were up-regulated in P-deficient plants. By contrast, no up-regulation was observed in the sucrose-induced root clusters of P-sufficient plants (Fig. 7), indicating that these roots were inactive in increased organic acid production.

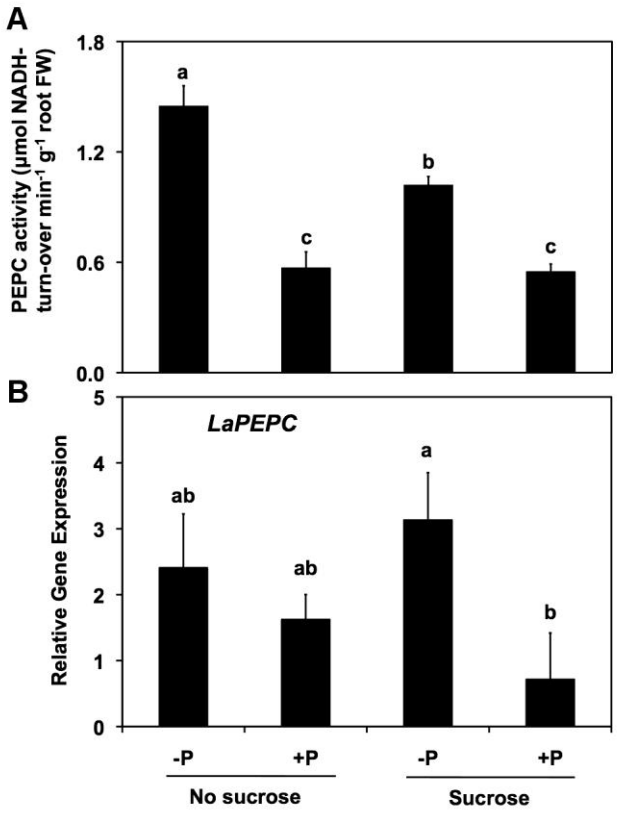

Fig. 7 *In-vitro* activities of phosphoenolpyruvate carboxylase (PEPC) (A) and expression of *LaPEPC* gene (B) of white lupin grown with and without phosphate (P) and sucrose (Suc) application. Plants were grown in sterile hydroponic culture containing 0 (-P) or 100 µM (+P) KH_2PO_4, supplemented with or without 25 mM sucrose (Suc) for 23 days after sowing (DAS). For (A), Means ± SE of three biological replicates are presented, different letters denote significant differences ($P < 0.05$); For (B), 2 cm excised apical root segments without root tips obtained from first-order laterals were harvested for +P –

Suc treatment, and mature cluster roots were harvested for -P –Suc, -P +Suc, +P +Suc treatments. Means ± SE of two to three biological replicates are presented. Gene expression was normalized to the *LaUbiquitin* expression levels of each sample.

Finally, the activity and expression of a gene encoding secretory acid phosphatase (*LaSAP*) were detectable only in P-deficient plants, but not in cluster roots induced by the sucrose application to +P control plants (Fig. 8).

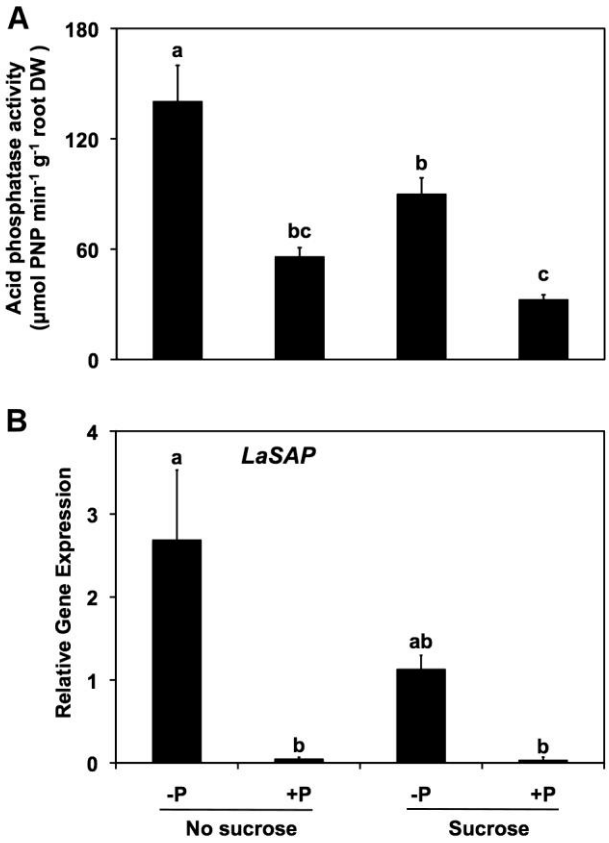

Fig. 8 Secretory acid phosphatase activity (A) and expression of *LaSAP* gene (B) of white lupin grown with and without phosphate (P) and sucrose (Suc) application. Plants were grown in sterile hydroponic culture containing 0 (-P) or 100 µM (+P) KH_2PO_4, supplemented with or without 25 mM sucrose (Suc) for 23 days after sowing (DAS). For (A), Means ± SE of three biological replicates are presented, different letters denote significant differences ($P < 0.05$); For (B), 2 cm excised apical root segments without root tips obtained from first-order laterals were harvested for +P –Suc treatment, and mature cluster roots were harvested for -P –Suc, -P +Suc, +P +Suc treatments. Means ± SE of two to three biological replicates are presented. Gene expression was normalized to the *LaUbiquitin* expression levels of each sample.

4.5 Discussion

A growing body of evidence suggests that sugars can act not only as carbon source, but also as signal molecules to control plant growth and development (Sheen *et al.*, 1999; Gibson, 2005; Rolland *et al.*, 2006; Smeekens *et al.*, 2010; Wind *et al.*, 2010). In higher plants, sensing and signalling pathways have been investigated, mainly for glucose (Smeekens *et al.*, 2010). More recent findings suggest that P starvation responses may be also mediated by sugar signalling (Jain *et al.*, 2007; Zhou *et al.*, 2008; Hammond & White, 2008, 2011; Lei *et al.*, 2011). In *Arabidopsis*, increased lateral root formation, proliferation of root hairs and inhibition of primary root growth are major root morphological adaptations for improved spatial P acquisition under P limitation (Williamson *et al.*, 2001; López-Bucio *et al.*, 2002). However, even with optimum P supply, these morphological changes were mimicked either by exogenous supply of sugars to the growth media or in mutants accumulating higher amounts of sucrose, both in shoot and root tissues, suggesting an important role of sucrose in mediating these root growth responses (Jain *et al.*, 2007; Karthikeyan *et al.*, 2007; Lei *et al.*, 2011). Furthermore, an increased sucrose concentration in the root tissue of P-deficient plants has been reported in various plant species including *Arabidopsis*, bean and soybean (Hammond & White, 2008; Lei *et al.*, 2011).

Similarly, the formation of CRs in *Lupinus albus* and in members of the Proteaceae (Neumann & Martinoia, 2002) is a special root morphological adaptation for improving P acquisition. This raises the question whether sugar (sucrose) signalling plays a comparable role also in this adaptive response. However, particularly in the case of cluster root formation, separating a putative signal function of sugars from substrate effects is not an easy experimental task, since both functions may act simultaneously. Moreover, in *Lupinus albus,* the formation of cluster roots starts earliest after a culture period of approximately two weeks without P supply (Neumann *et al.*, 2000). Therefore, the external application of sugars to the growth medium for signalling studies needs to be conducted over extended time periods. This approach requires an axenic culture system to avoid microbial contamination of the growth medium, which could in turn interfere with CR formation, e.g., by production of hormones.

In previous studies, sterile agarose media have been employed as growth substrates for axenic culture of lupin plants to study the effects of sucrose amendments on CR formation (Zhou *et al.*, 2008). However, plant growth on agar media over extended time periods bears the risk of depletion of nutrients and slow diffusion of oxygen (Barrett-Lennard & Dracup, 1988). Both factors are known to impact on root growth and morphology, e.g., by increased accumulation of ethylene (Torrey, 1976; Fukaki & Tasaka, 2009). Accordingly, the root morphology of lupin plants grown on agarose medium was strongly affected by the culture conditions, leading to inhibition of lateral root elongation, thickening of the primary root and only very limited formation of CRs in P-deficient plants (Zhou *et al.*, 2008). Less then five clusters per plant formed within an extended culture period of four weeks by 25 mM sucrose, suggesting that the CRs formation was strongly restricted by the culture conditions. By contrast, root systems of P-deficient *Lupinus albus* grown in aerated nutrient solutions are usually characterised by well-developed lateral roots and formation of 20-40 root clusters per plant within 3-4 weeks (Neumann *et al.*, 1999, 2000; Figs. 2, 3). Using such a sterile hydroponic culture system (Fig. 1), the supply of 25 mM sucrose to the growth medium was able to induce CRs formation in lupin plants even under P-sufficient conditions to a similar extent as under P limitation (Figs. 2, 3). However, this was associated with the inhibition of lateral root elongation and root thickening as also described by Zhou *et al.* (2008) (Figs. 2, 4F). This root morphological response is reminiscent and characteristic for the exposure to high levels of ethylene (Torrey, 1976; Qin *et al*., 2007) and was similarly observed after application of high-doses of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) in *Lupinus albus* (Chapter III). Accordingly, increased ethylene production induced by exogenous sucrose application in a dose-dependent manner was reported in rice and tobacco (Philosoph-Hadas *et al.*, 1985; Kobayashi & Saka, 2000). The observation that sucrose was less effective in stimulating CR formation of –P plants than in +P plants (Fig. 3A) may reflect even stronger ethylene-mediated inhibition of root growth as a consequence of increased ethylene production induced by both P limitation (Gilbert *et al*., 2000; Chapter I, III) and external sucrose supply (Philosoph-Hadas *et al*., 1985; Kobayashi & Saka, 2000).

In our study, the internal sucrose levels in the 2-3 cm sub-apical root zones of the first-order laterals was increased approximately two-fold in P-deficient plants, as compared with the respective root zones of the P-sufficient control (Table 1). In this

zone, the CR intiation (pre-emergent stage) starts. The increased sucrose concentration in this region reflected the well-documented increased shoot to root translocation of carbohydrates in response to P starvation (Marschner, 1995; Hammond & White, 2008; Lei *et al.*, 2011). However, the internal sucrose concentrations in P-sufficient plants supplied with 25 mM sucrose in the growth medium increased by 10-fold, as compared with the P-deficient control (Table 1). To investigate whether the formation of CRs in P-sufficient plants may be mainly attributed to artificially high intracellular concentrations of sucrose induced by high levels of external sucrose supply, we tested the induction of CRs over a range of external sucrose applications (0.25, 2.5, 12.5, 25.0 mM). Cluster root formation continuously increased even at the lowest external sucrose level and saturated at around 12.5 mM (Figs. 3D, 4). Lowering the external sucrose concentration that was used in the initial experiments (25 mM) by a factor of 10 (2.5 mM) mimicked the conditions in P-deficient plants (Table 1). This resulted in CR formation and root morphology indistinguishable from the P-deficient control (Fig. 4A, D). Under these conditions, root thickening or any inhibition of root elongation was absent (Fig. 4D, F). These findings demonstrate that CR formation in P-sufficient plants is not only induced by external sucrose application at artificially high levels, but occurs already at concentrations that are in the physiological range and which have been demonstrated to trigger P limitation-induced root responses (Hammond & White, 2008; Lei *et al.*, 2011).

In contrast to sucrose, the supply of the non-metabolizable sucrose analogue palatinose (Sinha *et al.*, 2002) in the growth medium failed to induce CR formation in P-sufficient plants (Fig. 3B). This indicates that CR formation depends on the presence of sucrose and cannot be attributed to osmotic effects. In agreement with that, Zhou *et al*. (2008) also failed to induce CR formation after application of 25 mM sorbitol to the growth media.

The disaccharide sucrose is easily hydrolysed to form glucose and fructose, but the combination of 12.5 mM glucose and 12.5 mM fructose was ineffective with respect to induction of cluster roots. This experiment differentiates between carbon source effect and signal functions of the sucrose molecule on CR formation, since glucose and fructose, as easily metabolizable carbon sourced, are easily taken up from the growth medium of P-sufficient plants but failed to induce CR formation. This

suggests that the induction of cluster roots is not primarily a carbon source effect and that sucrose is exclusively recognized as a signal compound. By contrast, Zhou *et al*. (2008) found a moderate induction of cluster roots also by combined glucose and fructose amendments, but this effect was confined to the presence of artificially high P concentrations (5 mM) in the growth medium (compared with 0.1 mM P in our study). Such high P concentration may induce even P toxicity symptoms in these highly P-efficient plant species.

The involvement of sucrose signalling in CR formation raises the question whether sucrose also plays a role in the regulation of CR function, which is characterised by an intense temporal secretion of root exudates, such as citrate, phenolics, protons and acid phosphatase. These exudates mediate the mobilisation of sparingly soluble soil P sources (Neumann & Martinoia, 2002). Therefore, we investigated the exudation of citrate and protons, the secretion of acid phosphatase and the activity of PEP carboxylase (PEPC), as a key enzyme for the biosynthesis of organic acids. These are all known to be up-regulated in CRs (Neumann & Martinoia, 2002). In complementary analyses, the expression of the underlying genes (*LaPEPC*, *LaSAP*, *LaMATE*) were measured in P-deficient plants and in P-sufficient plants with CRs induced by sucrose application. In both plants, we observed an acidification of the growth medium (Fig. 5) that indicates proton extrusion, but the proton extrusion was more rapid under P deficiency. The proton release, together with release of other cations (e.g., K^+, Na^+, Mg^{2+}), is usually associated with citrate exudation from CRs to maintain the charge balance (Neumann *et al.*, 2000; Zhu *et al*., 2005). However, citrate exudation was stimulated exclusively in the P-deficient plants, but not in the P-sufficient plants with sucrose amendments (Fig. 6A). Therefore, the acidification of the growth medium must be clearly attributed to processes other than citrate exudation. Accordingly, an increased gene expression and higher *in-vitro* activity of PEPC was detectable only in P-deficient plants, but not in P-sufficient plants with CRs induced by the sucrose treatment (Fig. 7). The same holds true also for the gene expression and the activity of secretory acid phosphatase (Fig. 8). Finally, the expression of a gene related to MATE transporters (*LaMATE*), which are known to be involved in root exudation of flavonoids and citrate (Neumann & Römheld, 2007), was induced exclusively by P limitation and not by external sucrose supply (Fig. 6B). In summary, these findings clearly indicate that sucrose signalling is

involved in the CR formation, but not in the processes regulating the functioning of CRs. This important finding is contradictory to the findings of Zhou *et al*. (2008), who reported the sucrose-induced expression of the secretory acid phosphatase gene (*LaSAP*) in P-sufficient plants and concluded functional CRs are induced by sucrose. The strongly contradictory results in both studies may be attributed to the highly artificial culture conditions used in former experiments, such as long-term agar culture with the risk of nutrients depletion and slow diffusion of oxygen, extremely high external P concentrations causing also visible changes in normal lupin root morphology.

Taken together, it can be concluded that under growth conditions excluding additional stress factors (except P limitation), CR formation and CR function are regulated independently. Indeed, the formation of cluster roots relies on a spatially and temporally interacting network of phytohormones that regulate the initiation of high-density lateral rootlet primordia, the outgrowth of the lateral rootlets and the maturation of the CRs. Only in mature CRs, massive secretion of P-mobilizing root exudates (protons, citrate, acid phosphatase and phenolics) is induced due to severe P limitation of this developmental stage, associated with P recycling to juvenile actively-growing tissues (Chapter I). Sucrose seems to be involved as a signal compound in the initiation and formation of CRs. The fact that only sucrose, but not glucose or fructose, was able to induce CR formation even in P-sufficient plants, underlines this signal function. This must be independent of the carbon source effect, although the carbon supply is of course also important for the production of root biomass, root respiration and finally root exudation mainly in mature CRs.

It is important to note that, in many other plant species sucrose application increases ethylene production in a concentration-dependent manner and has effects on various processes, such as anthocyanin production, flowering, and fruit ripening (Philosoph-Hadas *et al*., 1985; Kobayashi & Saka, 2000; Jeong *et al*., 2010). Furthermore, CR development is associated with increased expression of genes involved in ethylene biosynthesis and the production of ethylene (Gilbert *et al*., 2000; Uhde-Stone *et al*., 2003; O'Rourke *et al*., 2013; Chapter I). This raises the question, whether increased shoot to root translocation of sucrose under P-deficient conditions exerts a stimulatory effect on CR formation via ethylene signalling. Low levels of ethylene commonly promote lateral root initiation by stimulation of the auxin biosynthesis or

by the increasing auxin sensitivity of the responsive tissues (Fukaki & Tasaka, 2009). By contrast, high ethylene concentrations exert inhibitory effects on lateral root initiation and also on root elongation (Torrey, 1976). This fits with the observation that at lower concentrations of external sucrose (e.g., 2.5 mM) CR formation is stimulated, while at higher concentrations (e.g., 25 mM) lateral roots elongation is even inhibited and roots are thickened similar to lupin roots treated with ethylene precursor ACC (Chapter III). It has been speculated that sucrose may trigger ethylene production via increased liberation of auxins from auxin conjugates (Meir *et al*., 1989) and more recently, it was shown that phloem-located sucrose transporters (SUC1, SUT4) may exert regulatory functions (Jeong *et al*., 2010; Chincinska *et al*., 2013). Accordingly, a macromolecular analysis revealed also the presence of ethylene-biosynthetic components in the phloem sap of *Lupinus albus* (Rodriguez-Medina *et al*., 2011). Although these observations point to key functions of sucrose and ethylene within the signalling pathway that triggers CR formation, a detailed elucidation of the signalling network involved in these processes requires further investigations at the level of hormonal interactions and gene expression, which is presented in Chapter III.

4.6 Appendix

4.6.1 Authors' contributions

ZW carried out the experiments and data analysis. ZW and GN designed the experiments. ZW wrote the manuscript with final corrections provided by GN, UL and JS. All authors read and approved the final manuscript.

4.6.2 Acknowledgements

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5 Chapter III - Hormonal interactions during cluster root development in phosphate-deficient white lupin (*Lupinus albus* L.)

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5.1 Abstract

This study addresses hormonal interactions involved in cluster root (CR) development of phosphate (P_i)-deficient white lupin (*Lupinus albus*), which represents the most efficient plant strategy for root-induced mobilisation of sparingly soluble soil phosphorus (P) sources.

Shoot-to-root translocation of auxin was unaffected by P limitation, while strong stimulatory effects of external sucrose on CR formation, even in P-sufficient plants, suggest sucrose, rather than auxins, acts as a shoot-borne signal, triggering the induction of CR primordia. Ethylene may act as mediator of sucrose signal, as indicated by moderately increased expression of genes involved in ethylene biosynthesis in pre-emergent clusters and by strong inhibitory effects of ethylene antagonist $CoCl_2$ on CR formation induced by sucrose amendments or P limitation. As reported in other plants, moderately increased production of brassinosteroids (BRs) and cytokinin, in pre-emergent clusters, may be required for the formation of auxin gradients necessary for induction of CR primordia via interfering with auxin biosynthesis and transport. The well-documented inhibition of root elongation by high doses of ethylene may be involved in the inhibition of lateral rootlets growth during CR maturation, indicated by a massive increase of gene expression involved in ethylene production, associated with decline of transcripts with stimulatory effects (BR- and auxin-related genes).

5.2 Introduction

The formation of clustered-root structures (e.g., cluster roots or carrot-shaped dauciform roots densely covered with long root hairs) to increase the surface area for secretion of root exudates with nutrient-mobilising properties may be regarded as the third major adaptation for nutrient acquisition in terrestrial vascular sporophytes, following mycorrhizas and N_2-fixing nodules (Skene, 1998). The densely-spaced clusters of unbranched or even branched lateral rootlets, emerging from first-order lateral roots are characteristic for members of the Proteaceae, Casuarinaceae and some other plant species, adapted to infertile soils. The combined expression of adaptive changes in root exudation and root morphology, resulting in a massive increase of the secretory root surface area, represents the most efficient plant strategy for root-induced mobilisation of sparingly soluble soil nutrients (particularly phosphate, P_i). Nutrient mobilisation mediated by CRs has been characterised most comprehensively using white lupin (*Lupinus albus* L.) as a model plant (Watt & Evans, 1999a,b; Neumann *et al.*, 2000; Neumann & Martinoia, 2002; Cheng *et al.*, 2011; Chapter I).

Similar to lateral root formation, the lateral rootlets of CRs emerge from the pericycle opposite the protoxylem poles. However, CRs are characterised by extremely dense spacing of short laterals (50-1000 rootlets cm^{-1} root axis) with determinate growth. Inactivation of the meristems occurs when the lateral rootlets, densely covered with long root hairs, have reached a final length of 3-5 mm (Watt & Evans, 1999a,b; Neumann & Martinoia, 2002).

Individual clusters are emerging in rows with sequential patterns along the first-order laterals (Watt & Evans, 1999b) and exhibit only limited longevity of 2-3 weeks. During this time, CRs of P-deficient white lupin undergo distinct developmental stages, starting with induction of cluster root primordia, 2-3 cm behind the root tip of the first-order laterals (pre-emergent stage, PE), followed by outgrowth of juvenile (JU) clusters and reaching the mature (MA) stage when lateral rootlets exhibit no more growth activity. Root-induced chemical modifications of the rhizosphere for nutrient mobilisation are particularly expressed in the MA stage before senescence starts after 1-3 days and comprise secretion of citrate, phenolics,

protons and root secretory acid phosphatase (Neumann *et al.*, 1999, 2000; Watt & Evans, 1999b; Neumann & Martinoia, 2002).

Foliar P application can rapidly suppress formation of CR in P-deficient plants (Marschner *et al.*, 1987; Shane *et al.*, 2003), suggesting a regulatory function of the internal P status (Dinkelaker *et al.*, 1995; Shu *et al.*, 2007). Auxin (Gilbert *et al.*, 2000; Neumann *et al.*, 2000) and sucrose (Zhou *et al.*, 2008) have been discussed as shoot-borne signals mediating CR induction. However, based on experiments with removal of the shoot apex or shoot application of auxin transport inhibitors, Meng *et al.* (2013) postulated a central role of root-derived auxin. Expression studies of related genes and external application of hormones and hormone antagonists suggest a complex interplay of various hormonal or hormone-like factors, such as auxin, brassinosteroids (BRs), giberillic acid, cytokinin (CK), ethylene and nitric oxide (NO) (Gilbert *et al.*, 2000; Neumann *et al.*, 2000; Uhde-Stone *et al.*, 2003; Wang *et al.*, 2010; Meng *et al.*, 2012, 2013; O'Rourke *et al.*, 2013; Chapter I). On the other hand, CR formation seems to be stimulated also in the top-soil, characterised by increased levels of P and organic matter as compared with deeper soil layers (Purnell, 1960; Lamont, 1973; Skene, 1998) and similarly in split-root compartments with local high P supply (Shen *et al.*, 2005; Shu *et al.*, 2007), indicating an additional involvement of local signals (Neumann & Martinoia, 2002).

Using a combined approach of RT-qPCR based analysis of hormone-related gene expression, external application of hormones and hormone antagonists, as well as hormone transport analyses, this study addresses hormonal interactions during cluster root development in P-deficient white lupin.

5.3 Materials and methods

5.3.1 Hydroponic plant culture

Six seedlings of white lupin (*Lupinus albus* L. cv. Feodora) pre-germinated on filter paper were transferred into pots containing 2.5 L of aerated nutrient solution with 2 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe (III)-EDTA, 100 µM KH_2PO_4 at pH 5.6-5.8. Phosphate was omitted in the -P treatments. Replacement of nutrient solution was conducted every 4-5 days. Plant

culture was performed in a growth chamber with a light period of 16 h, light intensity of 200 µmol m^{-2} s^{-1}, 24/18 ℃ day/night temperature and a relative humidity of 60%.

5.3.2 Hydroponic plant culture under axenic conditions

Experiments with external sucrose supply required an axenic hydroponic culture system due to the rapid and massive microbial contamination of the growth medium in presence of sucrose. Seeds of white lupin were surface sterilized using 95% (v/v) ethanol for 10 min, washed 3 times with sterilized distilled water, followed by incubation in 10% (w/w) NaClO for 7 min and 6 times washing with sterile distilled water. Seeds were germinated on 1% (w/v) agar plates for four days in darkness, and then transferred to bottles containing 600 mL sterilized nutrient solution with 2 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe (III)-EDTA, with (+P) or without (-P) 100 µM KH_2PO_4 at pH 5.6-5.8, and with (+Suc, 25 mM) or without (-Suc) sucrose.

For sterile hydroponic culture, all bottles, nutrient solutions and other instruments were autoclaved before use. Sucrose was added by sterile filtration using 0.2 µm membrane filters (FP 30/0.2 CA-S, Whatman GmbH, Dassel, Germany). Also, inlet and outlet tubes for aeration were equipped with 0.2 µm sterile membrane filters. During pre-culture stage (9 days after sowing, DAS), plant shoots were covered with small beakers. Thereafter, the beakers were removed and bottles were sealed with sterilized paraffin at the entry point of the shoots. Plants were kept in a growth chamber with a light period of 16 h, and 24/18 ℃ day/night temperature, light intensity of 200 µmol m^{-2} s^{-1}, and relative humidity of 60%. Replacement of sterile nutrient solution was carried out every 5-7 days in a clean bench using the inlet and outlet aeration tubes after removal of the sterile filters.

5.3.3 Application of growth regulators

Ethylene and ethylene antagonists: At day 6 after sowing, treatments started with application of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) 0.1 µM for P-sufficient plants and 0.01, 0.1, 0.5 µM for P-deficient plants. The ethylene biosynthesis inhibitor $CoCl_2$ was applied at 10 µM for P-deficient plants.

The nutrient solution of different treatments was refreshed every five days. Plants were harvested at 25 DAS to analyse effects on root morphology and CR development.

For investigations of ethylene-sucrose interactions, sucrose (25 mM), ACC (1 µM) and $CoCl_2$ (10 µM) were supplied twice at 9 and 16 DAS during replacement of the nutrient solution. $CoCl_2$ was selected as ethylene antagonist since in contrast to other antagonistic compounds (e.g., $AgNO_3$) general plant development was not affected, except for the impact on CR formation. Harvest was performed at 23 DAS.

Brassinosteroids (BRs) and BR antagonists: Plants were cultivated in hydroponic culture until 12 DAS in 2.5 L pots. Thereafter, single plants were transferred to bottles containing 0.5 L nutrient solution (4 replicates) and the 24-epibrassinolide (EBR) was applied at 0.1 µM for P-sufficient and P-deficient plants, while brassinazole (Brz), as a BR antagonist, was applied to P-deficient plants only, at 1.5 and 3.0 µM. The treatments were conducted twice during replacement of the nutrient solution at 12 DAS and 16 DAS. Plants were harvested at 20 DAS to analyse effects on root morphology and CR development. The 24-epibrassinolide (EBR) and Brz were prepared as stock solutions (5 mM for EBR and 60 mM for Brz) in Dimethyl sulfoxide (DMSO) resulting in a final concentration of $\leq 0.05\%$ (v/v) DMSO in the nutrient solution which was also added to the control plants. Pilot experiments revealed that root growth and development were not affected by DMSO at the employed concentration level (data not shown).

Cytokinins: The synthetic cytokinin kinetin was applied to P-deficient plants during 24 days in hydroponic culture at concentration of 50 µg L^{-1} in intervals of 3-4 days during replacement of the nutrient solution. Plants were harvested at 28 DAS to analyse effects on root morphology and CR development.

5.3.4 Measurement of endogenous acropetal auxin transport

Indole acetic acid (IAA) export out of the shoot apices (diffusible IAA) of P-deficient and P-sufficient white lupin seedlings was measured as described by Li & Bangerth (1999). Stems with shoot apices were excised and the basal part of two shoots was immersed into 2.5 ml 2-(N-morpholino)ethanesulphonic acid (MES) buffer (50 mM, pH 6.2). The test was conducted with six replicates per treatment. Samplings were performed with plants grown in hydroponics in two-day intervals

over time period of 14 d starting at 8 DAS. The IAA that accumulated after 8 h in the buffer was adsorbed onto C-18 Sep-Pak cartridges (Waters, Eschborn, Germany) and washed with 0.20 and 40% (v/v) methanol in 0.1 M acetic acid. Indole acetic acid (IAA) in the 40% (v/v) methanol fraction was analysed after vacuum evaporation by radioimmunoassay using polyclonal antibodies as described by Bohner & Bangerth (1988).

5.3.5 Quantitative real-time PCR (RT-qPCR) of hormone-related genes

At 20 DAS, the root systems of P-deficient white lupin plants grown in hydroponic culture were harvested and separated into: pre-emergent root segments of first-order laterals without root tips (PE), juvenile CR (JU) which had not reached their final length, and mature CR (MA) located just basal to JU clusters (Fig. 1A, B, D). Dark-brown senescent clusters were not included. The root material was immediately frozen in liquid nitrogen and stored at -80 ℃ for further analysis. Total *RNA* of CR in different developmental stages was isolated with the innuPREP Plant *RNA* kit (Analytik Jena AG, Germany) using three independent biological replicates. Contaminating genomic *DNA* was removed with a RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. The *cDNA* was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Gene-specific primers were designed using Primer3Plus. Quantitative real-time PCR was performed as previously described (Straub *et al.*, 2013b). The gene expression level was indicated relative to reference genes. Primer sequences of selected reference genes (*PP2AA3*, *UBC*) and other genes can be found in Appendix Suppl. Table 1.

5.3.6 Statistical analysis

Data are shown as means ± SE. One-way ANOVA with least significant difference (LSD) test at 5% probability was used to test the differences. The software used was SAS for Windows Version 9.4 (SAS Institute Inc, Cary, NC, USA).

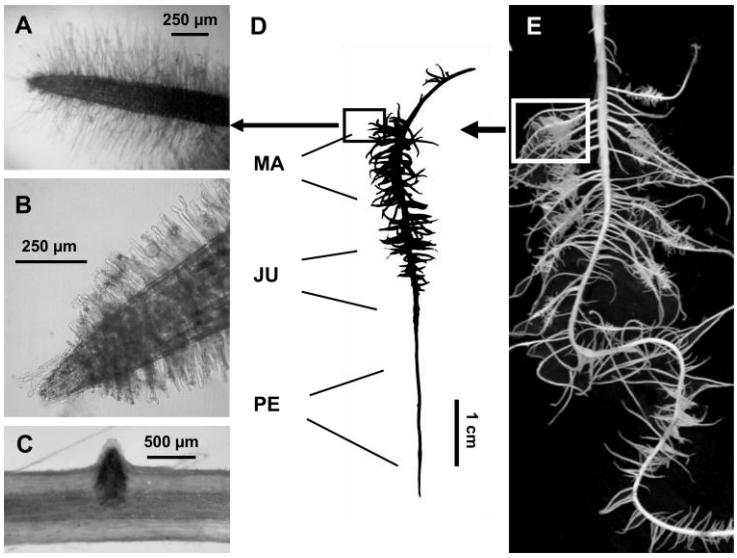

Fig. 1 Cluster roots (CRs) of P-deficient white lupin (*Lupinus albus* L.).
(A) Single lateral rootlet of a mature (MA) root cluster, densely covered with root hairs;
(B) Single lateral rootlet of a juvenile (JU) root cluster with growing root hairs;
(C) Outgrowth of a lateral rootlet primordium;
(D) Root clusters in different developmental stages along a first-order lateral root;
(E) Root system of P-deficient white lupin with CRs development.
PE, pre-emergent stage; JU, juvenile cluster; MA, mature cluster.

5.4 Results

5.4.1 Auxin

Shoot-to-root polar transport of auxin is a well-characterised determinant for induction of lateral root formation (Fukaki & Tasaka, 2009; Jung & McCouch, 2013) and may therefore act as a potential shoot-borne signal also for the induction of cluster roots (CRs; Watt & Evans, 1999a,b; Gilbert *et al.*, 2000; Neumann *et al.*, 2000). Based on the observation that the sequential emergence of individual root clusters along the first-order laterals in our hydroponic culture experiments coincided with the sequential formation and unfolding of young leaves, which serve as important sources of auxin production, it was hypothesised that sequential pulses of polar auxin transport originating from young leaves may be involved in the sequential induction of root clusters. Therefore, we determined the time courses of auxin translocation by measuring diffusible indole acetic acid (IAA) collected at the

shoot base of P-deficient and P-sufficient lupin plants over a time period of 14 days (Fig. 2).

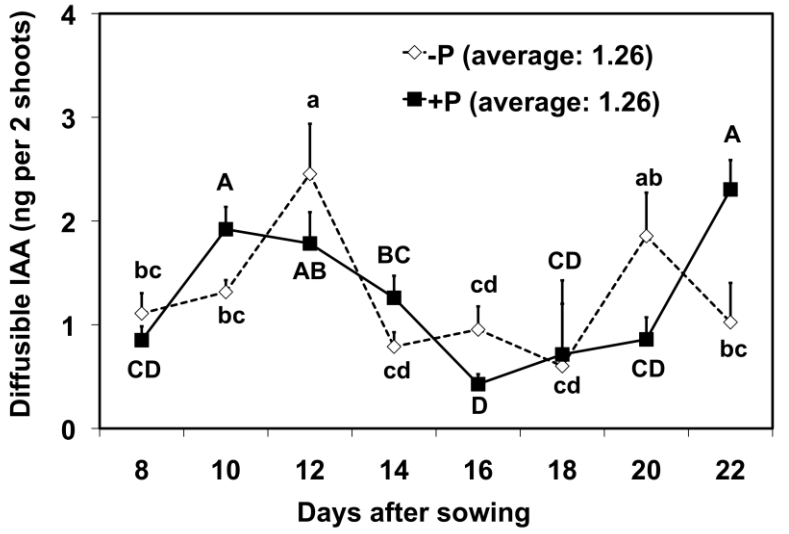

Fig. 2 Time course of polar indole acetic acid (IAA) transport in shoots of P-sufficient and P-deficient white lupin between 8 and 22 days after sowing (DAS). The phosphate treatments were started at day 6 after sowing. Data are shown as means ± SE (n = 4-6). Different lowercase letters denote significant differences ($P < 0.05$) in different dates of measurement for P-deficient plant; different capital letters denote significant differences ($P < 0.05$) in different dates of measurement for P-sufficient plant.

Despite considerable variability, diffusible auxin levels showed clear temporal, periodic fluctuations between 0.2 and 1.4 ng per plant within 4-5 days. In plants without P supply, formation of the first generation of CR was detected about 4 days after the first auxin maximum, consistent with the time window usually required for CR initiation (Watt & Evans, 1999b; Neumann & Martinoia, 2002). However, surprisingly there was no clear difference in the patterns of the auxin translocation between P-sufficient plants (which do not form clusters) and P-deficient plants with CR formation (Fig. 2). The average amounts of translocated auxins were also not significantly different (Fig. 2).

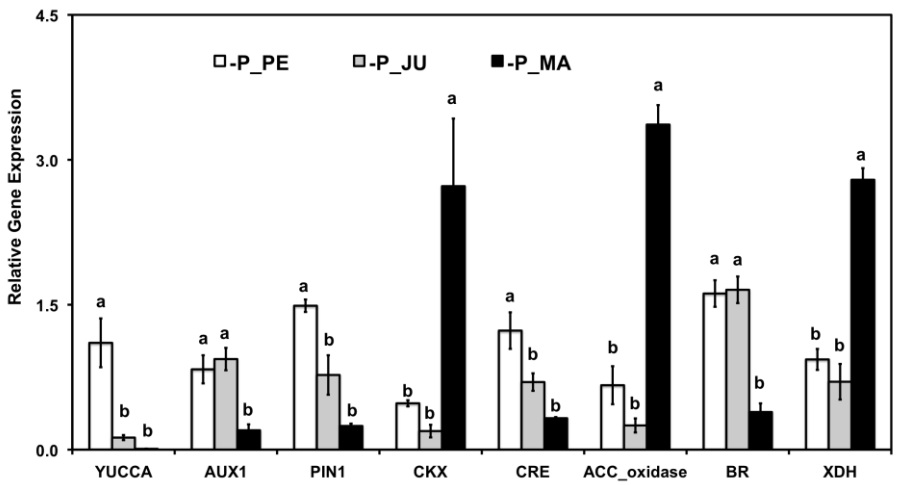

Fig. 3 Expression pattern of hormone-related genes evaluated by Quantitative real-time PCR (RT-qPCR). White lupin plants were grown for 20 days in hydroponic culture under P deficiency. The genes are involved in auxin biosynthesis *(YUCCA)*, auxin transport (*AUX1*, *PIN1*), cytokinin degradation (*CKX*), cytokinin receptors (*CRE*), ethylene biosynthesis (*ACC_oxidase*), brassinosteroid biosynthesis (*BR*) and nitric oxide formation (*XDH*). The gene expression level is indicated relative to reference genes. Data represent means ± SE (n = 2-3). Different lowercase letters denote significant differences ($P < 0.05$). PE, pre-emergent stage; JU, juvenile root clusters; MA, mature root clusters (modified after Wang *et al.*, 2014).

The potential importance of auxin for the induction of CR formation was underscored by the expression analysis of auxin-related genes. During different stages of cluster root development, RT-qPCR analyses revealed highest expression of the auxin influx carrier *AUX1* (Fukaki & Tasaka, 2009) in the pre-emergent (PE) stage and in juvenile (JU) clusters, while the expression of the efflux carrier *PIN1* (Fukaki & Tasaka, 2009) was most intense in PE clusters and declined continuously during CR maturation (Fig. 3). Apart from auxin transport, an intense expression of *YUCCA*, which was proposed as a central regulator of auxin biosynthesis (Zhao *et al.*, 2001), was detected in the PE stage. The expression declined dramatically in the later stages of CR development (Fig. 3).

5.4.2 Sucrose and ethylene

A common response to P limitation in higher plants is an increased shoot-to-root translocation of carbohydrates (sucrose) to enable adaptive changes in root growth

and root exudation for improved P acquisition (Hammond & White, 2008; Lei *et al.*, 2011).

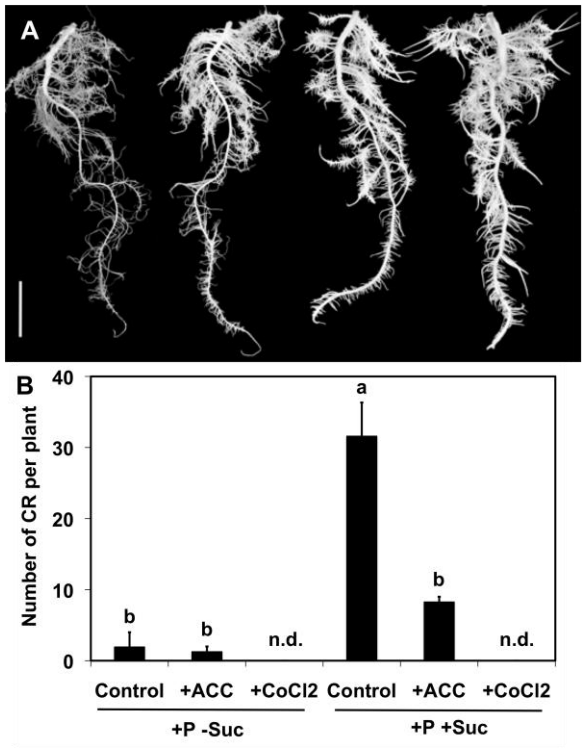

Fig. 4 (A) Effect of sucrose application on cluster root formation in white lupin. Plants were grown in sterile hydroponic culture for 23 days after sowing (DAS) under P-deficient (0 µM) and P-sufficient (100 µM) conditions, supplemented with (+Suc) or without (-Suc) 25 mM sucrose. From left to right: +P -Suc, -P -Suc, -P +Suc, +P +Suc. Scale bar: 5 cm; (B) Effect of sucrose, ACC and $CoCl_2$ on cluster root formation in white lupin. Sucrose (25 µM), ACC (1-aminocyclopropane-1-carboxylic acid, 1.0 µM) and $CoCl_2$ (10 µM) were added to the growth medium at 9 and 16 DAS. Means ± SE of three replicates are presented. Different letters indicate significant differences ($P < 0.05$). n.d. = not detected.

However, apart from carbon source effects, sucrose can also act as a signal compound, triggering P deficiency-induced root growth responses (Jain *et al.*, 2007; Karthikeyan *et al.*, 2007; Lei *et al.*, 2011) and also the formation of cluster roots (Zhou *et al.*, 2008). Accordingly, sucrose, but not glucose and fructose, application to the growth medium was able to induce cluster root formation even in P-sufficient white lupin (Fig. 4A; Chapter II). In various other plant species, sucrose application increased ethylene production in a concentration-dependent manner with effects on anthocyanin production and flowering (Philosoph-Hadas *et al.*, 1985; Kobayashi &

Saka, 2000; Jeong *et al.*, 2010). Since ethylene has also been implicated in lateral root formation (Fukaki & Tasaka, 2009), we tested the possibility that ethylene signalling may be similarly involved in the sucrose-induced formation of CRs by external applications of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the ethylene biosynthesis inhibitor $CoCl_2$ (Lau & Yang, 1976; Locke *et al.*, 2000). Increasing the ethylene levels in P-sufficient plants by ACC application alone had no effects on CR formation (Fig. 4B). However, sucrose-induced CR formation in P-sufficient plants was completely suppressed by the application of ethylene antagonist $CoCl_2$. A further increase of ethylene production by ACC application in sucrose-treated plants partially inhibited CR formation (Fig. 4B).

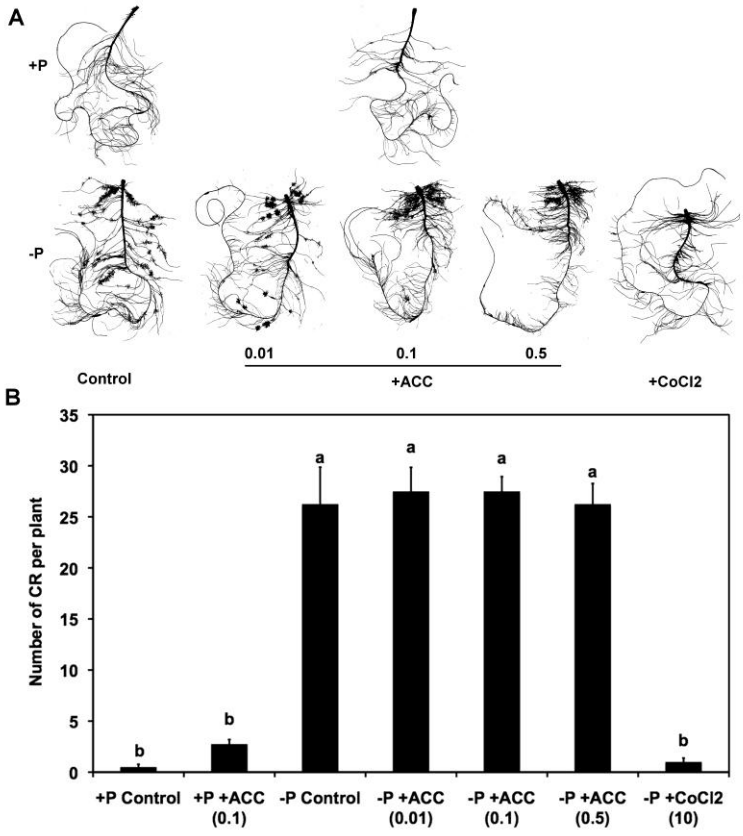

Fig. 5 Effects of ACC (1-aminocyclopropane-1-carboxylic acid) and $CoCl_2$ on cluster root formation of white lupin. Plants were grown in hydroponic culture for 25 days after sowing (DAS) under P-deficient (0 μM) and P-sufficient (100 μM) conditions. ACC (0.01, 0.1, 0.5 μM) or $CoCl_2$ (10 μM) were applied at 6, 11, 16, and 21 DAS. (A) Effects of ACC and $CoCl_2$ on root morphology of P-sufficient and P-deficient plants; (B) Effects

of ACC and $CoCl_2$ on cluster root formation. Means ± SE of four replicates are presented. Different letters indicate significant differences ($P < 0.05$).

In P-deficient plants, the inhibition of ethylene biosynthesis by application of $CoCl_2$ also completely suppressed CR formation (Fig. 5A, B), without further effects on root morphology (Fig. 5A). By contrast, external application of the ethylene precursor ACC to P-deficient plants had no stimulatory effects on CR formation (Fig. 5B), but the other root morphology was affected (Fig. 5A). In plants treated with higher ACC concentrations (0.1 and 0.5 µM), CR formation was restricted to the oldest first-order laterals covered with densely-spaced individual clusters (Fig. 5A). Apparently, a denser spacing of root clusters in ACC-treated plants as compared to the untreated control was the consequence of a general inhibition of lateral root elongation (Fig. 5A).

A direct determination of ethylene production in different stages of CR development was not included, since this would require extended incubation time of the excised CR segments, associated with excessive ethylene production as a response to wounding. Therefore, changes in ethylene production were monitored indirectly by analysing the expression of a gene encoding ACC oxidase as a key enzyme for ethylene biosynthesis in different stages of CR development, using root segments immediately frozen in liquid nitrogen after cutting, avoiding wounding responses. The expression analysis revealed a moderately higher abundance in the PE stage of cluster root development than in JU clusters. Thereafter, a dramatic increased expression occurred during cluster root maturation (Fig. 3), associated with inhibited elongation of the lateral rootlets and intense proliferation of root hairs (Fig. 1A, D). Similar to ACC oxidase, also transcripts of xanthine dehydrogenase, catalysing purine degradation and the biosynthesis of nitric oxide (NO), were highly expressed in MA clusters (Fig. 3).

5.4.3 Brassinosteroids and cytokinins

Apart from ethylene, also synergistic interactions of auxins with brassinosteroids (BRs) and cytokinins (CKs) have been implicated in the induction of lateral root formation (Fukaki & Tasaka, 2009; Jung & McCouch, 2013). Accordingly, the

expression of genes involved in the biosynthesis of brassinosteroids was predominant in the PE and JU stage of cluster root development (Fig. 3).

The external application of brassinazole (Brz), an inhibitor of BR biosynthesis (Asami *et al.*, 2000), to the nutrient solution of P-deficient white lupin during the whole culture period drastically inhibited plant growth. As a consequence, an assessment of its effects on CR formation was not possible. Therefore, Brz was applied at 12 DAS, when the first root clusters already emerged. The Brz treatments completely inhibited the development of additional CRs during the next eight days, until final harvest (Fig. 6). Brassinazole induced root thickening and inhibition of lateral root elongation (Fig. 6A), very similar to the changes in root morphology triggered by high doses of the ethylene precursor ACC (Fig. 5A). By contrast, the external application of the active BR, 24-epibrassinolide (EBR), into the nutrient solution had no significant effects on CR formation, neither in P-sufficient nor in P-deficient plants (Fig. 6B).

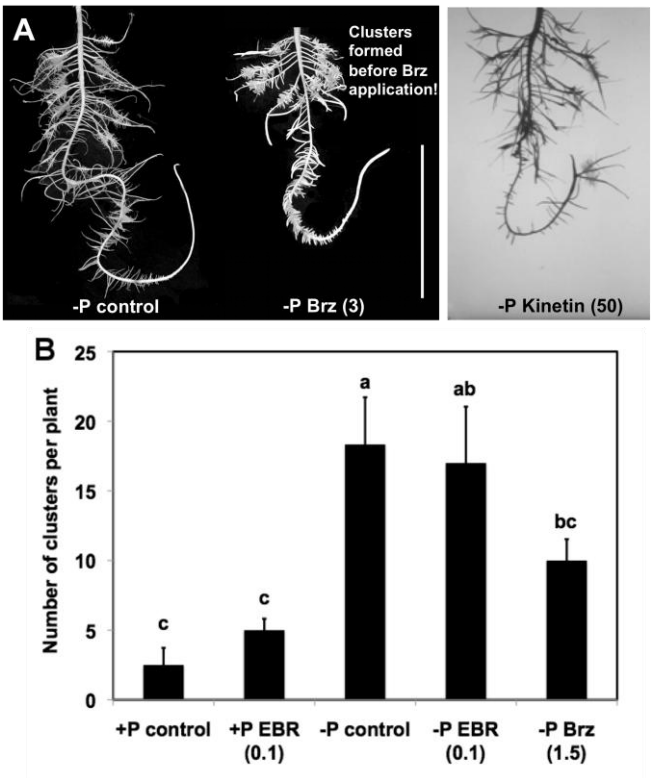

Fig. 6 (A) Effects of the brassinosteroid (BR) antagonist brassinazole (Brz, 3 µM) and the cytokinin analogue kinetin (50 µg L^{-1}) applied to the nutrient solution on root morphology of P-deficient white lupin, scale bar: 10 cm; (B) Effects of the 24-epibrassinolide (EBR, 0.1 µM) and the BR antagonist brassinazole (Brz, 1.5 µM) on CR formation of white

lupin grown in hydroponic culture under P-deficient (0 µM) and P-sufficient (100 µM) conditions. EBR and Brz were applied to the nutrient solution at 12 and 16 DAS. Means ± SE of four replicates are presented. Different letters indicate significant differences ($P < 0.05$).

The external application of the CK analogue kinetin exerted inhibitory effects on CR formation in P-deficient plants and inhibited root elongation similar to Brz treatments (Fig. 6A). Among CK-related genes, transcripts encoding CK receptor (*CRE*) were most prominently expressed in the pre-emergent stage of CR development and declined during CR maturation. The transcripts of CK oxidase (*CKX*), which mediates CK degradation, were most abundant in MA clusters (Fig. 3).

5.5 Discussion

5.5.1 Shoot-borne signals for cluster root formation

Based on the central role of auxins in lateral root development, Watt & Evans (1999a,b) suggested that polar auxin transport may act as a pulsed signal, which communicates changes in the shoot P status to the roots. These pulses of auxin transport may induce the sequential formation of CRs along the first-order laterals (Fig. 1D). Accordingly, our determinations of diffusible indole acetic acid (IAA), collected at the shoot base of P-deficient and P-sufficient lupin seedlings, revealed temporal fluctuations within a time period of 4-5 days. However, no differences were detectable between P-sufficient plants lacking CR formation and P-deficient plants with CRs. Furthermore, on average the same quantity of auxins (1.26 ng per two shoots) was translocated in both treatments (Fig. 2). This observation suggests that polar auxin transport alone is not the major signal mediating the characteristic patterns of CR formation in P-deficient plants. This is in line also with recent findings of Meng *et al.* (2013), showing that removal of shoot apices, as the main source of polar auxin transport, and application of auxin transport inhibitors via the shoot base had no effects on CR formation of P-deficient white lupin. However, CR formation was suppressed by the application of auxin transport inhibitors to the root medium (Gilbert *et al.*, 2000; Meng *et al.*, 2013). The authors concluded that in case of CR formation, root-borne auxins may be more important than polar auxin transport originating from the shoot. This view is further supported by our findings

that the transcripts encoding auxin transporters (*AUX1*, *PIN1*) and particularly the auxin biosynthesis-related gene *YUCCA* were most highly expressed in the 2-3 cm sub-apical root zones of the first-order laterals in P-deficient plants, representing the pre-emergent (PE) stage of CR development and the site of CR primordia formation (Fig. 3). Interestingly, a transcriptome analysis conducted prior to this experiment revealed that only *AUX1* and *PIN1* genes involved in auxin transport were detected to be differentially expressed during CR development (Chapter I), although an involvement of various other auxin transporters in lateral root formation has been reported in the literature (Fukaki & Tasaka, 2009). This finding points to a central role of *AUX1* and *PIN1* particularly for CR development.

Another process with potential for reporting the shoot P status to the root system could be shoot-to-root translocation of sucrose, which increases under P limitation (Hammond & White, 2008; Lei *et al.*, 2011). By using the described novel axenic hydroponic culture, it was possible to rapidly exchange or modify the growth solution and finally to evaluate the effects of sucrose and its interaction with phytohormones. Under non-axenic conditions, this is frequently not possible, due to the rapid and massive microbial contamination of the growth media after sucrose supply. External sucrose application to the roots of P-sufficient white lupin induced CR formation in a concentration-dependent manner, to a similar extent as P limitation (Fig. 4; Zhou *et al.*, 2008). This effect could not be mimicked by application of equal-molar mixture of glucose and fructose, as alternative carbon sources, pointing to a specific signal function of sucrose (Chapter II).

Skene (2000) proposed the so-called “primed-pericycle model” to explain the sequential induction of CR primordia in regular rows, based on a priming signal present in high concentrations in the vicinity of pericycle cells opposite the protoxylem poles and with low concentrations in between (Fig. 7C).

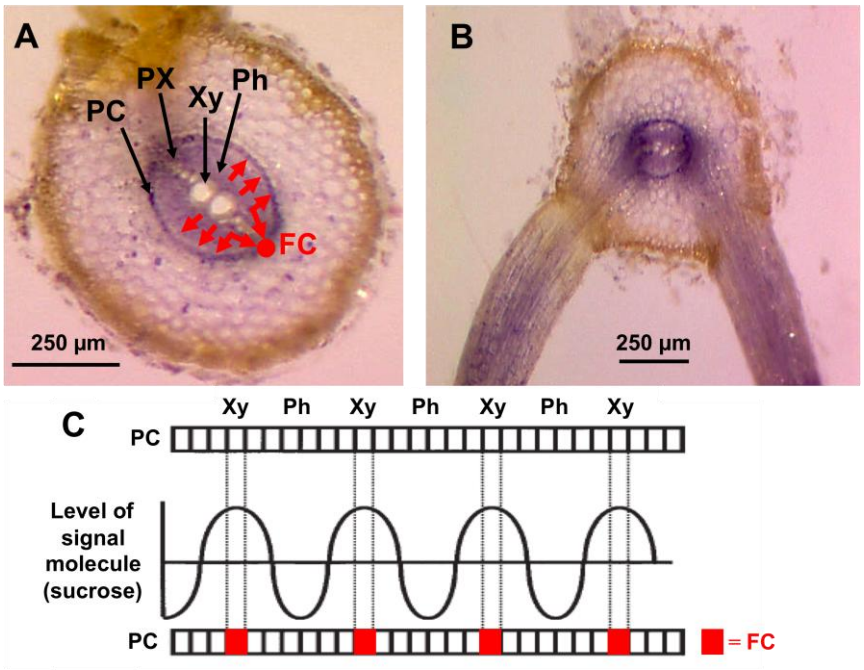

Fig. 7 (A) Cross-section in the pre-emergent (PE) cluster root zone of a first-order lateral root in P-deficient white lupin after formazan vital staining (Seligman & Rutenburg, 1951); (B) Cross section in the region of juvenile root clusters after formazan vital staining with two lateral rootlets emerging from the pericycle opposite protoxylem poles; (C) Schematic representation of the primed pericycle model, explaining the priming of founder cells for lateral root primordia (LRP) in the pericycle by local accumulation of signal compounds (Skene, 2000). Ph, phloem; Xy, xylem, PX, protoxylem; PC, pericycle; FC, LRP-founder cell. Red arrows indicate phloem sucrose export acting as FC priming signal in higher concentrations.

In *Lupinus albus,* organisation of the vascular bundles is characterised by two xylem strands (Fig. 7A), and consequently two rows or lateral rootlets are induced originating from the pericycle cells opposite the two protoxylem poles (Fig. 7B). Sucrose would meet the requirements for a signal to trigger the priming of pericycle cells, according to the model proposed by Skene (2000). In the radial direction, particularly high sucrose concentrations can be expected around the pericycle cells opposite to the protocylem poles, due to sucrose export originating from two phloem strands, located left and right from the xylem vessels (Fig. 7A). By contrast, the pericycle cells in between the protoxylem poles are supplied with sucrose only by one single phloem strand (Fig. 7A).

A central role of sugar signalling in P deficiency responses is also suggested by the *hps1* (*hypersensitive to phosphate starvation1*) *Arabidopsis* mutant caused by

overexpression of the *SUCROSE TRANSPORTER2* (*SUC2*) gene. This mutant showed enhanced sensitivity in almost all aspects of plant responses to P deficiency (Lei *et al.*, 2011). Sucrose may act in combination with *miR399*, identified as a systemic shoot-to-root signal under P deficiency (Liu & Vance, 2010; Chiou & Lin, 2011) directing the cleavage of *PHO2* mRNA, which is a negative regulator for P uptake into roots (Fujii *et al.*, 2005; Bari *et al.*, 2006; Chiou *et al.*, 2006). Although *miR399* has been identified in many plant species, its function has not yet been described for white lupin.

5.5.2 Signal perception in the root tissue

A link between increased tissue concentrations of sucrose and ethylene signalling is suggested by experiments with rice and tobacco, demonstrating a concentration-dependent increase of ethylene production in response to the exogenous application of sucrose (Philosoph-Hadas *et al.*, 1985; Kobayashi & Saka, 2000). This is particularly interesting, since low concentrations of ethylene exert stimulatory effects on lateral root formation (Fukaki & Tasaka, 2009). Accordingly, in our study, intense CR formation induced by external sucrose application was completely suppressed by simultaneous application of $COCl_2$ (Fig. 4B), as potent inhibitor of ethylene biosynthesis (Lau & Yang, 1976; Locke *et al.*, 2000). Moreover, $CoCl_2$ also suppressed P deficiency-induced CR formation (Fig. 5), while the expression of gene encoding ACC oxidase, a key enzyme for ethylene biosynthesis, was moderately increased in the pre-emergent (PE) stage of CR, as compared with juvenile (JU) root clusters (Fig. 3). This was associated with a doubling of the internal sucrose concentration in the PE stage as compared with the corresponding lateral root zones in P-sufficient plants (Chapter II). Taken together, these findings point to a sucrose-mediated stimulation of ethylene biosynthesis in the PE stage of CR development. Recently, Rodriguez-Medina *et al*. (2011) reported the presence of ethylene-biosynthetic components in the phloem sap of *Lupinus albus* and therefore, sucrose-mediated ethylene biosynthesis may take place already in the phloem, which is located in close proximity of the pericycle, the initiation site of CR primordia (Fig. 7A, B).

5.5.3 Signal transduction in the root tissue

Fukaki & Tasaka (2009) reported that moderately increased ethylene production can stimulate the biosynthesis of auxins, known to be responsible for the priming of founder cells in the pericycle, the first step for the induction of lateral root primordia. Accordingly, our RT-qPCR analysis revealed the highest expression of various genes involved in auxin biosynthesis and transport (*YUCCA, AUX1, PIN1*) in the PE stage, followed by a continuous decline during outgrowth and CR maturation (Fig. 3). This was associated with moderately increased expression of genes involved in ethylene biosynthesis in the PE stage of CR development (Fig. 3).

Apart from auxin and ethylene, also brassinosteroids and cytokinins have been implicated in the formation of auxin gradients required for the induction of lateral root primordia (Fukaki & Tasaka, 2009; Jung & McCouch, 2013). This fits with the observation that both expression of genes encoding cytokinin receptors and of genes involved in brassinosteroid synthesis were most highly expressed in PE and JU root clusters of *Lupinus albu*s (Fig. 3). A role of brassinosteroids in CR formation is further underlined by the finding that CR formation was completely suppressed by the external application of the brassinosteroid antagonist brassinazole (Fig. 6) which was associated with a general inhibition of lateral root elongation and root thickening. However, the additional application of cytokinins to the root medium resulted in the well-documented suppression of lateral root development (Fukaki & Tasaka, 2009; Jung & McCouch, 2013) and consequently, also of CR formation (Fig. 6A; Neumann *et al.*, 2000). Inhibition of PIN-mediated auxin transport has been discussed as a main reason for cytokinin-induced repression of lateral root development (Fukaki & Tasaka, 2009). Neumann *et al.* (2000) reported increased cytokinin concentration in the root tissue of P-deficient white lupin as compared with the P-sufficient control. This was attributed to the high number of young root tips present in JU root clusters as potential sources of cytokinin production. The authors speculated that the elevated internal cytokinin levels may be involved in growth inhibition of lateral rootlets during CR maturation.

Interestingly, the external application of hormonal growth regulators involved in CR formation, such as auxin (IAA), ethylene (ACC) and brassinosteroids (24-epibrassinolide), to P-sufficient lupin seedlings resulted only in a moderate stimulation of CR formation in comparison with P-deficient plants (Figs. 5, 6;

Neumann *et al.*, 2000). This may be explained by a limitation of simultaneous carbon supply due to lower shoot-to-root translocation of sucrose under P-sufficient conditions (Hammond & White, 2008; Lei *et al.*, 2011).

5.5.4 Hormonal interactions during maturation of cluster roots

During CR maturation, a massive up-regulation of genes involved in ethylene biosynthesis has been detected in P-deficient lupin plants and this was associated with the down-regulation of auxin- and brassinosteroid-related genes (Fig. 3). In contrast to the stimulatory effect of low ethylene concentrations on lateral root formation, higher levels of ethylene exert inhibitory effects on lateral root induction, inhibit root elongation and can stimulate the proliferation of root hairs (Torrey, 1976; Neumann & Römheld, 2002; Qin *et al.*, 2007), which are also characteristic for the morphology of mature (MA) root clusters (Neumann & Martinoia, 2002). Accordingly, external application of the ethylene precursor ACC in higher concentrations induced the inhibition of lateral root elongation, resulting in denser spacing of lateral rootlets within root clusters and the inhibition of CR formation in later stages of plant development (Fig. 5A). The increased ethylene production seems to be a general characteristic of P-deficient tissues (Lynch & Brown, 1997) and accordingly, Gilbert *et al.* (2000) reported increased production of ethylene also in root systems with CRs of P-deficient white lupin. Recently, this has been related with increased production of nitric oxide via xanthine dehydrogenase, which was up-regulated in MA clusters (Fig. 3; Wang *et al.*, 2010; Chapter I). This enzyme is thought to be involved in nucleotide degradation in the context with internal P recycling under P-deficient conditions. Nitric oxide can stimulate the biosynthesis of ethylene (García *et al.*, 2011), which may in turn induce the proliferation of root hairs and inhibit lateral rootlet elongation. These processes seem to be at least partially mediated via the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT), triggering similar responses also in adaptations to Fe deficiency (Strategy I) in dicots and non-graminaceous moncots (Lingam *et al.*, 2011; Chapter I). Moreover, NO seems to be also required for the induction of lateral root primordia in the pericycle by modulating the expression of regulatory genes of the cell cycle (Correa-Aragunde *et al.*, 2006). Accordingly, NO accumulation was also detected in PE root-clusters (Wang *et al.*, 2010).

In conclusion, the present study points to a central role of sucrose in CR formation simultaneously acting as a carbon source and as a long distance signal, reporting the shoot P status to the root system. In the root tissue, ethylene seems to have essential functions as a signal for both the CR formation and particularly the determination of CR morphology. The proposed interactions of regulatory factors involved in CR development are schematically summarised in Fig. 8.

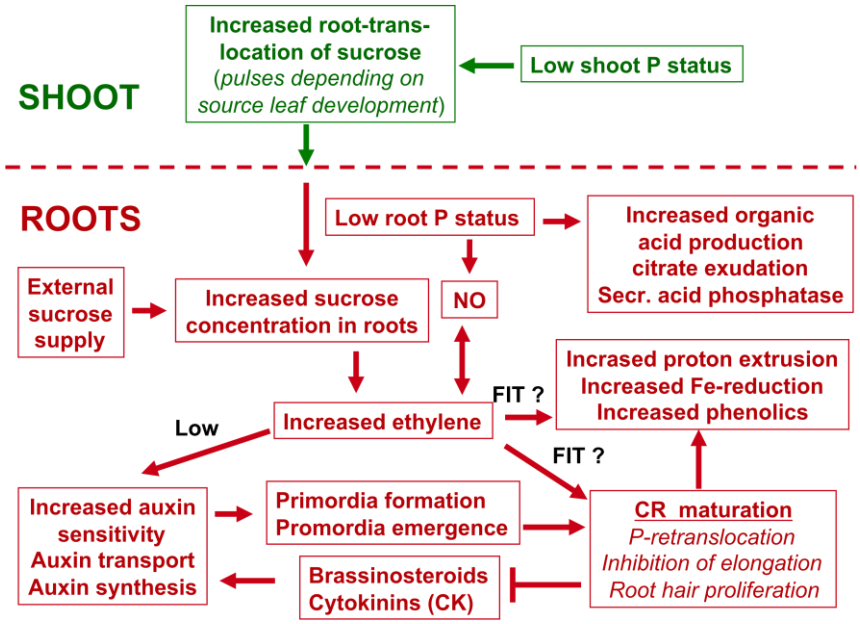

Fig. 8 Schematic model summarising hormonal interactions during cluster root formation in P-deficient white lupin (for detailed explanation see plain text).

5.6 Appendix

5.6.1 Authors' contributions

ZW carried out data analysis and most of the lab work, MR carried out the experiments related to brassinosteriods, GW performed the measurement for diffusible IAA translocation, ZW and GN designed the study, ZW wrote the manuscript with final corrections provided by GN, UL and JS. All authors read and approved the final manuscript.

5.6.2 Acknowledgements

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5.6.3 Supplementary materials

Suppl. Table 1 Primer pairs used in the RT-qPCR analyses.

Annotation	Sequence	
	Forward	Reverse
PP2AA3	TGCATGCATAGAGCACCAAG	CATTGTTGAGCTTGCTGAGG
UBC	TCGGCGGATCCTGATATTAC	CTGGAACAGAAAAGGCAAGC
YUCCA	TAGCTTCAATGTGGCAGCAC	ATGGCATAAGAGGGAGTTGG
AUX1	ATGCTGAGGGCAATGTTAGG	CACTTTTGGTGGACATGCTG
PIN1	CAAAGTGTTTGGGAGGGTTG	GGAGCAACTTGAGCAAAAGG
ACC_oxidase	TTGGGTAGGCTTGGTTTGTC	CTACAACCCAGCCAATGATG
BR	AATGGCGAGCACGCTTATAG	TCCCTCAAAAGATGGTCTCG
CKX	GGCACAACCTTCAGATTTGG	CCCAAATTGGCCTAATCCTC
CRE	ACTTCACATCGGCTCCAAAC	AGGGAAAGACATGCCTGATG
XDH	CTTTCAAGGCGAAGGATCTG	TCACGTGCCTTCACAAAGTC

6 Discussion and future perspectives

This study focuses on the regulation of cluster root (CR) development of white lupin (*Lupinus albus*) under phosphorus (P) deficiency, using for the first time a transcriptome sequencing approach with systematic comparison of different stages in CR development. In P-deficient white lupin, initiation, outgrowth, maturation and senescence of individual CR usually take place over a time period of less than two weeks, and CRs in the different developmental stages are simultaneously present in the same root system (Neumann & Martinoia, 2002). Due to internal orthophosphate (P_i) re-translocation, the zones of CR initiation and outgrowth are usually well supplied with phosphate even in P-deficient plants. By contrast, older root tissues such as mature (MA) clusters with strong expression of root-induced chemical changes for P mobilisation (exudation of citrate, phenolics, acid phosphatase and protons) are severely P-deficient (Massonneau *et al.*, 2001; Neumann & Martinoia, 2002). This constellation has the advantage that changes of gene expression involved in CR development and in metabolic adaptations to P limitation can be assessed simultaneously by comparative transcriptome sequencing of different stages in CR development. However, whole transcriptome sequencing studies are usually not valid as stand-alone approaches and require confirmation of gene expression data by PCR approaches and physiological analysis. During the last two decades, enormous progress has been achieved in the physiological characterisation of CR function using exactly the same comparison of different stages in cluster root development (see Neumann *et al.*, 1999, 2000; Massonneau *et al.*, 2001; Langlade *et al.*, 2002; Kania *et al.*, 2003; Wasaki *et al.*, 2005; Weisskopf *et al.*, 2006) in hypothesis-driven approaches. The transcriptome analysis offers the opportunity to validate these findings at the transcriptional level and additionally provides numerous novel indications for metabolic characteristics with potential functions in the regulation of CR development. Based on these findings, the present study developed a model for the key components in the regulatory network involved in CR formation, and functions of CRs in nutrient acquisition.

6.1 What are the factors controlling cluster root formation?

6.1.1 Hormonal interactions in cluster root initiation

Cluster roots are bottle-brush like lateral rootlets, with limited growth, densely covered by root hairs, that arise from the pericycle opposite the protoxylem poles in a sequential pattern along the first-order lateral roots (Skene, 1998; Neumann & Martinoia, 2002). The sequential development of CRs comprises: initiation of CR primordia, requiring pulses of priming signal(s) in the vicinity of pericycle cells opposite the protoxylem poles; outgrowth of lateral rootlets and maturation of CR. Split root experiments and studies with foliar P application suggested that the internal P status, particularly the shoot P status, is a systemic determinant for CR formation in white lupin (Marschner *et al.*, 1987; Shane *et al.*, 2003; Li *et al.*, 2008). Similar to the formation of conventional lateral roots, hormonal balances may play a curial role also in the regulation of cluster root formation. Auxin plays a key a role in the regulation of lateral root formation, starting with priming of founder cells in the pericycle, followed by induction, development and outgrowth of lateral root primordia, which involves PIN-mediated auxin transport, gradient formation and signalling of shoot and root-borne auxin (Chang *et al.*, 2013). Based on the central role on lateral root development, auxin has been suggested as a pulsed signal, which communicates changes in the shoot P status to the roots (Watt & Evans, 1999a,b). However, the present study, in line with findings by Meng *et al.* (2013), suggests that rather root-derived auxin and its redistribution along the roots play a crucial role for cluster root formation (Chapter III). Under P deficiency, increased sucrose concentrations have been detected in the root tissue of a wide range of plant species, such as *Arabidopsis*, bean, soybean and also white lupin (Hammond & White, 2008; Lei *et al.*, 2011; Chapter II). Meanwhile, sucrose has been identified as a signal molecule required for plant growth and development (Chiou & Bush, 1998). This signal has also been implicated in cluster root formation of white lupin (Liu *et al.*, 2005; Zhou *et al.*, 2008; Chapter II). Skene (2000) proposed the primed pericycle model to explain the sequential induction of CR primordia in regular rows, based on a priming signal present in high concentrations in the vicinity of pericycle cells opposite the protoxylem poles and with low concentrations in between. In *Lupinus albus,* organisation of the vascular bundles is characterised by two xylem strands and

consequently two rows of lateral rootlets are induced, emerging from the pericycle cells opposite the two protoxylem poles (Chapter III). This study presented a modified primed pericycle model based on the one given by Skene (2000), proposing sucrose instead of auxin, as the shoot-borne signal for priming the founder cells for lateral rootlet primordia formation (Chapter III). Increased tissue concentrations of sucrose can evoke ethylene production (Philosoph-Hadas *et al.*, 1985; Kobayashi & Saka, 2000), which is particularly interesting, since low concentrations of ethylene exert stimulatory effects on lateral root formation (Fukaki & Tasaka, 2009). Moderately increased ethylene production can stimulate biosynthesis of auxins, known to be responsible for the priming of founder cells in the pericycle, as a first step for the induction of lateral root primordia (Fukaki & Tasaka, 2009). Accordingly, the auxin biosynthesis-related gene (*YUCCA*) and also PIN-mediated transport (*PIN*) genes are both highly expressed in the pre-emergent cluster root (Chapter I). Brassinosteroids and cytokinins have been also implicated in the formation of auxin gradients required for the induction of lateral root primordia by directing auxin transport (Fukaki & Tasaka, 2009; Jung & McCouch, 2013). Moreover, nitric oxide (NO) seems to be required for the induction of lateral root primordia in the pericycle, by modulating the expression of regulatory genes of the cell cycle (Correa-Aragunde *et al.*, 2006). Accordingly, NO accumulation was also detected in PE root clusters (Wang *et al.*, 2010) and discussed as a common signal mediating P deficiency and Fe deficiency responses in white lupin probably acting via the scarecrow (*SCR*) transcription factor (Sbabou *et al.*, 2010; Meng *et al.*, 2012), which was predominantly expressed in pre-emergent clusters (Chapter I).

6.1.2 Hormonal interactions during cluster root maturation

During cluster root maturation, a massive up-regulation of genes involved in ethylene biosynthesis has been detected in P-deficient lupin plants, associated with down-regulation of auxin- and brassinosteroid-related genes (Chapter I). In contrast to the stimulatory effect of low ethylene concentrations on lateral root formation, higher levels of ethylene exert inhibitory effects on lateral root induction and root elongation and can stimulate proliferation of root hairs (Torrey, 1976; Fukaki & Tasaka, 2009; Jung & McCouch, 2013), characteristic for the morphology of mature root clusters. Recently, enhanced ethylene production in mature clusters has been

related with increased production of nitric oxide via xanthine dehydrogenase, which is thought to be involved in nucleotide degradation in context with internal P recycling under P-deficient conditions (Chapter I; Wang *et al.*, 2010). These processes seem to be at least partially mediated via the FIT transcription factor, triggering similar responses also in adaptations to Fe deficiency (Strategy I) in dicots and non-graminaceous moncots (Chapter I).

6.2 What are the mechanisms behind the cluster root function?

Under P deficiency, extrusion of huge amount of organic acids (mainly malate and citrate), protons, phenolics, together with increased expression of high-affinity P_i transporters, enhanced secretory acid phosphatase activity and ferric reduction capacity are key features of cluster roots function in white lupin (*Lupinus albus*) (Neumann & Martinoia, 2002). Cluster root maturation is typically characterised by a shift from malate, as major carboxylate accumulating in the juvenile clusters, to almost exclusive and extremely intense citrate accumulation in mature clusters (Neumann *et al.*, 2000; Neumann & Martinoia, 2002). In many soils, citrate is much efficient in mobilising P as compared with malate (Neumann & Römheld, 2007). Physiological measurements and gene expression studies revealed that citrate accumulation and exudation in mature clusters are a consequence of both increased biosynthesis and decreased degradation of citrate (Neumann & Martinoia, 2002; Chapter I). On one hand, due to severe P starvation, the P-requiring glycolytic pathway (via hexokinase, fructokinase, pyruvate kinase) is inhibited and replaced by the P-independent bypass reactions (via sucrose synthesis, PEPC), maintaining the carbon flows for citrate production; on the other hand, the activity of citrate degrading enzymes (aconitase, IDH) (Neumann *et al.*, 1999; Chapter I) in the TCA cycle is inhibited, probably due to the inhibited respiration and decreased nitrate uptake (Neumann *et al.*, 2000; Chapter I). Phosphorus deficiency-induced inhibition of the respiratory chain is a characteristic feature of mature cluster roots (Johnson *et al.*, 1994; Neumann *et al.*, 1999). Accordingly, the alternative oxidase (AOX), bypassing the energy-conserving steps in the normal cytochrome (Cyt) pathway, is induced in mature clusters to counteract overproduction of reactive oxygen species (ROS; Purvis & Shewfelt, 1993; Wagner & Krab, 1995).

Cluster root maturation is also accompanied by accumulation and exudation of phenolics (particularly isoflavonoids), with putative functions in P_i mobilisation (Tomasi *et al.*, 2008) and the protection of nutrient-mobilising root exudates against microbial degradation (Weisskopf *et al.*, 2006). Phenolic compounds are produced via the phenylpropanoid pathway (Dixon & Paiva, 1995). Accordingly, transcripts encoding two key enzymes of the phenylpropanoid pathway (PAL and 4CL) were highly expressed in mature clusters (Chapter I), reflecting the production of phenolic compounds at this stage.

Rhizosphere acidification is a result of proton extrusion to maintain the charge balance for the release of citrate anions and also a consequence of decreased net-uptake of nitrate (Neumann *et al.*, 1999).

The release of secretory acid phosphatase, mainly in mature clusters, is a crucial mechanism for mobilising organic P forms in soil (Ozawa *et al.*, 1995; Gilbert *et al.*, 1999; Neumann *et al.*, 1999; Miller *et al.*, 2001; Wasaki *et al.*, 2003). Sparingly soluble organic soil P forms can be mobilised via citrate exudation, and enzymatic hydrolysis is mediated by secretory acid phosphatases (Neumann & Römheld, 2007). Subsequently, the orthophosphate (P_i) can be taken up by plants via high-affinity P_i transporters.

6.3 Are there common regulation patterns in plants response to P and Fe deficiency?

One of the most interesting novel findings of this study was a strong up-regulation in the expression of the basic helix-loop-helix transcription factor FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) during CR maturation of P-deficient white lupin, even when the Fe status was sufficient (Chapter I). FIT is crucial for the coordinated regulation of Fe deficiency responses of dicotyledonous plant species and non-graminaceous monocots (Strategy I; Ling *et al.*, 2002; Colangelo & Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005; Bauer *et al.*, 2007), comprising characteristic adaptive responses, such as thickening of root tips, root hair proliferation, enhanced extrusion of protons and phenolics, increased ferric reductive (FRO) capacity and up-regulation of Fe^{2+} uptake mediated by IRT1 transporters (Eide *et al.*, 1996; Robinson *et al.*, 1999; Hagström *et al.*, 2001)

to improve Fe acquisition. Interestingly, very similar responses (proliferation of root hairs, increased release of phenolics and protons, up-regulation of *FRO* and the *IRT1* gene) appear also during CR maturation in P-deficient white lupin (*Lupinus albus*). Moreover, white lupin plants can form cluster roots, both under limitation of P and Fe, secrete huge amounts of citrate, and accumulate NO and ethylene known as major regulators of the FIT transcription factor (Hagström *et al.*, 2001; Meng *et al.*, 2012). The fact that white lupin plants exhibit many similar responses to P and Fe deficiency raises the question whether white lupin may have evolved a shared signalling pathway to overcome both nutrient limitations. From an evolutionary point of view, this could be a possible advantage particularly for acquisition of Fe-P in soils, since solubilising one will simultaneously increase the availability of the other nutrient. However, this would also bear the risk to induce Fe toxicity under conditions when P but not Fe is a limiting nutritional factor. Post-transcriptional down-regulation of Fe uptake could be a protective mechanism. This aspect as well as the interactions of ethylene and NO with FIT during CR maturation and the characterisation of processes regulated via FIT in CRs of P-deficient white lupin require a more derailed molecular and physiological characterisation. Of particular interest is the question whether common signalling pathways for P and Fe deficiency responses are a unique feature in *Lupinus albus* or a common principle also in other plant species. Analysis of P deficiency responses in FIT mutants of *Arabidopsis* could be a valuable approach to address this question.

6.4 Are there lessons to be learned for other crops?

Facing the global P crisis and environmental issues arising from the energy-intensive production and the extremely limited efficiency of mineral fertilizers in general, improving fertilizer use efficiency of future crops and cropping systems is an issue of increasing importance. Using plant species adapted to infertile soils with the potential to mobilise sparingly soluble soil nutrients, such as white lupin in intercropping systems, as green manure and also for suppression of pathogens is an approach with a long-lasting history in agriculture, reported already in the ancient cultures of Egypt, China, Greece, Rome and South America mainly based on empirical experience (Tisdale & Nelson, 1975). The scientific investigation of the underlying mechanisms started at the end of the 19th century (Prjanischnikow,

1934). The still ongoing issues as demonstrated by recent studies are improving growth and P uptake by wheat (*Triticum aestivum* L.) (Cu *et al.*, 2005) and increasing maize yield though inhibition of maize infestation by *Striga hermonthica* (Weisskopf *et al.*, 2009), when intercropped with white lupin (*Lupinus albus* L.). However, even in the most sophisticated cropping systems developed in the 1920's by Aereboe and von Wrangell or in modern organic farming systems, an adequate P supply to crops remains problematic and is meanwhile associated with a declining status of P fertility in many organic farming soils.

In the recent past, various approaches tried to use the knowledge on the mechanisms of root-induced nutrient mobilisation by root exudation or organic meal chelators, protons or hydrolysing enzymes for the development of transgenic plants overexpressing genes mediating the production or the release of the respective root exudates. While in case of Al-detoxification by root-induced malate exudation or release of Fe-mobilising phytosiderophores, impressive results even on the field scale have been achieved (Takahashi *et al*., 2001; Delhaize *et al*., 2004), these approaches were largely unsuccessful and not reproducible in case of root-induced P mobilisation (Koyama *et al.*, 2000; López-Bucio *et al.*, 2000; Delhaize *et al.* 2001; George *et al*., 2004). This may be attributed to the fact that the amount of mobilising root exudates required for sufficient mobilisation of the macronutrient P in the rhizosphere is much higher than for the micronutrient mobilisation or metal detoxification (Neumann & Römheld, 2007). In the most efficient plant species, such as *Lupinus albus*, the molecular basis of the release mechanism is still unclear and it needs to be established whether MATE transporters or ALMT-like transporters, both highly expressed in mature (MA) clusters with the highest secretory activity (Chapter I), are mediating the release of citrate into the rhizosphere as one of the most efficient carboxylates for P mobilisation in soils (Neumann & Römheld, 2007). Moreover, in a model calculation based on exudation rates determined for CRs, the amounts of carboxylates required for significant P mobilisation in soils and the morphological characteristics of CRs, Neumann & Römheld (2007) demonstrated that the secretory root surface extension induced by CR morphology seems to be quantitatively much more important for efficient P mobilisation in the rhizosphere than the release rates of carboxylates per unit root length or metabolic alterations (Fig. 1). In CRs of *Lupinus albus*, it was

demonstrated that a major function of metabolic alterations seems to be the production of root exudates with particularly high efficiency in P mobilisation (e.g., citrate instead of malate, efficient forms of acid phosphatases) (Neumann & Römheld, 2007) or root exudates with protective functions against microbial degradation of P-mobilising root exudates (Weisskopf *et al.*, 2006).

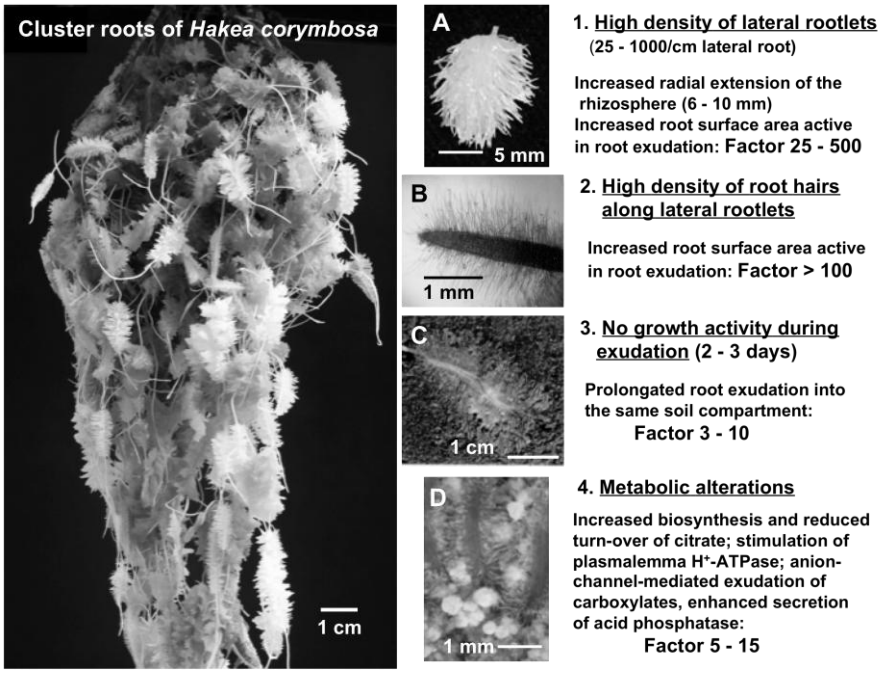

Fig. 1 Impact of cluster root characteristics on accumulation of P mobilising root exudates in the rhizosphere. Increase compared with normal lateral roots (modified after Neumann & Römheld, 2007).

If CR morphology is a major determinant for successful chemical P mobilisation in soils, one of the most interesting aspects is the question whether development of CR requires unique regulators, exclusively present only in cluster-rooted plant species. The fact that root clustering can also be observed in many other plant species under specific growth conditions, such as localized nutrient supply, exogenous hormone applications (Hinchee & Rost, 1992; Kaska *et al.*, 1999), inoculation with phytohormone-producing soil microorganisms (Kaska *et al.*, 1999; Sukumar *et al.*, 2013), points to the possibility that differences in the quantitative expression of general regulatory factors for root development may be the major determinants of cluster root morphology and function. Accordingly, in the present study no CR-specific regulatory features have been identified (Chapters I-III).

This may indicate that by a favourable combination of common gene expression and hormonal signalling, many crop roots may be able to form the highly complex CR-like structures, thereby improving nutrient acquisition. An impressive example in this direction has been recently published by Jing *et al.* (2010): comparing broadcast and localised field application of ammonium-based P fertilizers on a highly-buffered calcareous soil (pH 8.0). It was possible to induce root clustering in maize in the vicinity of the nutrient depot. Due to the effect of root clustering, rhizosphere acidification induced by ammonium uptake was intense enough to overcome the buffering capacity of the calcareous soil and to reduce the rhizosphere pH by more than 1.5 units, thereby improving the availability of P and micronutrients such as Fe, Zn and Mn. By contrast, in the variant with broadcast application without formation of root clusters, rhizosphere acidification was not intense enough to overcome the buffering capacity of the soil.

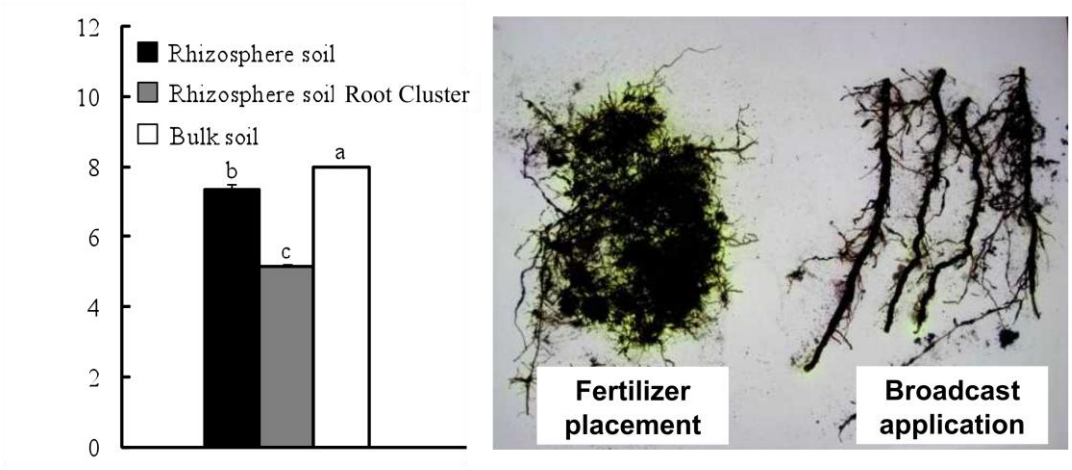

Fig. 2 Root clustering, induced by localised application of ammonium and P fertilizers with nitrification inhibitors can contribute to rhizosphere acidification of maize on highly buffered soils (modified after Jing *et al.*, 2010).

This example illustrates that CR formation can be an option to improve nutrient acquisition even in non cluster-rooted plant species. Therefore, an improved understanding of internal and external factors necessary for the induction and formation of CR can be a powerful tool to develop breeding targets, biotechnological strategies and management practices with the goal to improve nutrient acquisition efficiency by CR formation in crops. Of course this needs to be

always combined with intelligent fertilization strategies to minimize the risk of nutrient depletion by soil-nutrient mining.

6.5 Future perspectives

Taken together, the findings of this thesis, based on transcriptome sequencing, gene expression studies and hormone-related physiological data, identified key components of a network in hormonal interactions mediating the formation and function of CRs in P-deficient white lupin. Alterations in root translocation of shoot-borne sucrose act as a major signal reporting the shoot P status to the root system. The signal perception in the root tissue involves ethylene production triggering auxin biosynthesis and transport in a coordinated action with brassinosteroids and cytokinins to form auxin gradients required for the priming of root founder cells in the pericycle of the sub-apical zones in the first-order laterals.

For further validation of this model, it would be interesting to establish the time courses of sucrose translocation to the roots of lupin seedlings during the establishment of P limitation, as related to leaf development, photosynthetic activity, alterations of sucrose concentration in pre-emergent root zones and the development of CRs. To study the regulation of P deficiency-induced root translocation of sucrose, an interesting candidate to be investigated in this context would be the *PTF1* transcription factor recently identified to be involved in the regulation of sugar export via the phloem under P-deficient conditions (Li *et al.*, 2011).

A link between increased root tissue concentrations of sucrose under P limitation and ethylene signalling is suggested by the well-documented concentration-dependent increase of ethylene production in response to the exogenous sucrose application (Philosoph-Hadas *et al.*, 1985; Kobayashi & Saka, 2000). However, the perception of the sugar signal and the events leading to increased ethylene production remain to be elucidated. Recent studies suggest that some phloem sucrose exporters (SUT4) maybe involved in this process (Chincinska *et al.*, 2013). The potential interaction of sucrose signalling in combination with *miR399,* identified as another systemic shoot-to-root signal under P deficiency (Liu & Vance, 2010; Chiou & Lin, 2011) is another aspect which requires further investigation.

Apart from the involvement in CR initiation, the findings of this study also point to a central role of ethylene in CR maturation (root hair proliferation, inhibition of lateral

root elongation), and probably even in CR function via FIT-mediated activation of root exudation of phenolics, protons and increased ferric reductase activity (Chapter I). This view is supported by the massive increased expression of genes involved in biosynthesis of ethylene (Chapters I, III) and increased ethylene production during CR maturation. However, the reason for the increased ethylene production during CR maturation is still unknown. A likely candidate is the increased production of NO during CR maturation (Wang *et al.*, 2010) with known potential to trigger ethylene biosynthesis (Garcá *et al.*, 2011). This is probably a consequence of massive *RNA* degradation via xanthine dehydrogenase (Chapter I; Wang *et al.*, 2010) to mediate P_i re-mobilisation and re-translocation to young actively-growing clusters (Massonneau *et al.*, 2001). At the same time, NO may also influence CR activity by contributing to the down-regulation of citrate catabolism in the TCA cycle via inhibition of aconitase (Neumann *et al*., 2010; Wang *et al*., 2010; Gupta *et al.*, 2012). If this hypothesis is correct, a more detailed understanding of CR maturation and CR function would require elucidation of the regulatory mechanisms behind P recycling, and particularly of *RNA* degradation initiated during the CR maturation process and of the interactions between NO and ethylene production.

Gene knockdown and overexpression studies by using transgenic white lupin plants can be a powerful tool to identify key gene(s) regulating cluster root formation and function. This approach has been already proven as a successful tool by use of an *Agrobacterium rhizogenes*-based transformation system (Uhde-Stone *et al.*, 2005). The outcome of this thesis offers numerous novel target genes and processes to be investigated by application of this approach. It would be also worthwhile to conduct comparative gene expression analyses similar to the present study by comparing cluster-rooted (e.g., *Lupinis albus*) and non cluster-rooted Lupin species (e.g., *Lupinus angustifolius*) to identify the key processes required for CR formation. To facilitate crop improvement, an Australian genome sequencing project for narrow-leafed lupin (*Lupinus angustifolius*) is currently under way. A 12 × bacterial artificial chromosome (BAC) library was developed, and 13985 BAC end-sequences (BESs) were generated, covering approximately 1% of narrow-leafed lupin genome (Gao *et al.*, 2011). This approach will provide a valuable source of sequence information for further molecular studies on P acquisition.

7 Literature

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9 Curriculum vitae

Personal information

Name: Zhengrui Wang

Date of birth: 30.05.1984

Place of birth: Shandong, China

Nationality: Chinese

Academic profile

2009 – 2014:	PhD student, University of Hohenheim, Germany. Thesis title 'Transcriptomics and hormonal regulation of cluster root development in phosphate-deficient white lupin'. Supervisor: Prof. Dr. Günter Neumann
2007 – 2009:	M.Sc, China Agricultural University, China Thesis title 'Comparison on root growth, nitrogen uptake and assimilation in maize cultivars released from different years in China'. Supervisor: Prof. Dr. Jianbo Shen.
2006 – 2007:	B.Sc, College of Resources and Environment, Shandong Agricultural University, China.
2003 – 2006:	B.Sc, College of Life Sciences, Shandong Agricultural University, China.

Technical skills

Axenic hydroponic cultivation system for studying of sucrose signalling effect on cluster root formation of white lupin;
Measurement of organic acids (malate, citrate);
Determination of PEPC, acid phosphatase activity;
Determination of sucrose concentration;

Root morphology measurements using WhiRhizo software;
Plant RNA extraction;
Quantitative real-time PCR.

Computer skills MS-office, SAS, GIMP, MapMan, PageMan.

Language skills English (fluent), Chinese (mother tongue).

Conferences attended

06.2012 The 8th Symposium of the International Society of Root Research, Dundee, Scotland. Poster presentation.

08.2013 The 17th International Plant Nutrition Colloquium, Istanbul, Turkey. Oral presentation.

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List of publications

Zhengrui Wang, Daniel Straub, Huaiyu Yang, Angelika Kania, Jianbo Shen, Uwe Ludewig, Günter Neumann. 2014. The regulatory network of cluster-root function and development in phosphate-deficient white lupin (*Lupinus albus* L.) identified by transcriptome sequencing. *Physiologia Plantarum*, published online. doi: 10.1111/ppl.12187.

Zhengrui Wang, Jianbo Shen, Uwe Ludewig, and Günter Neumann. 2014. A re-assessment of sucrose signalling involved in cluster root formation and function in phosphate-deficient white lupin (*Lupinus albus* L.). *Physiologia Plantarum*, published online. doi: 10.1111/ppl.12311.

Zhengrui Wang, A.B.M. Moshiur Rahman, Guoying Wang, Uwe Ludewig, Jianbo Shen, Günter Neumann. 2014. Hormonal interactions during cluster root development in phosphate-deficient white lupin (*Lupinus albus* L.). *Journal of Plant Physiology*, Accepted.

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gemäß § 8 Absatz 2 der Promotionsordnung der Universität Hohenheim zum Dr.sc.agr.

1. Bei der eingereichten Dissertation zum Thema
   'Transcriptomics and hormonal regulation of cluster root development in phosphate-deficient white lupin'
   handelt es sich um meine eigenständig erbrachte Leistung.
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