New Phytologist

Proteaceae from severely phosphorus-impoverished soils extensively replace phospholipids with galactolipids and sulfolipids during leaf development to achieve a high photosynthetic phosphorus-use-efficiency

Hans Lambers[1], Gregory R. Cawthray[1], Patrick Giavalisco[2], John Kuo[3], Etienne Laliberté[1], Stuart J. Pearse[1], Wolf-Rüdiger Scheible[2,4], Mark Stitt[2], François Teste[1] and Benjamin L. Turner[1,5]

[1]School of Plant Biology, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009, Australia; [2]Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476, Potsdam-Golm, Germany; [3]Centre for Microscopy and Microanalysis, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009, Australia; [4]Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, OK, 73401, USA; [5]Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancón, Republic of Panama

Author for correspondence:
Hans Lambers
Tel: +61 8 64887381
Email: hans.lambers@uwa.edu.au

Received: *3 June 2012*
Accepted: *20 July 2012*

New Phytologist (2012) **196**: 1098–1108
doi: 10.1111/j.1469-8137.2012.04285.x

Key words: *Banksia*, galactolipids, *Hakea*, phospholipids, photosynthetic phosphorus-use-efficiency (PPUE), sulfolipids.

Summary

- Proteaceae species in south-western Australia occur on severely phosphorus (P)-impoverished soils. They have very low leaf P concentrations, but relatively fast rates of photosynthesis, thus exhibiting extremely high photosynthetic phosphorus-use-efficiency (PPUE). Although the mechanisms underpinning their high PPUE remain unknown, one possibility is that these species may be able to replace phospholipids with nonphospholipids during leaf development, without compromising photosynthesis.
- For six Proteaceae species, we measured soil and leaf P concentrations and rates of photosynthesis of both young expanding and mature leaves. We also assessed the investment in galactolipids, sulfolipids and phospholipids in young and mature leaves, and compared these results with those on *Arabidopsis thaliana*, grown under both P-sufficient and P-deficient conditions.
- In all Proteaceae species, phospholipid levels strongly decreased during leaf development, whereas those of galactolipids and sulfolipids strongly increased. Photosynthetic rates increased from young to mature leaves. This shows that these species extensively replace phospholipids with nonphospholipids during leaf development, without compromising photosynthesis. A considerably less pronounced shift was observed in *A. thaliana*.
- Our results clearly show that a low investment in phospholipids, relative to nonphospholipids, offers a partial explanation for a high photosynthetic rate per unit leaf P in Proteaceae adapted to P-impoverished soils.

Introduction

South-western Australia is an ancient region known for its severely nutrient-impoverished soils (McArthur, 1991; Lambers *et al.*, 2012) and exceptionally high plant biodiversity (Hopper, 2009). Among the macronutrients, phosphorus (P) is the least available nutrient in this region, as a consequence of prolonged soil weathering (Lambers *et al.*, 2010; Laliberté *et al.*, 2012). Sulfur is one of the few macronutrients that is found at concentrations similar to that considered adequate for growth of crop plants in *Banksia* (Proteaceae) species in this region (Denton *et al.*, 2007). On the most severely P-impoverished soils, nonmycorrhizal Proteaceae are an important component of the vegetation (Pate & Bell, 1999). Under low-P conditions, plant species in this family typically form cluster roots that effectively 'mine' P by releasing large amounts of low-molecular-weight carboxylates (Lambers *et al.*, 2008).

P-starved leaves tend to have low rates of photosynthesis per unit leaf area, at least in crop plants (Brooks *et al.*, 1988; Rao & Terry, 1989; Fredeen *et al.*, 1990). Leaves of Proteaceae species from south-western Australia, however, exhibit relatively fast rates of photosynthesis, despite having extremely low leaf P concentrations ([P]) (Denton *et al.*, 2007). Consequently, some of these species exhibit a very high photosynthetic P-use-efficiency (PPUE) (Denton *et al.*, 2007; Lambers *et al.*, 2010). In view of dwindling phosphate rock reserves and increasing prices of P fertilizers (Gilbert, 2009), understanding the biochemical basis of this high PPUE would allow us to explore whether there are lessons for developing P-efficient crops (Lambers *et al.*, 2011).

In barley (*Hordeum vulgare*) grown in nutrient solution at a growth-limiting P supply, the major P fractions in leaves are nucleic acids (30%), free orthophosphate (26%), P-containing metabolites (26%) and phospholipids (17%) (Chapin & Bieleski, 1982). Phospholipids are a component of the plasmalemma and of tonoplast, chloroplast, and mitochondrial membranes (Härtel *et al.*, 2000; Andersson *et al.*, 2003; Jouhet *et al.*, 2004; Andersson *et al.*, 2005). Phospholipids also play a role in signalling during plant development and in plant responses to stress (Cowan, 2006). Therefore, when considering changes in P distribution that could affect PPUE in mature leaves, changes in the concentrations of orthophosphate, P-containing metabolites and nucleic acids and membrane lipid composition are the most likely candidates (Veneklaas *et al.*, 2012).

There is good evidence that rapid rates of photosynthesis require a fine balance between the concentrations of free phosphate and phosphorylated intermediates, and that photosynthesis is inhibited when free phosphate is depleted (Heldt *et al.*, 1977; Stitt & Quick, 1989; see Stitt *et al.*, 2010 for a recent review). The total concentration of P, adenine nucleotides and phosphorylated intermediates is constrained by the amount of phosphate in the cytoplasm. While there is evidence that shortage of phosphate in the cytosol and chloroplast can lead to remobilization of phosphate from the vacuole (Sharkey *et al.*, 1986; Mimura, 1995), little is known about how this process is regulated. Eudicots tend to accumulate orthophosphate in epidermal cells (Conn & Gilliham, 2010); however, *Hakea prostrata* R. Br. (Proteaceae) accumulates P in its mesophyll cells (Shane *et al.*, 2004). The accumulation of P in mesophyll cells may allow more efficient use of P for photosynthesis, which occurs in the mesophyll cells. Except for some studies indicating that enzyme concentrations of UDP-glucose pyrophosphorylase may increase in P-deficient plants (Ciereszko *et al.*, 2001), little is known about how photosynthesis can be optimized to maintain flux when the total amount of P available for intermediary metabolism is decreased.

In a recent paper (Lambers *et al.*, 2011), we hypothesized that a high PPUE might be partly attributable to a replacement of phospholipids by galactolipids or sulfolipids, which do not contain P. Upon P starvation of *Arabidopsis thaliana* plants, the phospholipid fraction in leaves declines from 36 to 19% (Dörmann & Benning, 2002) with a concomitant increase of galactolipids and sulfolipids. In P-replete plants, the thylakoid and the inner envelope membrane already contain quite high galactolipid concentrations, but other cellular membranes contain mainly phospholipids. During P-starvation, galactolipids are substituted for phospholipids in these extrachloroplastidic membranes (Härtel *et al.*, 2000; Dörmann, 2007). The replacement of phospholipids by other lipids in several membranes in response to P starvation is a dynamic and reversible process (Andersson *et al.*, 2003; Cruz-Ramírez *et al.*, 2006; Gaude *et al.*, 2008) and is seen in many plant species, including barley, oats (*Avena sativa*) and maize (*Zea mays*) (Tjellström *et al.*, 2008). However, replacement of phospholipids by other lipids, while preventing leaf death under severe P limitation, might inexorably lead to a decline in the rate of photosynthesis (Brooks *et al.*, 1988; Rao & Terry, 1989; Fredeen *et al.*, 1990).

Here we test the hypothesis that mature leaves of Proteaceae that occur naturally on severely P-impoverished soils and exhibit a very high PPUE (Denton *et al.*, 2007) invest relatively little P in phospholipids and predominantly use galactolipids and sulfolipids instead. We chose to test this hypothesis in a location that is well known for its high plant biodiversity (particularly Proteaceae) and its ancient, nutrient-impoverished soils, Lesueur National Park in south-western Australia (Hopper & Gioia, 2004) (Fig. 1). We compare the results on relative lipid composition in six Proteaceae species with those obtained on the model plant *Arabidopsis thaliana*, grown under both P-sufficient and P-starved conditions. This allows a comparison of the response of Proteaceae species from severely P-impoverished soils with that of a species commonly found in a relatively nutrient-rich habitat.

Materials and Methods

Site and species description

All sites were located in the Arrowsmith Region in Lesueur National Park (30°S, 115°E), north-east of Jurien Bay (220 km north of Perth) in south-western Australia (Figs 1, 2). Geological formations within this region are of Early Jurassic (e.g. Cockleshell Gully Formation) to Middle to Late Triassic (e.g. Lesueur Sandstone) age (Playford *et al.*, 1976). Soils on the uplands in Lesueur National Park are a complex mixture of siliceous sands,

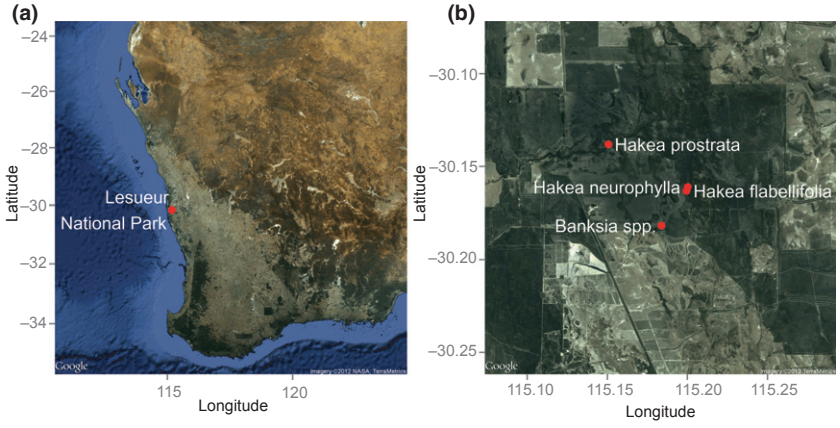

Fig. 1 Location of (a) Lesueur National Park in Western Australia and (b) the sites in Lesueur National Park where three *Banksia* and three *Hakea* species were sampled.

lateritic gravels, yellow texture-contrast soils, yellow massive earths and brown mottled cracking clays (Griffin & Burbidge, 1990); all are strongly weathered and invariably low in major plant nutrients, particularly P (McArthur, 1991). Lesueur National Park is well known for its high plant species diversity, particularly Proteaceae (Burbidge *et al.*, 1990); < 27 000 ha contain > 820 higher plant taxa (http://www.dec.wa.gov.au/). We collected leaves and soil in the pristine habitat of three *Banksia* and three *Hakea* species. Three *Banksia* species were sampled in the Lesueur Dissected Uplands (30.1836°S; 115.1524°E): *Banksia candolleana* Meisn., *Banksia attenuata* R.Br. and *Banksia menziesii* R.Br. (Figs. 1b, 2a). Two *Hakea* species were sampled in the Banovich Uplands (30.1621°S; 115.1993°E): *Hakea flabellifolia* Meisn. and *Hakea neurophylla* Meisn. (Figs. 1b, 2). A third *Hakea* species, *Hakea prostrata*, was sampled in the Lesueur Dissected Uplands, close to Cockleshell Gully (30.13907°S; 115.1507°E; Fig. 1b). All leaves had a healthy appearance and showed no visual signs of nutrient deficiency (such as leaf yellowing or anthocyanin accumulation), despite the extremely low soil P availability.

Soil sampling and analyses

In November 2010, soil was sampled close to the studied plants, but outside the main rooting zone, typically 1 m from the base of the stem. After digging a small 25-cm-deep pit, soil was sampled from the side of the pit, at three depths: 0–5, 5–10 and 10–15 cm. The samples were air-dried and then stored at 4°C in plastic bags, before being sent to the Smithsonian Tropical Research Institute in Panama for chemical analyses.

Soil pH was determined in a 1 : 2 soil to solution ratio in both water and 10 mM $CaCl_2$ using a glass electrode (Hendershot *et al.*, 2008). Total carbon (C) and nitrogen (N) were determined by automated combustion and thermal conductivity detection on a Thermo Flash EA112 analyser (CE Elantech, Lakewood, NJ, USA).

Total P was determined by ignition (550°C for 1 h) and acid extraction (1 M H_2SO_4 for 16 h), with detection by automated molybdate colourimetry on a Lachat Quickchem 8500 (Hach Ltd, Loveland, CO, USA). Readily exchangeable phosphate (resin P) was determined by extraction with anion-exchange membranes (Turner & Romero, 2009). It is assumed that this fraction is easily available for most plants given that it is readily exchangeable; it includes free phosphate in solution, phosphate sorbed to surfaces that can exchange with anions on the resin, and some acid-labile organic and condensed inorganic phosphates (Cheesman *et al.*, 2010).

Leaf gas exchange

Gas exchange was measured on young, expanding leaves and on mature, fully expanded leaves, after which the leaves were quickly frozen in liquid N_2 for subsequent analysis of phospholipids and other lipids. We measured at least four intact, attached, young, expanding (current year) and mature (previous year) leaves of at least three replicate plants each, using an LI-6400 portable gas-exchange system (Li-Cor, Lincoln, NE, USA) at ambient pCO_2 and temperature (400 μmol mol^{-1} and 25–30°C, respectively). Photosynthetically active radiation was set at 1500 μmol quanta m^{-2} s^{-1} (LI-6400-02B red-blue light source; Li-Cor). Measurements were taken on two separate days in November and December 2010. All *Banksia* species and *H. flabellifolia* were measured in November only, whereas *H. neurophylla* was measured both in November and December. No significant differences were found between the gas exchange measurements of the one species (*H. neurophylla*) that were taken on the separate days. Following the gas-exchange measurements, leaves were harvested to determine leaf area, then oven-dried for total leaf P analysis.

Leaf P analyses

The dry mass of leaves that were used for leaf gas-exchange measurements was determined after drying for 48 h at 70°C. The material was then finely ground with a stainless steel ball mill and subsamples were digested in concentrated HNO_3 : $HClO_4$ (3 : 1) which was then analysed for P using the malachite-green method (Motomizu *et al.*, 1983).

Lipid analyses

Leaf material collected from the same plants as those used for gas-exchange measurements was snap-frozen in liquid N immediately after field collection. Fresh weights were determined and the samples were transferred on dry ice to the Max Planck Institute of Molecular Plant Physiology (Potsdam, Germany). Approximately 40 mg of fresh weight material (range 35.6–49.9 mg) was ground to a fine powder using a Mixer Mill (MM300; Retsch GmbH, Haan, Germany). The lipid extraction, UPLC-FT-MS (Ultra Performance Liquid Chromatography-Fourier Transform-Mass Spectrometry) analysis and peak extraction were essentially performed as previously described (Giavalisco *et al.*, 2011; Hummel *et al.*, 2011). The peak signal intensities of the annotated

Fig. 2 Habitat of the investigated *Banksia* and *Hakea* species in Lesueur National Park, near Jurien Bay in Western Australia (Photos: Marion Cambridge).

lipids were finally normalized against an internal standard as well as the fresh weights of the individual samples, so that the arbitrary units represent signal intensity per unit fresh weight and are a proxy for lipid concentrations.

Growth of *Arabidopsis thaliana*

To compare the results for highly P-efficient Proteaceae with those for a model species, P-sufficient and P-starved *Arabidopsis thaliana* (L.) Heynh. plants for lipid analysis were grown at the Max Planck Institute of Molecular Plant Physiology (Potsdam, Germany). Seeds of the Columbia (Col-0) accession were germinated and seedlings grown for 1 wk in a 16-h light (250 μmol photons m^{-2} s^{-1}, 20°C and 75% relative humidity (RH)) 8-h dark (6°C and 75% RH) regime in a standard peat-vermiculite-sand (6 : 3 : 1) substrate (Stender AG, Luckau, Germany). After another 1 wk in an 8-h light (160 μmol photons m^{-2} s^{-1}, 20°C and 60% RH), 16-h dark (16°C and 75% RH) regime, individual plantlets were transferred to pots (6 cm diameter) filled with either standard substrate or a P-poor substrate (Kausek Gartenbau, Mittenwalde, FRG), and placed for another 4 wk in a Percival AR-36L2 growth chamber (Percival-Scientific, Perry, IA, USA) set to the same environmental conditions. The pots were irrigated twice a week with deionized water. All analysed leaf samples were harvested on the same day and within 1 h during the middle of the light period, by snap-freezing in liquid N. Lipid analysis was performed as already described.

Electron and fluorescence microscopy

Fresh, healthy and intact mature (young) leaves, cut into 3–5-mm-long pieces with a double-edged razor blade, were fixed in 2.5% (v/v) glutaraldehyde in phosphate-buffered saline (PBS) for 24 h. The fixed tissues were dehydrated in an ethanol series (70–95–100% dry ethanol), critical point-dried, mounted on SEM aluminium stubs, and coated with gold. Images were captured with a Zeiss 1555 field-emission variable-pressure scanning electron microscope (VP-FESEM; Carl Zeiss, Oberkochen, Germany) at 5 kV.

Leaf blades of *B. menziesii* and *H. prostata* were cross-sectioned with a double-edged razor blade, critical-pointed dried, and coated with gold. Images were taken with a Zeiss 1555 VP-FESEM at 5 Kv. The hand sections of freshly collected leaf blades were photographed under an excitation filter (G365) and an emission filter (LP 420) inserted into a beam of incident light from a mercury vapour lamp with a Zeiss Axioplan Microscope equipped with a Zeiss Axiocam digital camera.

Statistical analysis

Differences in leaf [P] and photosynthetic rates between leaf development stages and plant species were tested using linear mixed-effect models (Pinheiro & Bates, 2000), with random intercepts per individual plant (because more than one leaf was sampled from the same plant). The significance of differences in total signal intensities of phospholipids, galactolipids and sulfolipids between young and mature leaves was assessed using linear mixed-effect models, with random intercepts per plant species. In all cases, residuals were visually inspected for heteroscedasticity and different variance structures were specified if they significantly improved the models, as evaluated via likelihood ratio tests (Pinheiro & Bates, 2000). Analyses were conducted in the R Environment, using the 'nlme' package (Pinheiro & Bates, 2000).

Results

Soil analyses

As expected, concentrations of 'available' and total P in soil collected next to sampled plants were extremely low (Fig. 3). Resin-P values were <1 mg P kg^{-1} dry soil in the top 0–5 cm and further down the profile, with the exception of the top 0–5 cm at the site of *H. prostrata* which showed slightly higher values (1.7 mg P kg^{-1} dry soil) (Fig. 3a). *Hakea neurophylla* grows on a rocky substrate, exploiting cracks, and hence the collection of soil at depth was not always feasible.

Resin-P concentrations in soil collected from the Peron Slopes and Spearwood dunes (>120 000 yr old) (McArthur & Bettenay, 1974), where some of the sampled species occur, were similar to those shown in Fig. 3(a). For comparison, resin-P concentrations

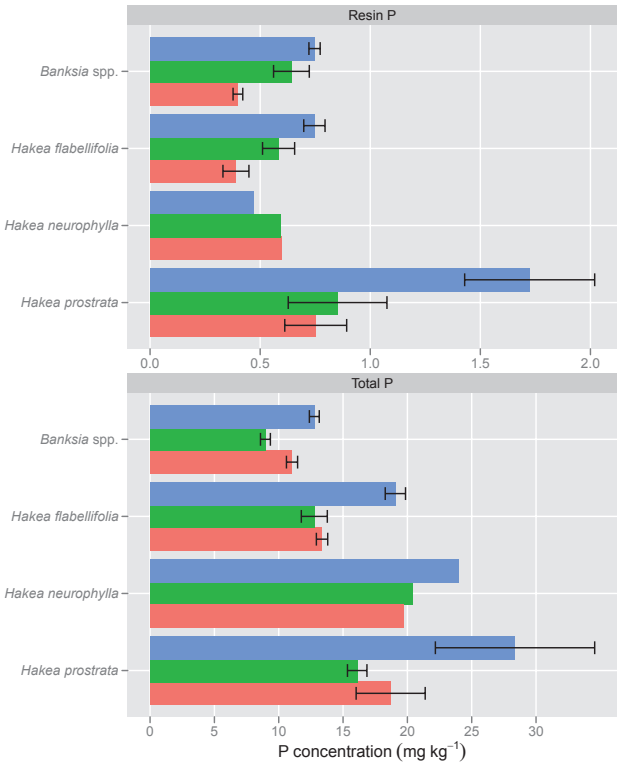

Fig. 3 Soil resin phosphorus (P) and total P from three depths (0–5 cm (blue), 5–10 cm (green) and 10–20 cm (red)) at the sampling sites (Fig. 1b) where the *Banksia* and *Hakea* species occur in their natural habitat. Resin P is considered the fraction that is readily available for most plants; however, Proteaceae species have access to a larger pool, as a result of their carboxylate-releasing cluster roots. Values are means ± SE ($n = 3$). *Hakea neurophylla* grows on a rocky substrate, exploiting cracks, and hence the collection of soil at depth was not always feasible; soil collected in three samples was pooled, and hence for this site no standard errors could be calculated.

in unfertilized crop and pasture soils are typically in the range of 20–40 mg P kg^{-1} dry soil (Hedley *et al.*, 1982).

Total P concentrations in the sampled soils were invariably very low, ranging from 9 to 25 mg kg^{-1} dry soil (Fig. 3b). For comparison, total-P concentrations in unfertilized crop and pasture soils are typically in the range of 550–770 mg P kg^{-1} dry soil (Hedley *et al.*, 1982).

All soils collected at locations where the studied species were sampled (Fig. 1b) were acidic, with a pH ($CaCl_2$) of 4–5, irrespective of location or soil depth.

Total [P] in mature and expanding leaves

Mature leaf [P] values of all six species were typically *c.* 200 μg g^{-1} leaf dry weight (Fig. 4), as found before for a range of *Banksia* and *Hakea* species growing in their natural habitat (Wright *et al.*, 2004; Denton *et al.*, 2007) and for *H. prostrata* grown in a glasshouse in soil collected from its native habitat (Shane & Lambers, 2005). Young expanding leaves had significantly greater ($P \leq 0.0001$) leaf [P] than mature leaves. This difference was consistent across all six species (species × leaf development stage interaction; $P = 0.087$). There were also significant differences in leaf [P] among species ($P = 0.025$), and *post hoc* Tukey tests showed that this was attributable to *H. prostrata* having significantly greater ($P = 0.039$) leaf [P] than *H. flabellifolia*, reflecting the higher availability of soil P in the habitat of *H. prostrata* (Fig. 3).

Photosynthesis

Differences in photosynthetic rate between young and mature leaves depended on species (species × leaf development stage interaction; $P \leq 0.0001$). However, in all six species, photosynthetic rates in young leaves were significantly ($P \leq 0.05$) lower than in mature leaves (Fig. 5).

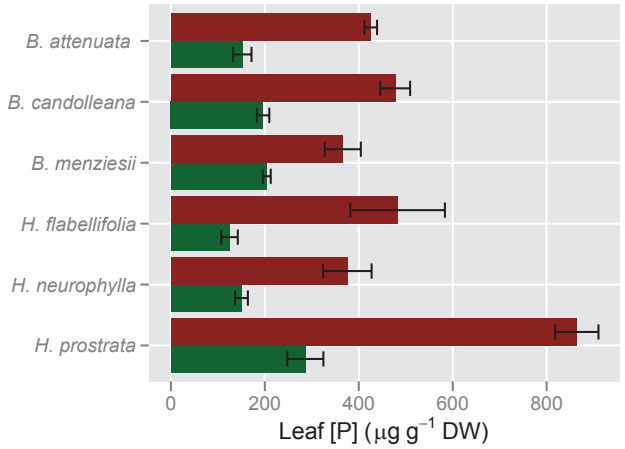

Fig. 4 Phosphorus (P) concentrations in young, expanding leaves (red bars) and in fully expanded, mature leaves (green bars) of *Banksia* and *Hakea* species growing in their natural habitat. Young expanding leaves had significantly greater ($P \leq 0.0001$) leaf [P] than mature leaves. Mature leaves were produced in the preceding year but were not senescent, because leaves of the investigated species continue to function for 2 yr or more. Values are means ± SE ($n = 3$).

Rates of photosynthesis on a leaf area basis of mature leaves of the three *Banksia* species (Fig. 5) were similar to published values for *Banksia* plants in their natural habitat in south-western Australia (Wright *et al.*, 2004; Denton *et al.*, 2007). Rates of photosynthesis of mature leaves of *H. prostrata* (Fig. 5) were similar to those of glasshouse plants grown in a natural soil without P addition (Shane & Lambers, 2005).

Young, expanding *Banksia* leaves were brown and pubescent, with abundant trichomes on both adaxial and abaxial leaf surfaces (Fig. 6), while expanding leaves of the *Hakea* species were reddish or brownish. Young expanding leaves either showed a net CO_2 release (*B. attenuata*, *B. candolleana* and *H. prostrata*) or significantly lower rates of net CO_2 uptake than mature leaves (*B. menziesii*, *H. flabellifolia* and *H. neurophylla*) (Fig. 5).

Lipid composition

The lipids in mature leaves, on average, comprised 77.7% galactolipids (sum of eight digalactosyldiacylglycerol and seven monogalactosyldiacylglycerol species) and 12.8% sulfolipids (six sulfoquinovosyl diacylglycerol compounds), and only 9.6% phospholipids (sum of nine phosphatidylcholine and seven phosphatidyl ethanolamine species) (Fig. 7). This contrasts markedly with the composition in young, expanding leaves, which contained less galactolipids (46.5%) and sulfolipids (7.5%), and markedly more phospholipids (46.0%). The fraction of phospholipids was especially high in young leaves of the three *Banksia* species (49–56%), while the fraction of phospholipids in young leaves of *Hakea* species leaves was 33–43%. The change in lipid composition did not simply reflect some dilution by lipids other than phospholipids, because signal intensities for total phospholipids normalized for fresh weight decreased significantly ($P \leq 0.0001$) from young to mature leaves, whereas those for galactolipids and sulfolipids significantly increased ($P \leq 0.0001$; Fig. 7).

We also compared changes in lipid concentrations between plant species and leaf development stages (young vs mature) for the four most important individual lipid species in each group (phospholipids, galactolipids and sulfolipids) (Fig. 8). Signal intensities (a proxy for lipid concentration) of the most important phospholipid species in mature leaves were invariably lower than those in young leaves in all six plant species (Fig. 8). Conversely, signal intensities of the most important galactolipids and sulfolipids were always greater in mature leaves, with the exception of SQDG 36:4; this sulfolipid compound, however, showed very low signal intensities overall, compared with the other three (Fig. 8). This confirms that the relative changes in lipid composition are not simply attributable to dilution, but are a result of the extensive replacement of phospholipids by galactolipids and sulfolipids during leaf development.

Lipid distribution in soil-grown *A. thaliana* wild-type Col-0 plants

To compare the present results with those for a species that typically grows in a relatively nutrient-rich habitat and which has

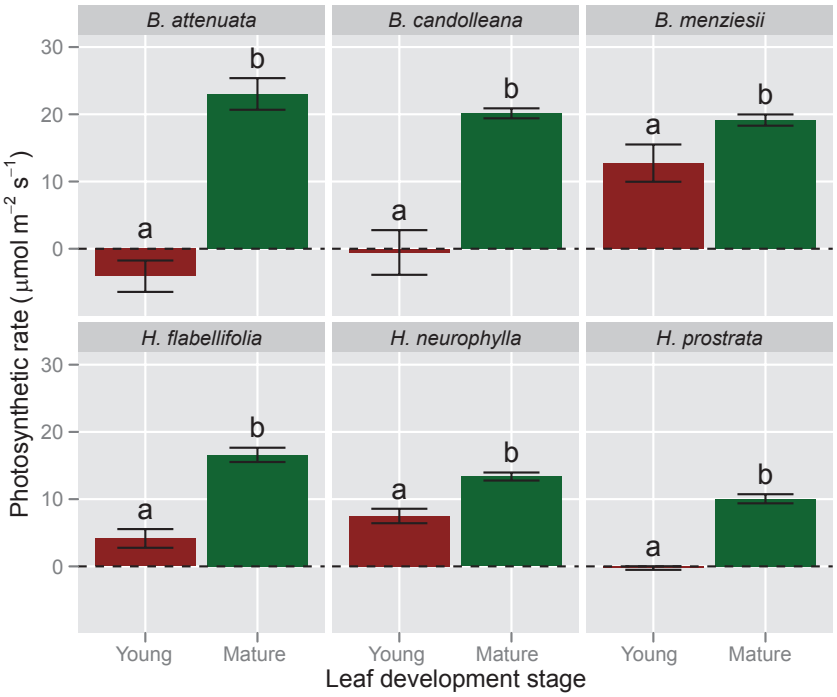

Fig. 5 Area-based rates of photosynthesis of young, expanding leaves (red bars) and of fully expanded, mature leaves (green bars) of *Banksia* and *Hakea* species growing in their natural habitat. Mature leaves were produced in the preceding year but were not senescent, because leaves of the investigated species continue to function for 2 yr or more. Values are means ± SE; at least three measurements were taken on at least three plants. For each species, different letters indicate significant ($P \leq 0.05$) differences following *post hoc* Tukey tests.

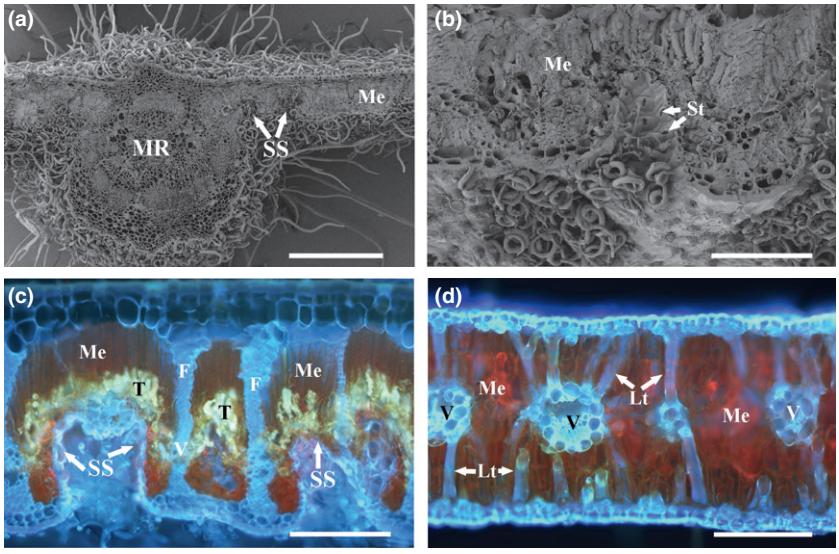

Fig. 6 (a) Scanning electron microscopy (SEM) image of a cross-section of a young *Banksia attenuata* leaf blade showing a midrib (MR), dense trichomes covering both surfaces and sunken stomata (SS) on the abaxial surface; bar, 500 μm. (b) SEM image of a group of stomata (St) in a stomatal crypt of a *Banksia menziesii* leaf; bar, 200 μm. (c) Fluorescent image of a cross-section of a *B. menziesii* leaf blade showing sunken stomata (SS), tannin-rich materials (T) appearing as yellow in mesophyll tissues (Me). Note that lignified and thick-walled fibrous bundles (F) are dividing mesophyll tissue (Me) into segments; bar, 200 μm. (d) Fluorescent image of a cross-section of a leaf blade of *Hakea prostrata* showing laticifer-like structures (Lt) distributed among mesophyll (Me), which appear to be connecting vascular bundles (V) and the epidermis; bar, 200 μm.

been previously investigated, we grew *A. thaliana* under both P-sufficient and P-limited conditions and analysed lipid composition in very young and mature leaves (Fig. 9). While the expected decrease in the phospholipid fraction occurred when *A. thaliana* was grown under P-deficient conditions, even the oldest leaves of plants grown in severely P-limiting conditions still showed *c.* 41% of phospholipids, as opposed to an average of 10% in the mature leaves of the Proteaceae species. Leaves of P-sufficient plants showed a decrease in the fraction of phospholipids with leaf development, but this is far less drastic than the change with development in Proteaceae species. The proportional decrease in phospholipids under P-deficient conditions was largely accounted for by an increase in galactolipids and sulfolipids (Fig. 9). The results clearly demonstrate that the shift from phospholipids to other lipids is far more pronounced in the six Proteaceae species than in *A. thaliana*.

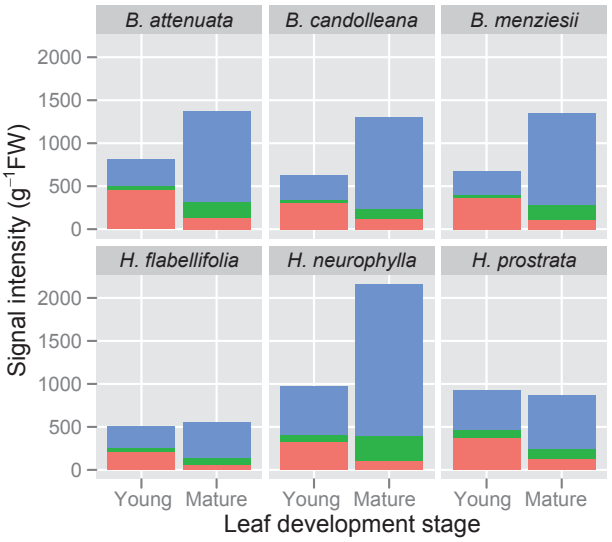

Fig. 7 Total signal intensity, normalized for fresh weight, for groups of lipids in (left) young, expanding, and (right) mature leaves of *Banksia* and *Hakea* species. Mature leaves were produced in the preceding year but were not senescent, because leaves of the investigated species continue to function for 2 yr or more. The signal intensities of the different components of the three groups of lipids (galactolipids, blue; sulfolipids, green; phospholipids, red) were summed. The justification for such summation is that all components of the groups show the same trends, as illustrated in Fig. 8.

Discussion

Soils in the natural habitat of six Proteaceae were severely P-impoverished, mature leaf [P] was very low, and PPUE was high, as previous studies had shown (Denton *et al.*, 2007; Lambers *et al.*, 2010). Most importantly, our results show, for the first time, that these Proteaceae species extensively replace phospholipids in mature leaves with lipids that do not contain P (i.e. galactolipids and sulfolipids), thus demonstrating that savings can be made in this P pool to a previously unknown extent, and offering a partial molecular explanation for their extremely high PPUE.

Total leaf [P]

As observed for south-western Australian *Banksia* and *Hakea* species (Wright *et al.*, 2004; Shane & Lambers, 2005; Denton *et al.*, 2007), mature leaf [P] was very low, without any visual signs of P deficiency. Interestingly, [P] of expanding leaves was about twice as high as that in mature leaves. This is partly accounted for by an increase in sclerenchymatic tissue that is associated with increasing leaf toughness during leaf development; that is, by 'dilution'. However, there was probably also a change in chemical composition during leaf development, when the prominently present brownish or reddish pigments in soft expanding leaves disappeared. These pigments may have been metabolized or they could be masked by the increase in chlorophyll concentration. This change in colour and the gradual build-up of photosynthetic capacity are similar to the phenomenon of 'delayed greening' in rainforest plants (Kursar & Coley, 1992).

The decline in leaf [P] following full leaf expansion agrees with the 'growth rate hypothesis' (Elser *et al.*, 2003). The demand for ribosomal RNA, and thus for P, would be relatively high during leaf expansion when rates of protein synthesis are higher. In contrast, in mature leaves, ribosomal RNA is required only to sustain protein turnover and hence investment in this fraction can be less (Lambers *et al.*, 2010; Veneklaas *et al.*, 2012). This aspect clearly requires further investigation.

Photosynthesis

Rates of photosynthesis were relatively high, with values for *Banksia* species being typically higher than those for *Hakea* species. As shown in Fig. 6, *B. menziesii* has sunken stomata as typically found for thick-leaved *Banksia* species; the depth of the stomatal crypt is closely correlated with the thickness of the leaf (Hassiotou *et al.*, 2009). Conversely, the thick leaves of *H. prostrata* do not have stomatal crypts (Fig. 6). Unlike *Banksia* species, which commonly have several stomata located in crypts, *Hakea* species only occasionally have sunken stomata in shallow individual crypts (Groom *et al.*, 1997; Jordan *et al.*, 2008). The absence of deep stomatal crypts with multiple stomata leads to a longer path for CO_2 diffusion from the air to the chloroplasts (Roth-Nebelsick *et al.*, 2009). The lack of deep stomatal crypts possibly accounts for the lower rates of photosynthesis in *Hakea* species compared with those in *Banksia* (Fig. 5).

Given the relatively high rates of photosynthesis and very low mature leaf [P], PPUE values would be high (Lambers *et al.*, 2010, 2011). We estimated PPUE by combining data on photosynthesis per unit leaf area (Fig. 5), data on leaf [P] per unit leaf dry weight, obtained for similar leaves of nearby plants (Fig. 4), and values for leaf area per unit dry weight, also for similar leaves of nearby plants. Although PPUE should ideally be estimated for the same leaves, the average estimated PPUE value for the six Proteaceae species was 305 μmol CO_2 g^{-1} P s^{-1}, with values as high as 488 μmol CO_2 g^{-1} P s^{-1} for *B. attenuata*, the lowest being 169 μmol CO_2 g^{-1} P s^{-1} for *H. prostrata*. These rates of photosynthesis expressed per unit leaf P are remarkably high, as found before for Proteaceae from south-western Australia (Lambers *et al.*, 2010). Global average values for PPUE, as determined under field conditions, are 103 μmol CO_2 g^{-1} P s^{-1} (Wright *et al.*, 2004). Values for PPUE vary by an order of magnitude at any value for leaf mass per unit leaf area (LMA), and this correlated with variation in leaf N concentration (Reich *et al.*, 2009). Mean values for PPUE are 59 mol CO_2 g^{-1} s^{-1} for leaves with N : P < 15, whereas PPUE is 129 mol CO_2 g^{-1} s^{-1} for leaves with N : P > 15 (Wright *et al.*, 2004), but the biochemical basis of the N-linked difference in PPUE remains unclear.

The extensive replacement of phospholipids by galactolipids and sulfolipids offers a partial explanation for the high PPUE values of the present Proteaceae. However, as phospholipids represent *c.* 20% of all P in leaves of plants grown at a limiting P supply (Chapin & Bieleski, 1982; Poirier *et al.*, 1991), additional factors must play an important role as well (Veneklaas *et al.*, 2012). Preferential allocation of orthophosphate to mesophyll cells in *H. prostrata* (Proteaceae) (Shane *et al.*, 2004), close to where photosynthesis occurs, may also contribute to a high PPUE. This allocation pattern differs from what is generally found in eudicots,

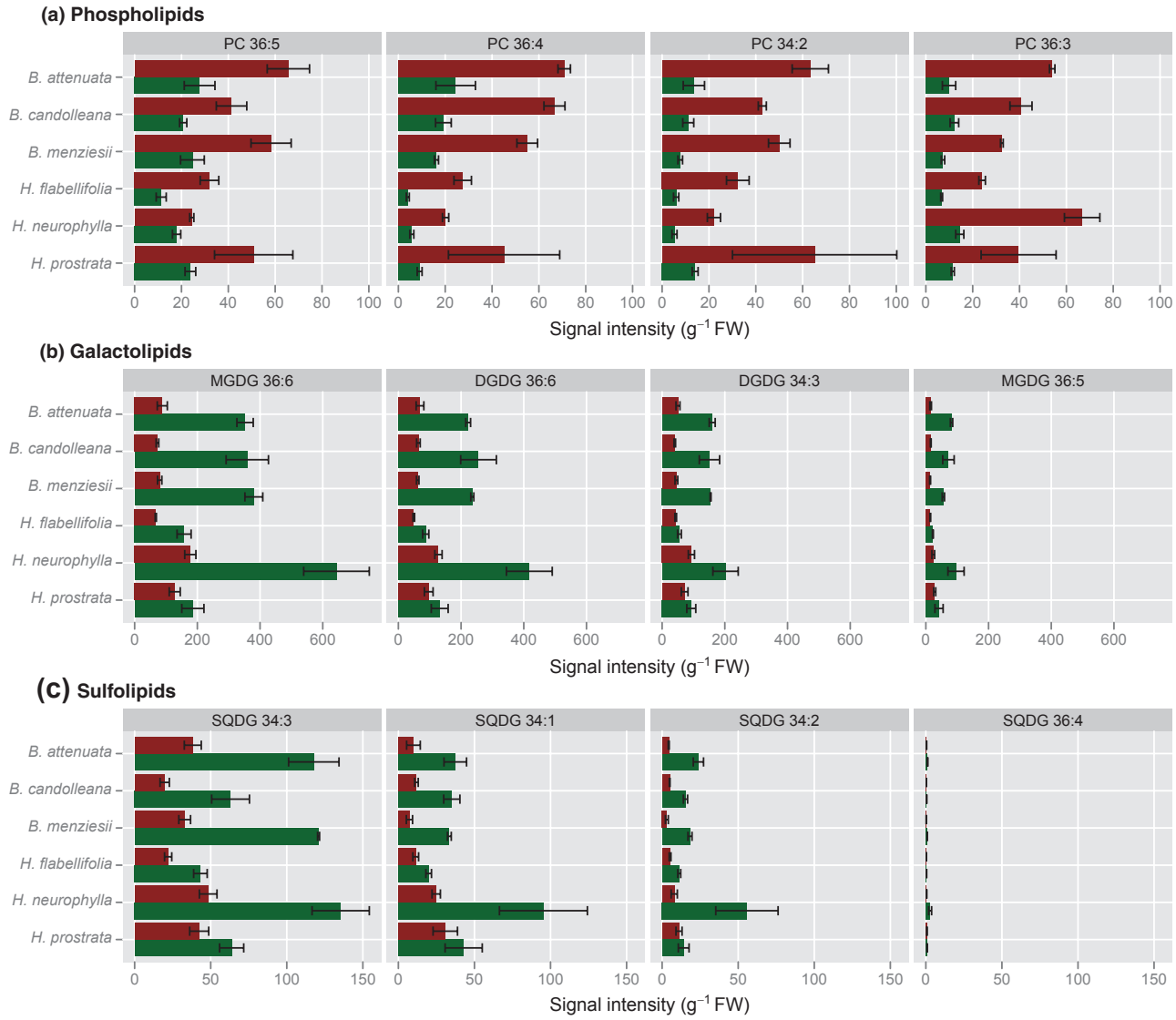

Fig. 8 Signal intensity, normalized for fresh weight, for the four most important individual (a) phospholipids, (b) galactolipids, and (c) sulfolipids in young and mature leaves of six *Banksia* and *Hakea* species. Values are means ± SE ($2 \leq n \leq 4$). Mature leaves were produced in the preceding year but were not senescent, because leaves of the investigated species continue to function for 2 yr or more. The values for young and mature leaves are shown as red and green bars, respectively, and the order of the Proteaceae species in each panel is given in the top left panel. Numbers refer to fatty acid chain length and number of double bonds in the chain, respectively.

which tend to accumulate orthophosphate in epidermal cells (Conn & Gilliham, 2010). The significance of both the phosphorylated intermediates and the ribosomal RNA fraction (Lambers *et al.*, 2010; Veneklaas *et al.*, 2012) is currently being studied.

Lipids

During leaf development, the fraction of phospholipids declined three- to five-fold (dependent on the Proteaceae species), and phospholipids were replaced to a major extent by galactolipids and to a lesser extent by sulfolipids. Replacement of phospholipids by galactolipids or sulfolipids is also considered a hallmark of P starvation (Tjellström *et al.*, 2008) and has been described for a range of species upon P starvation, including *A. thaliana*, barley, oats and maize (Dörmann & Benning, 2002; Tjellström *et al.*, 2008). The transcription of genes involved in the synthesis of galactolipids and sulfolipids is up-regulated rapidly under P starvation in leaves of *A. thaliana* (e.g. Hammond *et al.*, 2003; Morcuende *et al.*, 2007) and other plant species (e.g. Hammond *et al.*, 2011). The three- to four-fold decline of the phospholipid fraction during leaf development, for example, 56.2–9.6% in *B. attenuata* (Fig. 6), is much greater than what is observed in the comparison of young and mature leaves of P-stressed *A. thaliana*, where the phospholipid fraction declined by less than two-fold, from *c.* 59 to 41% (Fig. 9). Moreover, the decline of phospholipids observed in the comparison of mature leaves from P-sufficient and P-starved *A. thaliana* plants is much smaller: *c.* 63 to 41% (Fig. 9) or 36–19% (Dörmann & Benning, 2002). Also remarkable is that the replacement in the present six Proteaceae species occurred without any signs of P deficiency of the leaves and while

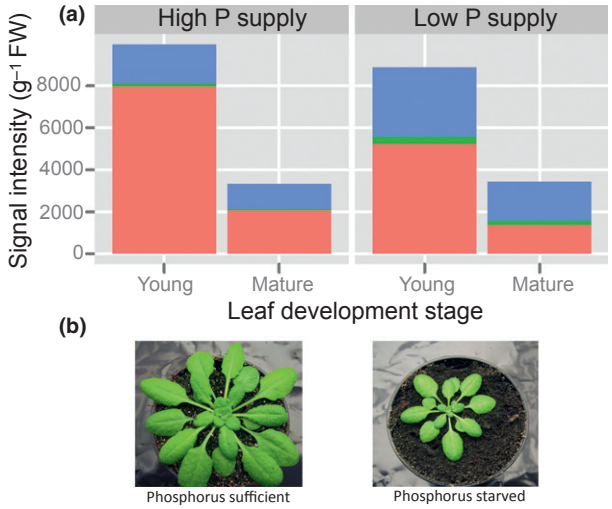

Fig. 9 (a) Total signal intensity for groups of lipids in (left) young and (right) old leaves of 6-wk old *Arabidopsis thaliana* plants grown under P-sufficient (left) or P-starved (right) conditions. Galactolipids, blue; sulfolipids, green; phospholipids, red. (b) Leaf developmental stages of 6-wk-old *Arabidopsis thaliana* plants grown under phosphorus-sufficient (left) and phosphorus-starved (right) conditions.

maintaining high photosynthetic activities (Fig. 5), whereas rates of photosynthesis decrease dramatically in barley leaves when plants are grown with a limiting P supply (Foyer & Spencer, 1986). In fact, increasing the P supply to *H. prostrata* grown in a natural soil collected from its native habitat to that commonly used for crop plants only marginally increases rates of photosynthesis of glasshouse-grown plants, and markedly reduces it when the P supply is increased further, when P-toxicity symptoms develop (Shane & Lambers, 2005).

Why would phospholipids be a major component in expanding leaves and then be replaced or diluted by other lipids at a later developmental stage? Phosphorus deficiency causes phospholipid replacement in membranes in a range of species (Härtel *et al.*, 2000; Dörmann & Benning, 2002; Dörmann, 2007; Tjellström *et al.*, 2008). Considering the very low rates of photosynthesis in young, expanding leaves compared with mature ones, this shift may reflect increased investment in chloroplast membranes (Forde & Steer, 1976). Galactolipids are a major and phospholipids only a minor component of chloroplast membranes (Bahl *et al.*, 1976; Dörmann, 2007). Increased investment of galactolipids and sulfolipids in chloroplast membranes of fully expanded leaves cannot entirely explain the shift we observed. During development, organelles other than chloroplasts are actively built up, and in their membranes phospholipid must have been replaced by other lipids as a result of P shortage. It is likely that Proteaceae have adapted to this situation, and that membrane perturbation deriving from phospholipid replacement is minimized in a manner that deserves further investigation. In addition, phospholipids play a role in signalling during plant development and this may require greater investment in phospholipids during leaf expansion (Cowan, 2006); however, it is not clear if that signalling component is quantitatively important. The plasma membrane leaflet facing the apoplast (probably the major water permeability barrier) contains only trace amounts of galactolipids (Tjellström *et al.*, 2010). Phospholipids possibly play a vital role in the plasma membrane and tonoplast when they require a high degree of lipid order, during leaf expansion. This aspect deserves further study, if we wish to exploit this trait linked to a high PPUE in P-efficient crop plants.

Concluding remarks

In south-western Australia, Proteaceae are very successful at growing on the world's most P-impoverished soils. They exhibit very low mature leaf [P] and very high PPUE. While the lipid fraction of young, expanding leaves of the studied species, on average, contains 46.0% phospholipids, mature leaves show as little as 9.6% phospholipids. This shift is much greater than what is known for other species and we clearly showed that it is not simply attributable to dilution by other lipids during normal leaf development. The reduction in the phospholipid fraction from young to mature leaves indicates that these Proteaceae species extensively replace phospholipids with nonphospholipids during leaf development. This coincides with relatively high rates of photosynthesis and no signs of P deficiency of mature leaves. This P investment pattern offers a partial explanation for the high PPUE of the investigated species. Further research is warranted to explore whether this mechanism to increase PPUE is worth applying in future crop plants, in view of dwindling rock phosphate reserves and increasing P-fertilizer prices (Gilbert, 2009).

Acknowledgements

We thank the Western Australia Department of Environment and Conservation (DEC) for their permission to collect leaf and soil samples. HL was supported by the Australian Research Council (ARC), and EL was supported by Research Fellowships from the University of Western Australia and the ARC. WRS, PG and MS acknowledge support from the Max-Planck Society. Graham Zemunik helped with field work and Dana Schindelasch (Max-Planck Institute of Molecular Plant Physiology) with *A. thaliana* plant growth. We thank John Raven for his critical comments on an earlier version of this manuscript and for providing valuable background information. We also thank the anonymous reviewers for their valuable input, and the suggestion to add a couple of sentences in the section on phospholipids and possible adaptations in Proteaceae.

References

Andersson MX, Larsson KE, Tjellström H, Liljenberg C, Sandelius AS. 2005. Phosphate-limited oat. *Journal of Biological Chemistry* **280**: 27578–27586.

Andersson MX, Stridh MH, Larsson KE, Liljenberg C, Sandelius AS. 2003. Phosphate-deficient oat replaces a major portion of the plasma membrane phospholipids with the galactolipid digalactosyldiacylglycerol. *FEBS Letters* **537**: 128–132.

Bahl J, Francke B, Monéger R. 1976. Lipid composition of envelopes, prolamellar bodies and other plastid membranes in etiolated, green and greening wheat leaves. *Planta* **129**: 193–201.

Brooks A, Woo KC, Wong SC. 1988. Effects of phosphorus nutrition on the response of photosynthesis to CO_2 and O_2, activation of ribulose bisphosphate carboxylase and amounts of ribulose bisphosphate and 3-phosphoglycerate in spinach leaves. *Photosynthesis Research* **15**: 133–141.

Burbidge AA, Hopper SD, Van Leeuwen S, eds. 1990. *A report to the Environmental Protection Authority from the Department of Conservation and Land Management.* Bulletin 424. Perth, Australia: Environmental Protection Authority.

Chapin FS, Bieleski RL. 1982. Mild phosphorus stress in barley and a related low-phosphorus-adapted barleygrass: phosphorus fractions and phosphate absorption in relation to growth. *Physiologia Plantarum* **54**: 309–317.

Cheesman AW, Turner BL, Reddy KR. 2010. Interaction of phosphorus compounds with anion-exchange membranes: implications for soil analysis. *Soil Science Society of America Journal* **74**: 1607–1612.

Ciereszko I, Johansson H, Hurry V, Kleczkowski LA. 2001. Phosphate status affects the gene expression, protein content and enzymatic activity of UDP-glucose pyrophosphorylase in wild-type and *pho* mutants of *Arabidopsis*. *Planta* **212**: 598–605.

Conn S, Gilliham M. 2010. Comparative physiology of elemental distributions in plants. *Annals of Botany* **105**: 1081–1102.

Cowan A. 2006. Phospholipids as plant growth regulators. *Plant Growth Regulation* **48**: 97–109.

Cruz-Ramírez A, Oropeza-Aburto A, Razo-Hernández F, Ramírez-Chávez E, Herrera-Estrella L. 2006. Phospholipase DZ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in *Arabidopsis* roots. *Proceedings of the National Academy of Sciences, USA* **103**: 6765–6770.

Denton MD, Veneklaas EJ, Freimoser FM, Lambers H. 2007. *Banksia* species (Proteaceae) from severely phosphorus-impoverished soils exhibit extreme efficiency in the use and re-mobilization of phosphorus. *Plant, Cell & Environment* **30**: 1557–1565.

Dörmann P 2007. *Galactolipids in plant membranes.* Encyclopedia of Life Sciences: eLS. Chichester, UK: John Wiley & Sons Ltd. http://www.els.net. doi: 10.1002/9780470015902.a0020100.

Dörmann P, Benning C. 2002. Galactolipids rule in seed plants. *Trends in Plant Science* **7**: 112–118.

Elser JJ, Acharya K, Kyle M, Cotner J, Makino W, Markow T, Watts T, Hobbie S, Fagan W, Schade J *et al.* 2003. Growth rate – stoichiometry couplings in diverse biota. *Ecology Letters* **6**: 936–943.

Forde J, Steer MW. 1976. The use of quantitative electron microscopy in the study of lipid composition of membranes. *Journal of Experimental Botany* **27**: 1137–1141.

Foyer C, Spencer C. 1986. The relationship between phosphate status and photosynthesis in leaves. Effects on intracellular orthophosphate distribution, photosynthesis and assimilate partitioning. *Planta* **167**: 369–375.

Fredeen AL, Raab TK, Rao IM, Terry N. 1990. Effects of phosphorus nutrition on photosynthesis in *Glycine max* (L.) Merr. *Planta* **181**: 399–405.

Gaude N, Nakamura Y, Scheible W-R, Ohta H, Dörmann P. 2008. Phospholipase C5 (NPC5) is involved in galactolipid accumulation during phosphate limitation in leaves of *Arabidopsis*. *Plant Journal* **56**: 28–39.

Giavalisco P, Li Y, Matthes A, Eckhardt A, Hubberten H-M, Hesse H, Segu S, Hummel J, Köhl K, Willmitzer L. 2011. Elemental formula annotation of polar and lipophilic metabolites using ^{13}C, ^{15}N and ^{34}S isotope labelling, in combination with high-resolution mass spectrometry. *The Plant Journal* **68**: 364–376.

Gilbert N. 2009. The disappearing nutrient. *Nature* **461**: 716–718.

Griffin EA, Burbidge AA 1990. Vegetation. In: Burbidge AA, Hopper SD, Van Leeuwen D eds. *A report to the Environmental Protection Authority from the Department of Conservation and Land Management.* Bulletin 424. Perth, Australia: Environmental Protection Authority, 15–24.

Groom PK, Lamont BB, Markey AS. 1997. Influence of leaf type and plant age on leaf structure and sclerophylly in *Hakea* (Proteaceae). *Australian Journal of Botany* **45**: 827–838.

Hammond JP, Bennett MJ, Bowen HC, Broadley MR, Eastwood DC, May ST, Rahn C, Swarup R, Woolaway KE, White PJ. 2003. Changes in gene expression in *Arabidopsis* shoots during phosphate starvation and the potential for developing smart plants. *Plant Physiology* **132**: 578–596.

Hammond JP, Broadley MR, Bowen HC, Spracklen WP, Hayden RM, White PJ. 2011. Gene expression changes in phosphorus deficient potato (*Solanum tuberosum* L.) leaves and the potential for diagnostic gene expression Markers. *PLoS ONE* **6**: e24606.

Härtel H, Dörmann P, Benning C. 2000. DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **97**: 10649–10654.

Hassiotou F, Evans JR, Ludwig M, Veneklaas EJ. 2009. Stomatal crypts may facilitate diffusion of CO_2 to adaxial mesophyll cells in thick sclerophylls. *Plant, Cell & Environment* **32**: 1596–1611.

Hedley MJ, Stewart JWB, Chauhan BS. 1982. Changes in inorganic and organic soil phosphorus fractions induced by cultivation practices and by laboratory incubations. *Soil Science Society of America Journal* **46**: 970–976.

Heldt HW, Chon CJ, Maronde D, Herold A, Stankovic ZS, Walker DA, Kraminer A, Kirk MR, Heber U. 1977. Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiology* **59**: 1146–1155.

Hendershot WH, Lalande H, Duquette M 2008. Chapter 16: Soil reaction and exchangeable acidity. In: Carter MR, Gregorich EG, eds. *Soil sampling and methods of analysis, 2nd edn.* Boca Raton, FL, USA: Canadian Society of Soil Science and CRC Press, 173–178.

Hopper SD. 2009. OCBIL theory: towards an integrated understanding of the evolution, ecology and conservation of biodiversity on old, climatically buffered, infertile landscapes. *Plant and Soil* **322**: 49–86.

Hopper SD, Gioia P. 2004. The Southwest Australian Floristic Region: evolution and conservation of a global hotspot of biodiversity. *Annual Review of Ecology, Evolution and Systematics* **35**: 623–650.

Hummel J, Segu S, Li Y, Irgang S, Jueppner J, Giavalisco P. 2011. Ultra performance liquid chromatography and high resolution mass spectrometry for the analysis of plant lipids. *Frontiers in Plant Science* **2**: 1–17.

Jordan GJ, Weston PH, Carpenter RJ, Dillon RA, Brodribb TJ. 2008. The evolutionary relations of sunken, covered, and encrypted stomata to dry habitats in Proteaceae. *American Journal of Botany* **95**: 521–530.

Jouhet J, Maréchal E, Baldan B, Bligny R, Joyard J, Block MA. 2004. Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria. *The Journal of Cell Biology* **167**: 863–874.

Kursar TA, Coley PD. 1992. Delayed greening in tropical leaves: an antiherbivore defense? *Biotropica* **24**: 256–262.

Laliberté E, Turner BL, Costes T, Pearse SJ, Wyrwolll K-H, Zemunik G, Lambers H. 2012. Experimental assessment of nutrient limitation along a 2-million year dune chronosequence in the south-western Australia biodiversity hotspot. *Journal of Ecology* **100**: 631–642.

Lambers H, Bishop JG, Hopper SD, Laliberté E, Zúñiga-Feest A. 2012. Phosphorus-mobilisation ecosystem engineering: the roles of cluster roots and carboxylate exudation in young P-limited ecosystems. *Annals of Botany* **110**: 329–348.

Lambers H, Brundrett MC, Raven JA, Hopper SD. 2010. Plant mineral nutrition in ancient landscapes: high plant species diversity on infertile soils is linked to functional diversity for nutritional strategies. *Plant and Soil* **334**: 11–31.

Lambers H, Finnegan PM, Laliberté E, Pearse SJ, Ryan MH, Shane MW, Veneklaas EJ. 2011. Phosphorus nutrition of Proteaceae in severely phosphorus-impoverished soils: are there lessons to be learned for future crops? *Plant Physiology* **156**: 1058–1066.

Lambers H, Raven JA, Shaver GR, Smith SE. 2008. Plant nutrient-acquisition strategies change with soil age. *Trends in Ecology and Evolution* **23**: 95–103.

McArthur WM. 1991. *Reference soils of south-western Australia.* South Perth, Australia: Department of Agriculture Western Australia.

McArthur WM, Bettenay E. 1974. *Development and distribution of soils of the Swan Coastal Plain, Western Australia.* Melbourne, Australia: CSIRO.

Mimura T. 1995. Homeostasis and transport of inorganic phosphate in plants. *Plant and Cell Physiology* **36**: 1–7.

Morcuende R, Bari R, Gibon Y, Zheng W, Pant BD, Blasing O, Usadel B, Czechowski T, Udvardi MK, Stitt M, *et al.* 2007. Genome-wide reprogramming of metabolism and regulatory networks of *Arabidopsis* in response to phosphorus. *Plant, Cell & Environment* **30**: 85–112.

Motomizu S, Wakimoto T, Toei K. 1983. Spectrophotometric determination of phosphate in river waters with molybdate and malachite green. *Analyst* **108**: 361–367.

Pate JS, Bell TL. 1999. Application of the ecosystem mimic concept to the species-rich *Banksia* woodlands of Western Australia. *Agroforestry Systems* **45**: 303–341.

Pinheiro JC, Bates DM. 2000. *Mixed-effects models in S and S-PLUS.* New York, NY, USA: Springer.

Playford PE, Cockbain AE, Low GH. 1976. *Geology of the Perth Basin, Western Australia.* Perth, Australia: Geological Survey, Western Australia.

Poirier Y, Thoma S, Somerville C, Schiefelbein J. 1991. Mutant of *Arabidopsis* deficient in xylem loading of phosphate. *Plant Physiology* **97**: 1087–1093.

Rao IM, Terry N. 1989. Leaf phosphate status, photosynthesis, and carbon partitioning in sugar beet: I. Changes in growth, gas exchange, and Calvin cycle Enzymes. *Plant Physiology* **90**: 814–819.

Reich PB, Oleksyn J, Wright IJ. 2009. Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia* **160**: 207–212.

Roth-Nebelsick A, Hassiotou F, Veneklaas EJ. 2009. Stomatal crypts have small effects on transpiration: a numerical model analysis. *Plant Physiology* **151**: 2018–2027.

Shane MW, Lambers H. 2005. Manganese accumulation in leaves of Hakea prostrata(Proteaceae) and the significance of cluster roots for micronutrient uptake as dependent on phosphorus supply. *Physiologia Plantarum* **124**: 441–450.

Shane MW, McCully ME, Lambers H. 2004. Tissue and cellular phosphorus storage during development of phosphorus toxicity in *Hakea prostrata* (Proteaceae). *Journal of Experimental Botany* **55**: 1033–1044.

Sharkey TD, Stitt M, Heineke D, Gerhardt R, Raschke K, Heldt HW. 1986. Limitation of photosynthesis by carbon metabolism: II. O_2-insensitive CO_2 uptake results from limitation of triose phosphate utilization. *Plant Physiology* **81**: 1123–1129.

Stitt M, Lunn J, Usadel B. 2010. *Arabidopsis* and primary photosynthetic metabolism – more than the icing on the cake. *The Plant Journal* **61**: 1067–1091.

Stitt M, Quick WP. 1989. Photosynthetic carbon partitioning: its regulation and possibilities for manipulation. *Physiologia Plantarum* **77**: 633–641.

Tjellström H, Andersson MX, Larsson KE, Sandelius AS. 2008. Membrane phospholipids as a phosphate reserve: the dynamic nature of phospholipid-to-digalactosyl diacylglycerol exchange in higher plants. *Plant, Cell & Environment* **31**: 1388–1398.

Tjellström H, Hellgren LI, Wieslander Å, Sandelius AS. 2010. Lipid asymmetry in plant plasma membranes: phosphate deficiency-induced phospholipid replacement is restricted to the cytosolic leaflet. *The FASEB Journal* **24**: 1128–1138.

Turner BL, Romero TE. 2009. Short-term changes in extractable inorganic nutrients during storage of tropical rain forest soils. *Soil Science Society of America Journal* **73**: 1972–1979.

Veneklaas EJ, Lambers H, Bragg J, Finnegan PM, Lovelock CE, Plaxton WC, Price C, Scheible W-R, Shane MW, White PJ, *et al.* 2012. Opportunities for improving phosphorus-use efficiency in crop plants. *New Phytologist* **195**: 306–320.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC, Diemer M, *et al.* 2004. The worldwide leaf economics spectrum. *Nature* **428**: 821–827.