Lipid Biosynthesis and Protein Concentration Respond Uniquely to Phosphate Supply during Leaf Development in Highly Phosphorus-Efficient *Hakea prostrata*[1][W][OPEN]

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Hakea prostrata (Proteaceae) is adapted to severely phosphorus-impoverished soils and extensively replaces phospholipids during leaf development. We investigated how polar lipid profiles change during leaf development and in response to external phosphate supply. Leaf size was unaffected by a moderate increase in phosphate supply. However, leaf protein concentration increased by more than 2-fold in young and mature leaves, indicating that phosphate stimulates protein synthesis. Orthologs of known lipid-remodeling genes in Arabidopsis (*Arabidopsis thaliana*) were identified in the *H. prostrata* transcriptome. Their transcript profiles in young and mature leaves were analyzed in response to phosphate supply alongside changes in polar lipid fractions. In young leaves of phosphate-limited plants, phosphatidylcholine/phosphatidylethanolamine and associated transcript levels were higher, while phosphatidylglycerol and sulfolipid levels were lower than in mature leaves, consistent with low photosynthetic rates and delayed chloroplast development. Phosphate reduced galactolipid and increased phospholipid concentrations in mature leaves, with concomitant changes in the expression of only four *H. prostrata* genes, *GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE1*, *N-METHYLTRANSFERASE2*, *NONSPECIFIC PHOSPHOLIPASE C4*, and *MONOGALACTOSYLDIACYLGLYCEROL3*. Remarkably, phosphatidylglycerol levels decreased with increasing phosphate supply and were associated with lower photosynthetic rates. Levels of polar lipids with highly unsaturated 32:x (x = number of double bonds in hydrocarbon chain) and 34:x acyl chains increased. We conclude that a regulatory network with a small number of central hubs underpins extensive phospholipid replacement during leaf development in *H. prostrata*. This hard-wired regulatory framework allows increased photosynthetic phosphorus use efficiency and growth in a low-phosphate environment. This may have rendered *H. prostrata* lipid metabolism unable to adjust to higher internal phosphate concentrations.

Many Australian Proteaceae spp. are adapted to severely phosphorus (P)-impoverished soils and operate at very low leaf P status without compromising rates of photosynthesis (Denton et al., 2007; Lambers et al., 2012b; Sulpice et al., 2014). A large number of these species are endemic to the South West Australian Floristic Region, which features highly weathered, P-impoverished soils (Hopper, 2009; Laliberté et al., 2012; Hayes et al., 2014). Not only do southwestern Australian Proteaceae spp. use P-mining strategies to acquire inorganic phosphate (Pi; Shane et al., 2003; Shane and Lambers, 2005; Lambers et al., 2012a), but they also use leaf P very efficiently. They achieve this efficiency by allocating P to mesophyll cells rather than to epidermal cells (Shane et al., 2004; Lambers et al., 2015), as is common for other dicotyledons (Conn and Gilliham, 2010), by extensively replacing phospholipids by galactolipids and sulfolipids during leaf development (Lambers et al., 2012b) and by operating at very low ribosomal RNA (rRNA) levels in their leaves (Sulpice et al., 2014). The low abundance of rRNA economizes on P and also decreases the rate of protein synthesis and the resulting demand for P. In addition, many southwestern Australian Proteaceae spp. show delayed greening (Lambers et al., 2012b). Their young leaves have especially low levels of plastidic rRNA and a very low photosynthetic capacity. As the leaves develop, the levels of plastidic rRNA increase, concomitant with an increase in photosynthetic capacity, while their cytosolic rRNA levels decline (Sulpice et al., 2014).

The three major classes of polar membrane lipids in plants are phospholipids, glycoglycerolipids, and sphingolipids. During normal plant development, phospholipids are the dominant polar lipid fraction in plasma membranes, while the galactoglycerolipids

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monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are found exclusively in plastidic membranes (Moellering and Benning, 2011). Phospholipids are predominantly synthesized in the endoplasmic reticulum (ER; Nerlich et al., 2007; Gibellini and Smith, 2010) and constitute about one-third of the organic P pool (Bieleski, 1973; Poirier et al., 1991). Plants with low P status show lipid remodeling and replace some of their phospholipids with galactolipids and sulfolipids (Benning et al., 1995; Andersson et al., 2005; Hölzl and Dörmann, 2007; Tjellström et al., 2008; Shimojima and Ohta, 2011; Lambers et al., 2012b; Yuzawa et al., 2012; Shimojima et al., 2013). Genes involved in galactolipid and sulfolipid synthesis are strongly induced in response to low Pi supply in Arabidopsis (*Arabidopsis thaliana*), while those involved in de novo synthesis of phospholipids are repressed (Hammond et al., 2003; Misson et al., 2005; Morcuende et al., 2007; Müller et al., 2007; Lan et al., 2012; Woo et al., 2012).

During P-limited growth, DGDG is found in extraplastidic membranes, including plasma membranes, mitochondrial membranes, and the tonoplast (Härtel et al., 2000; Jouhet et al., 2004; Andersson et al., 2005). However, this extraplastidic pool of DGDG differs from the large pool associated with the chloroplasts (Douce and Joyard, 1990). In Arabidopsis, the plastidic pool is synthesized by the sequential activities of the type A MGDG synthase MGD1, in the inner envelope membrane, and the DGDG synthase DGD1, in the outer envelope membrane (Dörmann et al., 1999; Dubots et al., 2010). The extraplastidic pool is synthesized via the inducible type B MGDG synthases MGD2 and MGD3 as well as DGD2, which are all found in the outer envelope membrane (Kelly and Dörmann, 2002; Kelly et al., 2003; Benning and Ohta, 2005; Kobayashi et al., 2009b). For this low-P-dependent lipid remodeling to occur, extraplastidic phospholipids are hydrolyzed by either PHOSPHOLIPASE C (PLC) or PLD to produce the substrate for MGDG synthases, diacylglycerol (DAG). In the case of PLD, this occurs via phosphatidic acid (PA) and its subsequent dephosphorylation by either LIPID PHOSPHATE PHOSPHATASE (LPP) or PHOSPHATIDIC ACID PHOSPHOHYDROLASE (PAH) enzymes (Pierrugues et al., 2001; Qin and Wang, 2002; Jouhet et al., 2003; Andersson et al., 2005; Nakamura et al., 2007, 2009; Pokotylo et al., 2013). PLC action produces DAG and P-containing lipid head groups. To release Pi for use in other metabolic pathways, the activity of a unique phosphoethanolamine/phosphocholine phosphatase of the haloacid dehalogenase family, PECP1, is required, which is strongly induced in P-limited Arabidopsis (Misson et al., 2005; May et al., 2012). Transcripts encoding Arabidopsis PLDς2, and to a lesser extent PLDς1, isoforms are induced by Pi deprivation, and both enzymes function redundantly in phosphatidylcholine (PC) hydrolysis, contributing to DGDG accumulation in roots of P-limited plants but not in their shoots (Cruz-Ramírez et al., 2006; Li et al., 2006). The Arabidopsis PLC isoforms that show transcriptional responses to the plant's P status in roots and leaves are *NONSPECIFIC PHOSPHOLIPASE C4* (*NPC4*) and *NPC5*, respectively (Misson et al., 2005; Nakamura et al., 2005; Morcuende et al., 2007; Gaude et al., 2008). Alternatively, phospholipids can be deacylated by either phospholipase A in combination with lysophospholipase, phospholipase B, or lipid acyl hydrolase activities (Matos and Pham-Thi, 2009). GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE (GDPD) subsequently hydrolyzes glycerophosphodiesters such as glycerophosphocholine into glycerol-3-phosphate and choline (Cheng et al., 2011).

In addition to neutral galactolipids, anionic sulfolipids may also replace phospholipids in plastids of P-limited plants (Essigmann et al., 1998). Transcripts encoding all three enzymes in the sulfolipid biosynthetic pathway identified so far, the plastidic UDP-GLUCOSE PYROPHOSPHORYLASE3 (UGP3), the UDP-sulfoquinovose synthase SQD1, and the sulfoquinovosyldiacylglycerol (SQDG) synthase SQD2, are coordinately induced in Arabidopsis plants with low P status (Essigmann et al., 1998; Yu et al., 2002; Hammond et al., 2003; Shimojima, 2011). SQDG species play a role under P-limiting conditions, as demonstrated by the reduced growth of the *sqd2* mutant under Pi deficiency (Yu et al., 2002). Since SQDG is not able to fully compensate the photosynthetic impairment and growth inhibition in the Arabidopsis *plastidic phosphatidylglycerolphosphate synthase1* mutant, which features a 30% reduction in phosphatidylglycerol (PG) levels, it is not clear how SQDG contributes to maintaining metabolic functions in plants with low P status (Xu et al., 2002; Frentzen, 2004).

While the ability to replace phospholipids with galactolipids and sulfolipids in response to low P availability is highly conserved in plants (Yuzawa et al., 2012), little is known about the importance of this trait for the functioning of species in severely P-impoverished environments or the molecular mechanisms that allow lipid remodeling in these species. Here, we address the question of how this trait may contribute to the high P-use efficiency of a southwestern Australian Proteaceae species, *Hakea prostrata*. This is one of six Proteaceae species we previously studied in their natural habitat (Lambers et al., 2012b; Sulpice et al., 2014). Here, we analyzed changes in lipid profiles and the levels of transcripts encoding lipid remodeling enzymes during leaf development in glasshouse-grown *H. prostrata* plants. In addition, we assessed how lipid and associated transcript profiles respond to Pi availability. Our aim was to address the following questions: How are lipid-remodeling pathways regulated during leaf development in *H. prostrata*? Do components of these pathways in *H. prostrata* respond to external Pi availability? and Do the observed metabolite and transcript profiles of this species suggest unique adaptive responses to a severely P-impoverished environment?

RESULTS

Plant Biomass Is Not Responsive to Short-Term Changes in External Pi Supply in *H. prostrata*

We first analyzed the impact of the external Pi supply on plant growth. Growth at a standard application rate of 6 μmol Pi plant^{-1} d^{-1} induced cluster-root production

in all seedlings. When this application rate was continued, one-third of the plants produced new cluster roots during the 21-d treatment period. When Pi was withdrawn from the nutrient solution, plants had an average of 6 ± 2 cluster roots per plant at harvest. At the intermediate and high Pi supplies (23 and 171 μmol Pi plant^{-1} d^{-1}, respectively), no cluster roots were produced, and newly formed white root tips were much thicker and shorter than at lower Pi supplies. Shoot-to-root dry weight ratios were similar across the four Pi treatments, ranging from 3.1 ± 0.5 for P-limited plants that received no Pi during the treatment period to 2.5 ± 0.2 for plants growing at the highest Pi supply. Interestingly, despite showing clear signs of P toxicity, plants at the highest Pi supply produced the same number of leaves as plants under the standard and intermediate application rates (9 ± 3 leaves plant^{-1}), while plants without continuous Pi supply tended to produce fewer new leaves (5 ± 2 leaves plant^{-1}). Young leaf fresh weight tended to be greater at the intermediate Pi supply, indicating faster growth rates of young leaves. However, there was no final trend in the weights of individual mature and senescing leaves across treatments (Fig. 1A). Total plant biomass was similar across treatments (Fig. 1B). Plants at the highest Pi supply showed 36% lower chlorophyll concentrations on a leaf area basis (Supplemental Fig. S1A). Irrespective of Pi supply, anthocyanins accumulated in young leaves and senescing leaves but not in mature leaves (Supplemental Fig. S1B).

Leaf Pi Accumulation and Inhibition of Its Conversion into Organic P Compounds

Plants under our standard, intermediate, and highest Pi treatments took up all, more than 82%, and 62% of the available Pi, respectively, at the first solution exchange (Fig. 2A). Plants without Pi supply did not release Pi into the growth medium. Net Pi uptake rates remained the same over the remaining 18 d for plants at the intermediate Pi supply. By contrast, plants at the highest Pi supply reduced their Pi uptake rate by a further 50% over the following 13 d, and between 16 to 22 d they took up only about 35% of the provided Pi. Nevertheless, their final uptake rate of 55 μmol Pi plant^{-1} d^{-1} was still three times greater than that of plants at intermediate Pi supply. The rapid Pi uptake at the highest Pi supply led to significant Pi accumulation of about 45 μmol Pi g^{-1} fresh weight in both mature and senescing leaves (Fig. 2B).

Young leaves showed an increase in Pi concentration at higher external Pi supplies, but this increase was much smaller than that in mature or senescing leaves. This was most likely due to the greater ability of young leaves to convert Pi into organic phosphate (Po) compounds (Fig. 2B). In plants that did not receive any Pi during the treatment phase, the Po fraction in young leaves was three times greater (15 μmol P g^{-1} fresh weight) than in mature leaves (5 μmol P g^{-1} fresh weight, Fig. 2B; $P < 0.001$), indicating that *H. prostrata* reallocated resources to growing organs under these conditions (Lambers et al., 2012b). The Po concentration in young leaves increased by up to 3-fold in high external Pi treatments. This is similar to the increase in protein concentration in these leaves (see below). Mature and senescing leaves of P-limited *H. prostrata* plants had a free Pi concentration (1 μmol P g^{-1} fresh weight) that was approximately 50% less than that in young leaves ($P < 0.001$). While the free Pi pool in mature and senescing leaves increased more than 40-fold when high external Pi was supplied, their Po pool was much smaller than that in young leaves under these conditions.

The contribution of Po to the total P pool in young leaves was 85% across treatments, except at the highest Pi supply, where it was 74%. In mature and senescing leaves, the contribution of Po to the total P pool decreased with increasing Pi supply, from 84% to 25% and from 83% to 36%, respectively (Fig. 2B).

Leaf RNA, Protein, Photosynthetic Pigment, and Starch Concentrations

Consistent with higher Po concentrations, young leaves contained 285 ± 19 μg total RNA g^{-1} fresh weight, about twice as much as mature leaves (139 ± 9 μg g^{-1} fresh weight; Fig. 3A). RNA concentrations were extremely low in senescing leaves (Fig. 3A). The RNA level, however, was largely independent of the Pi supply in

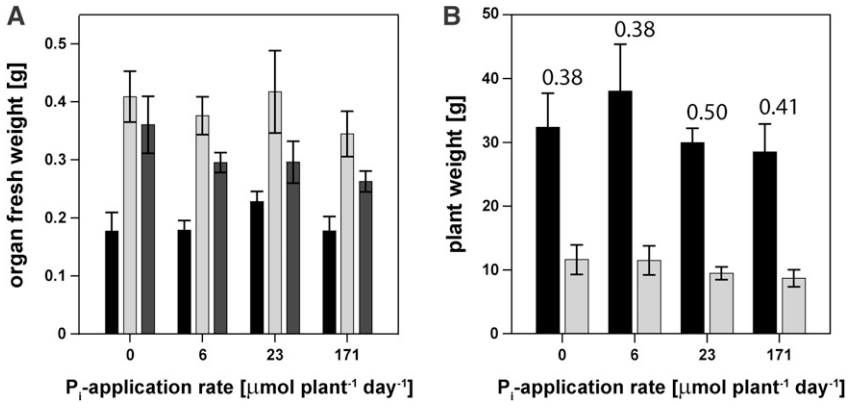

Figure 1. Biomass accumulation of *H. prostrata* plants grown at a range of Pi supplies. A, Biomass of young (black bars), mature (light gray bars), and senescing (dark gray bars) leaves. B, Total plant fresh weight (black bars) and dry weight (gray bars) after 28 weeks of treatment with the root-to-shoot dry weight ratios given above the columns. Four-month-old nursery-supplied seedlings were grown in nutrient solution for 12 weeks before being exposed to the indicated Pi supplies (experiment A). After 3 weeks of treatment, a destructive harvest was undertaken. Fresh weights are means ± SE; $n = 3$ (young leaves) to 6. No significant differences were found between treatments.

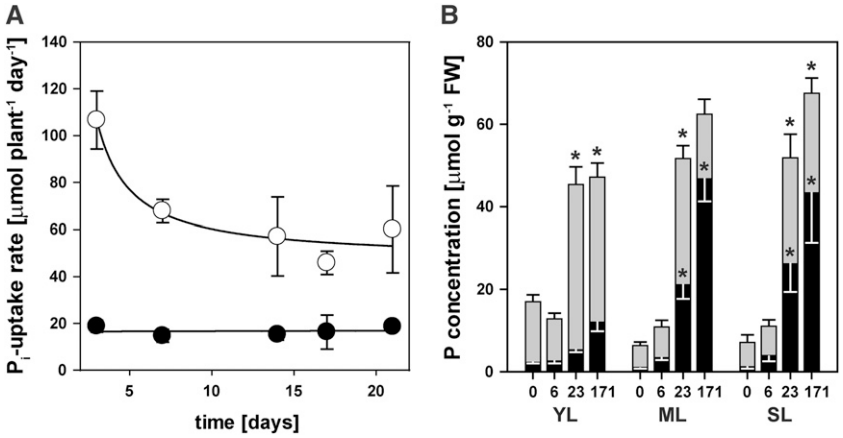

Figure 2. P relations in *H. prostrata* plants grown at a range of Pi supplies. A, Whole-plant net Pi uptake rates for plants grown at a daily supply rate of 23 μmol Pi plant^{-1} (black circles) or 171 μmol plant^{-1} (white circles). Values shown are means ± SE; $n = 3$. The curve was fitted using an exponential decay function [$f = a \times \exp(b/(x + c))$; $r^2 = 0.971$]. B, Accumulation of Pi (black bars) or Po (gray bars; Po = total P – Pi) in young (YL), mature (ML), and senescing (SL) leaves of plants exposed to different Pi supplies (0, 6, 23, or 171 μmol plant^{-1} d^{-1}) in experiment A. FW, Fresh weight. Values shown are means ± SE; $n = 4$ (young leaves) to 6. Significant differences between treatments for each organ relative to P-limited plants are indicated with asterisks at $P < 0.001$.

young, mature, and senescing leaves. Given that the majority of total RNA is rRNA, the change in RNA concentration during leaf development in these glasshouse-grown seedlings resembled the decrease in rRNA that was found between young and mature leaves of *H. prostrata* in the field (Sulpice et al., 2014).

Total protein levels in P-limited plants were almost twice as high in young leaves as those at other leaf developmental stages (Fig. 3B). This resembled observations on *H. prostrata* plants in the field (Sulpice et al., 2014). The developmental changes in leaf protein concentrations closely tracked the changes in total RNA (see above). However, leaf protein concentration responded more strongly than leaf RNA concentration to the Pi supply. In young leaves, an increase in the Pi supply resulted in a 3-fold increase in total protein concentration. In healthy mature leaves, total protein concentrations were highest at 40 μmol Pi plant^{-1} d^{-1}. Such large increases in leaf protein concentration with increasing Pi supply have not been reported in other plants, where changes in protein concentrations are relatively small (Lauer et al., 1989; Rao and Terry, 1989). It was also noteworthy that in *H. prostrata*, increased Pi supply did not lead to an increase in leaf fresh weight (Fig. 1A) but instead to an increase in protein concentration. Total protein concentration was very low in leaves showing P-toxicity symptoms (Fig. 3B). The protein concentration in senescing leaves was generally lower than that in the other leaves and did not change markedly with external Pi supply.

Levels of starch in young leaves at midday were generally low and did not respond to Pi supply (Fig. 3C). Starch concentrations were highest in mature and senescing leaves of P-limited plants and decreased with increasing Pi supply, confirming that the plants grown at the low Pi supply were indeed P limited (Rao and Terry, 1995; Ciereszko et al., 2005; Wissuwa et al., 2005). However, the marginal stimulation of leaf growth and overall plant biomass production by increased Pi supply in *H. prostrata* raises the question of why leaf starch concentrations decreased.

In young leaves, chlorophyll levels were lower than those in mature leaves and unresponsive to Pi supply (Fig. 3D). Surprisingly, the chlorophyll degradation intermediate pheophytin was 10-fold more abundant than chlorophylls in young leaves and showed a negative correlation with Pi availability (Fig. 3D). The opposite was true for mature leaves, where pheophytin levels were low and unresponsive to Pi supply, while chlorophyll concentrations were highest in mature leaves of P-limited plants and decreased with increasing Pi supply. In senescing leaves, chlorophylls and pheophytin were unresponsive to moderate increases in Pi supply. As an early indicator of P toxicity, chlorophyll levels were lowest in mature and senescing leaves of plants at the highest Pi supply, with a concomitant increase in the chlorophyll degradation intermediate pheophytin.

H. prostrata Lacks Orthologs of Arabidopsis Lipid-Remodeling Genes That Are Induced by P Limitation

In the *H. prostrata* transcriptome data set derived from leaves and roots of P-limited plants, transcripts for orthologs of P-responsive lipid-remodeling genes in Arabidopsis were largely absent, apart from those that are single-copy genes in Arabidopsis (Table I). However, a paralog encoding the strongly Pi starvation-inducible type B MGDG synthase MGD3 was present in the *H. prostrata* transcriptome assembly. From the selection of predicted *H. prostrata* complementary DNA (cDNA) contigs that encode putative orthologs of P-responsive lipid-remodeling genes in Arabidopsis (Table I), we chose

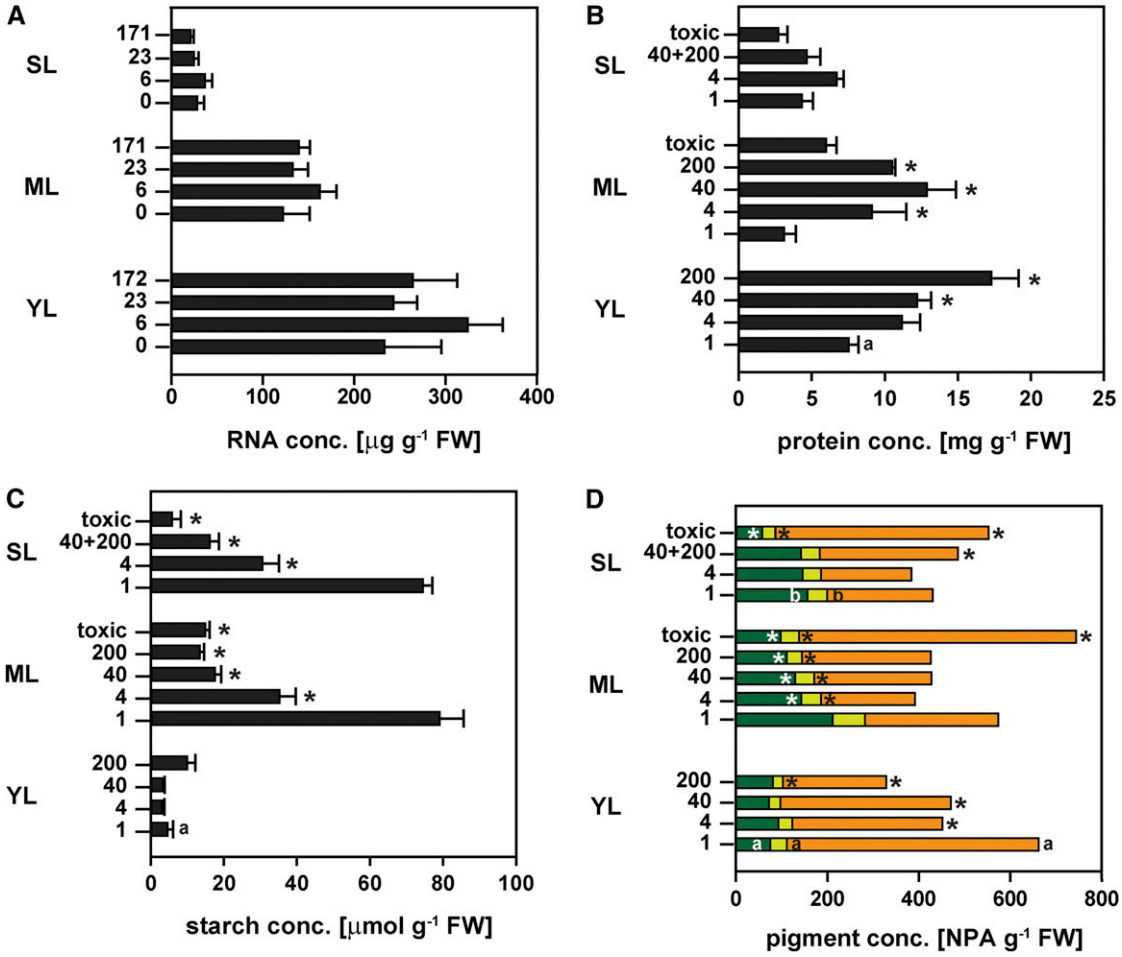

Figure 3. Dependence of key metabolite abundance on Pi supply during leaf development of *H. prostrata*. A, Total RNA concentration. B, Total protein concentration. C, Starch concentration. D, Concentrations of chlorophyll *a* (dark green bars), chlorophyll *b* (light green bars), and pheophytin (orange bars). RNA, protein, and starch concentrations (A–C) and normalized signal intensities (D; NPA, normalized peak area) g^{-1} fresh weight (FW) are plotted against the leaf developmental stage (YL, young leaf; ML, mature leaf; SL, senescing leaf) at the indicated Pi supply (0, 6, 23, or 171 μmol plant^{-1} d^{-1} for experiment A, in A; 1, 4, 40, or 200 μmol plant^{-1} d^{-1} for experiment B, in B–D). Data for healthy senescing leaves of plants grown at 40 and 200 μmol plant^{-1} d^{-1} were combined (40+200). The label toxic designates leaves with visible P-toxicity symptoms from plants at the two highest Pi application rates. Values shown are means ± SE; $n = 2$ to 3 (A) or $n = 3$ to 6 (B–D). Significant leaf stage-specific differences relative to leaves of plants receiving 1 μmol plant^{-1} d^{-1} are shown (*$P < 0.05$). Metabolites with significant differences between young and mature leaves as well as between senescing and mature leaves of P-limited plants are also shown (a and b, respectively).

a subset for relative transcript expression analysis (Supplemental Table S1). Observed differences in relative transcript abundance suggest that de novo phospholipid biosynthesis involving *H. prostrata N-METHYLTRANSFERASE2* (*HpNMT2*) was suppressed in mature leaves compared with that in young leaves of P-limited plants (Fig. 4, column ML–no Pi; for individual transcript profiles, see Supplemental Table S2). At the same time, *HpPECP1* transcripts were 5-fold more abundant, suggesting an increased release of Pi from phosphocholine and phosphoethanolamine, two phospholipid head groups. Consistent with the replacement of phospholipids by sulfolipids during leaf development, the abundance of *H. prostrata SULFITE REDUCTASE* (*HpSIR*) transcripts encoding plastid-localized sulfite reductase was 5-fold higher. This may indicate a higher demand for sulfate reduction, a rate-limiting step for sulfolipid biosynthesis in Arabidopsis (Khan et al., 2010; Shimojima, 2011). By contrast, the gene encoding the first committed step in the pathway, *HpUGP3*, showed a less than 2-fold induction, and the transcript abundance of *HpSQD1* and *HpSQD2*, which were already very highly expressed in young leaves, did not change as the leaves matured. Galactolipid biosynthesis was probably more active in mature leaf chloroplasts, with a 2-fold increase in the transcript abundance for *HpMGD1*.

The phospholipase NPC4 has been associated with P-dependent lipid remodeling in Arabidopsis roots

Table I. *Key lipid-remodeling genes in Arabidopsis and their presence in the H. prostrata transcriptome*

Genes in regular lightface do not have an ortholog in the *H. prostrata* data set. Genes highlighted in boldface are those with *H. prostrata* orthologs that were selected for quantitative reverse transcription-PCR. For more information, see Supplemental Table S1. EM, Endomembranes; FPKM, fragments per kilobase of exon per million fragments mapped; IEM, chloroplast inner envelope membrane; N/A, not applicable; ND, not determined; OEM, chloroplast outer envelope membrane; PM, plasma membrane.

Pathway	Arabidopsis Genome Initiative Code	Gene	Subcellular Localization (The Arabidopsis Information Resource; Joyard et al., 2010)	Reference	Fold Induction (Misson et al., 2005)	*H. prostrata* Ortholog?	FPKM
Galactolipid biosynthesis	**At4g31780**	***MGD1***	**IEM**	**Benning and Ohta (2005)**	**ND**	**Yes**	**13**
	At5g20410	***MGD2***	**OEM**	**Benning and Ohta (2005)**	**24.8**	**Yes**	**14.1**
	At2g11810	***MGD3***	**OEM**	**Benning and Ohta (2005)**	**55.7**	**Yes**	**6.5**
	At3g11670	***DGD1***	**OEM**	**Benning and Ohta (2005)**	**3.6**	**Yes**	**29.1**
	At4g00550	***DGD2***	**OEM**	**Benning and Ohta (2005)**	**ND**	**Yes**	**18.6**
Sulfolipid biosynthesis	**At5g04590**	***SIR***	**Stroma**	**Khan et al. (2010)**	**ND**	**Yes**	**35.4**
	At3g56040	***UGP3***	**Stroma**	**Okazaki et al. (2009)**	**10.2**	**Yes**	**11.3**
	At4g33030	***SQD1***	**Stroma**	**Sanda et al. (2001)**	**4.4**	**Yes**	**50.8**
	At5g01220	***SQD2***	**IEM**	**Yu et al. (2002)**	**14.6**	**Yes**	**73.5**
Phospholipid biosynthesis	At3g18000	*NMT1*	Cytosol	Cruz-Ramirez et al. (2004)	3.7	ND	N/A
	At1g48600	***NMT2***	**Cytosol**	**BeGora et al. (2010)**	**ND**	**Yes**	**17.2**
	At1g73600	*NMT3*	Cytosol	BeGora et al. (2010)	0.09	ND	N/A
	At2g32260	***CCT1***	**Cytosol**	**Inatsugi et al. (2009)**	**2.7**	**Yes**	**0.1**
	At4g15130	*CCT2*	Cytosol	Inatsugi et al. (2009)	ND	ND	N/A
	At1g13560	***AAPT1***	**PM, Golgi**	**Goode and Dewey (1999)**	**ND**	**Yes**	**28.3**
	At3g25585	*AAPT2*	PM	Goode and Dewey (1999)	ND	ND	N/A
Phospholipid degradation phospholipase C	**At1g07230**	***NPC1***	**PM, EM**	**Pokotylo et al. (2013)**	**ND**	**Yes**	**74.9**
	At2g26870	***NPC2***	**PM, EM**	**Pokotylo et al. (2013)**	**ND**	**Yes**	**40**
	At3g03520	*NPC3*	PM, tonoplast	Pokotylo et al. (2013)	ND	ND	N/A
	At3g03530	***NPC4***	**PM**	**Nakamura et al. (2005)**	**ND**	**Yes**	**4.6**
	At3g03540	*NPC5*	Cytosol	Gaude et al. (2008)	241.6	ND	N/A
	At3g48610	*NPC6*	PM (?)	Pokotylo et al. (2013)	ND	Yes	
Phospholipase D	At3g15730	*PLDα1*	PM, EM	Li et al. (2008)	ND	Yes	152.6
	At1g52570	***PLDα2***	**OEM (?)**	**Eliáš et al. (2002)**	**ND**	**ND**	**5.7**
	At5g25370	*PLDα3*	PM associated	Hong et al. (2008)	ND	ND	N/A
	At1g55180	***PLDα4/ε***	**PM associated**	**Hong et al. (2009)**	**ND**	**Yes**	**3.3**
	At2g42010	*PLDβ1*	PM, nuclear (?)	Qin et al. (1997)	ND	Yes	11.3
	At4g00240	*PLDβ2*	PM, nuclear (?)	Qin et al. (1997)	ND	ND	N/A
	At4g11850	*PLDγ1*	EM associated	Hong et al. (2009)	ND	ND	N/A
	At4g11830	*PLDγ2*	OEM, PM (?)	Hong et al. (2009)	ND	ND	N/A
	At4g11840	*PLDγ3*	PM	Qin et al. (1997)	ND	ND	N/A
	At4g35790	*PLDδ*	PM associated	Li et al. (2008)	ND	Yes	12.5
	At3g16785	***PLDζ1***	**PM, cytosol**	**Li et al. (2006)**	**ND**	**Yes**	**2**
	At3g05630	*PLDζ2*	Tonoplast	Li et al. (2006)	54.1	ND	N/A

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Pathway	Arabidopsis Genome Initiative Code	Gene	Subcellular Localization (The Arabidopsis Information Resource; Joyard et al., 2010)	Reference	Fold Induction (Misson et al., 2005)	*H. prostrata* Ortholog?	FPKM
Glycerophosphodiester phosphodiesterase	**At3g02040**	***GDPD1***	**Stroma**	**Cheng et al. (2011)**	**14.2**	**Yes**	**7.2**
	At5g41080	*GDPD2*	Cytosol	Cheng et al. (2011)	ND	ND	N/A
	At5g43300	*GDPD3*	Cytosol	Cheng et al. (2011)	ND	ND	N/A
	At1g71340	*GDPD4*	Mitochondrion	Cheng et al. (2011)	ND	Yes	4.8
	At1g74210	*GDPD5*	PM, tonoplast	Cheng et al. (2011)	6.7	Yes	25.4
	At5g08030	*GDPD6*	PM, ER	Cheng et al. (2011)	156.1	ND	N/A
	At1g66970	***GDPDL1***	**IEM associated**	**Cheng et al. (2011)**	**ND**	**Yes**	**12.7**
	At1g66980	*GDPDL2*	PM associated	Cheng et al. (2011)	ND	ND	N/A
	At4g26690	*GDPDL3*	PM associated	Cheng et al. (2011)	ND	ND	N/A
	At5g55480	*GDPDL4*	PM associated	Cheng et al. (2011)	ND	Yes	12.7
	At3g20520	*GDPDL5*	PM associated	Cheng et al. (2011)	ND	ND	N/A
	At5g58050	*GDPDL6*	PM associated	Cheng et al. (2011)	ND	ND	N/A
	At5g58170	*GDPDL7*	PM associated	Cheng et al. (2011)	ND	ND	N/A
Phosphatidate phosphatase lipins (Mg^{2+} dependent)	At2g01180	*LPPα1*	PM	Pierrugues et al. (2001)	ND	ND	N/A
	At1g15080	***LPPα2***	**PM**	**Pierrugues et al. (2001)**	**ND**	**Yes**	**23.3**
	At3g02600	***LPPα3***	**PM**	**Pierrugues et al. (2001)**	**ND**	**Yes**	**4**
	At3g18220	*LPPα4*	PM	Katagiri et al. (2005)	ND	ND	N/A
	At4g22550	*LPPβ*	PM	Nakamura et al. (2007)	ND	Yes	5.2
	At5g03080	***LPPγ***	**IEM**	**Nakamura et al. (2007)**	**ND**	**Yes**	**27.5**
	At3g58490	***LPPδ / SPP1***	ER	**Nakagawa et al. (2012)**	**ND**	**Yes**	**13.4**
	At3g50920	***LPPε1***	**IEM**	**Nakamura et al. (2007)**	**ND**	**Yes**	**11.1**
	At5g66450	***LPPε2***	**IEM**	**Nakamura et al. (2007)**	**ND**	**Yes**	**11.1**
	At3g09560	***PAH1***	**ER associated**	**Nakamura et al. (2009)**	**ND**	**Yes**	**5.6**
	At5g42870	***PAH2***	**ER associated**	**Nakamura et al. (2009)**	**ND**	**Yes**	**4.2**
Phosphocholine/phosphoethanolamine phosphatase	**At1g17710**	***PECP1***	**Cytosol**	**May et al. (2012)**	**45.2**	**Yes**	**166.9**

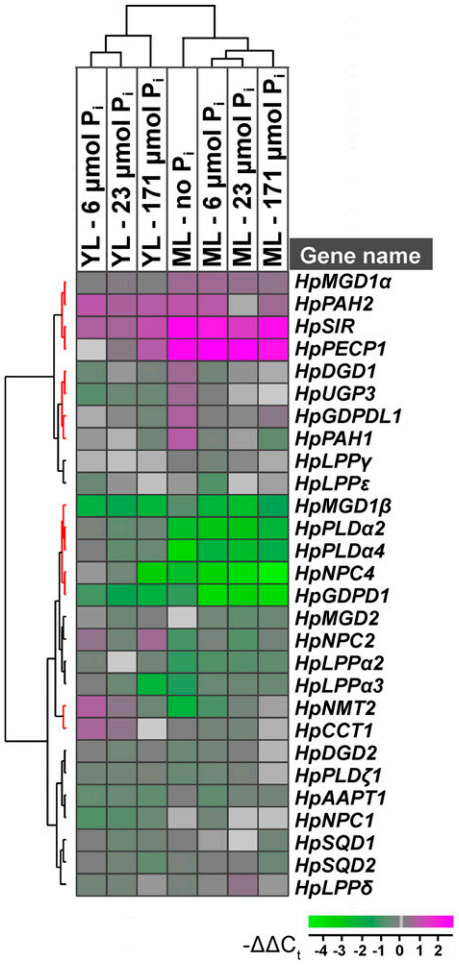

Figure 4. Hierarchical cluster analysis of relative transcript abundance from lipid-remodeling genes as dependent on P status across *H. prostrata* leaf development. Mean $\log_2$ expression ratios (−ΔΔCt [difference in threshold cycle number relative to reference gene and control sample]) relative to the normalized expression in young leaves from P-limited plants with three biological replicates for each leaf stage are shown (experiment A). Data were normalized against the transcript abundance of *HpACTIN7* and *H. prostrata YELLOW-LEAF-SPECIFIC GENE8* (*HpYLS8*) reference genes. The clusters were generated using squared Euclidean distance and complete linkage (Dysvik and Jonassen, 2001). The linkage groups highlighted in red are described further in "Results." Details on individual transcript expression patterns can be found in Supplemental Table S2. YL, Young leaf; ML, mature leaf.

(Nakamura et al., 2005). Here, phospholipases *HpNPC2*, *HpNPC4*, *HpPLDα2*, *HpPLDα4*, and *HpLPPα2* were all suppressed in mature compared with young leaves of P-limited *H. prostrata*, indicating that they are not involved in the developmentally regulated lipid remodeling.

Transcript Abundances for Genes Involved in Lipid Remodeling Show Very Little Response to Pi Availability or Internal P Status in *H. prostrata*

Out of the 28 putative lipid-remodeling genes tested, very few showed P-dependent expression profiles in leaves similar to those described previously in Arabidopsis (Misson et al., 2005; Müller et al., 2007). In mature leaves, relative transcript abundance of *HpNMT2* showed a positive correlation with Pi supply (Fig. 3). Consistent with this, *HpGDPD1*, *HpMGD3*, and *HpSIR* transcripts that were highly abundant in mature leaves of P-limited plants showed a lower abundance with increasing Pi availability. *HpPECP1* transcript amounts remained high in mature leaves, irrespective of Pi supply. *HpPLDα2*, *HpPLDα4*, and *HpLPPα2* transcripts showed an overall lower abundance in mature leaves, irrespective of the plant's P status. *HpNPC4* transcript abundance, which was already low in mature leaves of P-limited plants, decreased further with increasing Pi supply and was 4-fold lower in mature leaves of plants with P-toxicity symptoms. By contrast, *HpMGD3*, *HpSIR*, and *HpLPPα2* had higher transcript abundance in mature leaves of plants at the highest Pi supply than in those of plants at intermediate Pi supply. In young leaves that otherwise showed even less P-dependent transcriptional regulation than mature leaves, *HpMGD3*, *HpNPC4*, *HpGDPD1*, and *HpLPPα3* transcript amounts were lowest in plants given the highest Pi supply.

Polar Lipid Profiles Change during Leaf Development and in Response to Pi Supply

Given the relatively small changes in transcript abundance described above, we investigated how the lipid composition changed during leaf development and in response to Pi supply in *H. prostrata*. For these analyses, we used plants grown in experiment B (see "Materials and Methods").

Free fatty acid levels were similar across leaf developmental stages. Mature leaves of P-limited plants had higher free fatty acid levels than leaves of P-sufficient plants. At the highest Pi supply, fatty acid levels were lower in young leaves (Supplemental Table S3). Mature and senescing leaves with P-toxicity symptoms had higher levels of free fatty acids. Lyso-PC levels were highest in young leaves and showed little response to external Pi availability, but these lipid species accumulated in mature and senescing leaves of plants showing P-toxicity symptoms (Supplemental Table S3).

Concentrations of galactolipids decreased by as much as 50% in young and mature leaves with increasing Pi supply, while they did not respond to Pi availability in senescing leaves (Fig. 5A). MGDG and DGDG levels were higher in mature leaves compared with young and senescing leaves of P-limited plants. DGDG levels were also higher in mature leaves showing P-toxicity symptoms than in those without symptoms. The majority of galactolipids were found in group II (b) of the hierarchical cluster analysis (Supplemental Fig. S2). This group was most likely associated with lipid remodeling under P-limiting conditions, with a strong negative correlation to Pi supply.

Sulfolipid concentrations were more than 2-fold higher in mature and senescing leaves than in young

leaves (Fig. 5B). Unlike galactolipids, concentrations of sulfolipids did not decrease with increasing Pi supply, but they were higher in mature leaves showing P-toxicity symptoms (Fig. 5B). While some of the most abundant sulfolipids were also found in the lipid remodeling group II (b), SQDG 34:1 and SQDG 36:3 associated with group II (a) alongside all three PG species detected in this study (Supplemental Fig. S2). The fact that these lipids are predominantly found in chloroplast membranes (Marechal et al., 1997) could associate group II (a) with delayed greening in *H. prostrata*.

The most abundant glycoglycerolipid species in leaves were MGDG 36:6, DGDG 36:6, and SQDG 36:6 (Supplemental Table S3). MGDG 34:3, DGDG 34:3, and SQDG 34:3 were also very strongly represented in all three leaf stages. MGDG 34:6 species were detected, while DGDG 34:6 and SQDG 34:6 were either not present or below the detection limit, consistent with the current model for the desaturation of acyl chains of thylakoid lipids in the chloroplast (Li-Beisson et al., 2010; Boudière et al., 2014).

Concentrations of the two main phospholipid classes in *H. prostrata*, PC and phosphatidylethanolamine (PE), were higher in young leaves of P-limited plants than in mature and senescing leaves (Fig. 5, C and D). This was mostly due to a decline in 36:6 PC species carrying 18-C acyl chains with a higher degree of unsaturation in older leaves (Supplemental Table S3). Consistent with the P-dependent lipid remodeling observed in other plants, PC species in mature and senescing leaves showed a positive correlation with Pi supply (group IV in Supplemental Fig. S2), but their levels were constant in young leaves (Fig. 5C). PE concentrations gradually increased with increasing Pi supply across all leaf developmental stages (Fig. 5D). At the highest Pi supply, however, both PC and PE levels dropped significantly in young leaves.

In contrast to the other phospholipids, concentrations of PG, the main phospholipid in the chloroplast inner envelope and thylakoid membranes (Marechal et al., 1997), were highest in mature and senescing leaves and lowest in young leaves of P-limited plants (Fig. 5E). All PG species clustered together in group II (a) and, therefore, may primarily be involved in chloroplast and more specifically thylakoid membrane development during delayed greening in *H. prostrata* (Supplemental Fig. S2). In young leaves, PG showed a positive correlation with Pi supply. However, in mature leaves, PG concentrations showed a negative correlation with Pi supply. PG levels were also more than 50% lower in senescent leaves that showed P-toxicity symptoms.

Consistent with a high demand for transient carbon storage during leaf development, triacylglycerol (TAG) levels were highest in young leaves of *H. prostrata* and tended to decline with increasing Pi availability (Fig. 5F; group I in Supplemental Fig. S2). TAG species with long acyl chains and a higher degree of unsaturation were almost exclusively found in young leaves (Supplemental Fig. S2). In mature and senescing leaves, normalized signal intensities of TAG were lowest at 4 μmol Pi plant^{-1} d^{-1}, but TAG levels were considerably higher in leaves with toxicity symptoms. This profile, therefore, was very similar to the one observed for galactolipids. The lipid species that accumulated in leaves with P-toxicity symptoms differed from the ones found in young leaves or in mature leaves of P-limited plants and were mostly part of group III (Supplemental Fig. S2).

A Group of Polar Lipids Associated with the Chloroplast Is Highly Sensitive to Excess Pi Accumulation in Leaves

One group of lipids stood out from all others in the hierarchical cluster analysis. Group III was similar to the PG group II (a) (Fig. 6A; Supplemental Fig. S2). The 34:x (x = number of double bonds in hydrocarbon chain) sulfolipids (SQDG 34:1–34:4), 34:x PC species (34:1–34:4), PE 34:4, as well as the 34:x PG species (34:2–34:4) in group II (a) were all very low in abundance in young leaves compared with mature leaves (Supplemental Table S3). In a complete reversal of the normal Pi-dependent lipid-remodeling profile, PG levels decreased with increasing Pi supply in mature and senescing leaves, while at the same time, 34:x SQDG species increased. The 34:x PC species, on the other hand, showed the expected positive correlation with Pi supply. In contrast with group II (a), however, all of the group III lipids showed a very strong accumulation in leaves with P-toxicity symptoms; MGDG 34:4 and DGDG 34:4 were 8- to 15-fold more abundant than in leaves of P-limited plants (Fig. 6B). Similarly, sulfolipid SQDG 34:4 as well as its more saturated precursors SQDG 34:1, 34:2, and 34:3 in groups II (a) and III (b) increased 4- to 6-fold in both mature and senescing leaves at the two highest Pi supplies, regardless of whether the leaves were healthy or had P-toxicity symptoms (Fig. 6B; Supplemental Fig. S2). Interestingly, group III (a) also included free palmitoleic acid as well as a number of TAG species (TAG 50:4, 50:5, and 52:7) that showed the same profile as the select group of glycerolipids. They were more than 10-fold more abundant in leaves with P-toxicity symptoms than in comparable asymptomatic leaves (Fig. 6A; Supplemental Table S3).

While SQDG 34:1 to 34:3 profiles clustered in groups II (a) and III (b), the more saturated precursors of PE and galactolipids with 34:1 to 34:3 acyl chains were much more abundant in young than in mature leaves. These lipids showed the classic lipid-remodeling response, with MGDG 34:1 and DGDG 34:1 decreasing in abundance and PE 34:1 increasing in abundance with increasing Pi supply. Unlike PE 34:1, the 34:1 galactolipids were also quite abundant in mature leaves of P-limited plants and decreased with increasing Pi supply.

DISCUSSION

Increased Pi Supply Does Not Greatly Alter Leaf Growth Rates But Does Alter Leaf Metabolite Composition, Including a Large Increase in Protein Concentration

Proteaceae spp. like *H. prostrata* grow with a very low P availability in their natural habitat (Lambers et al.,

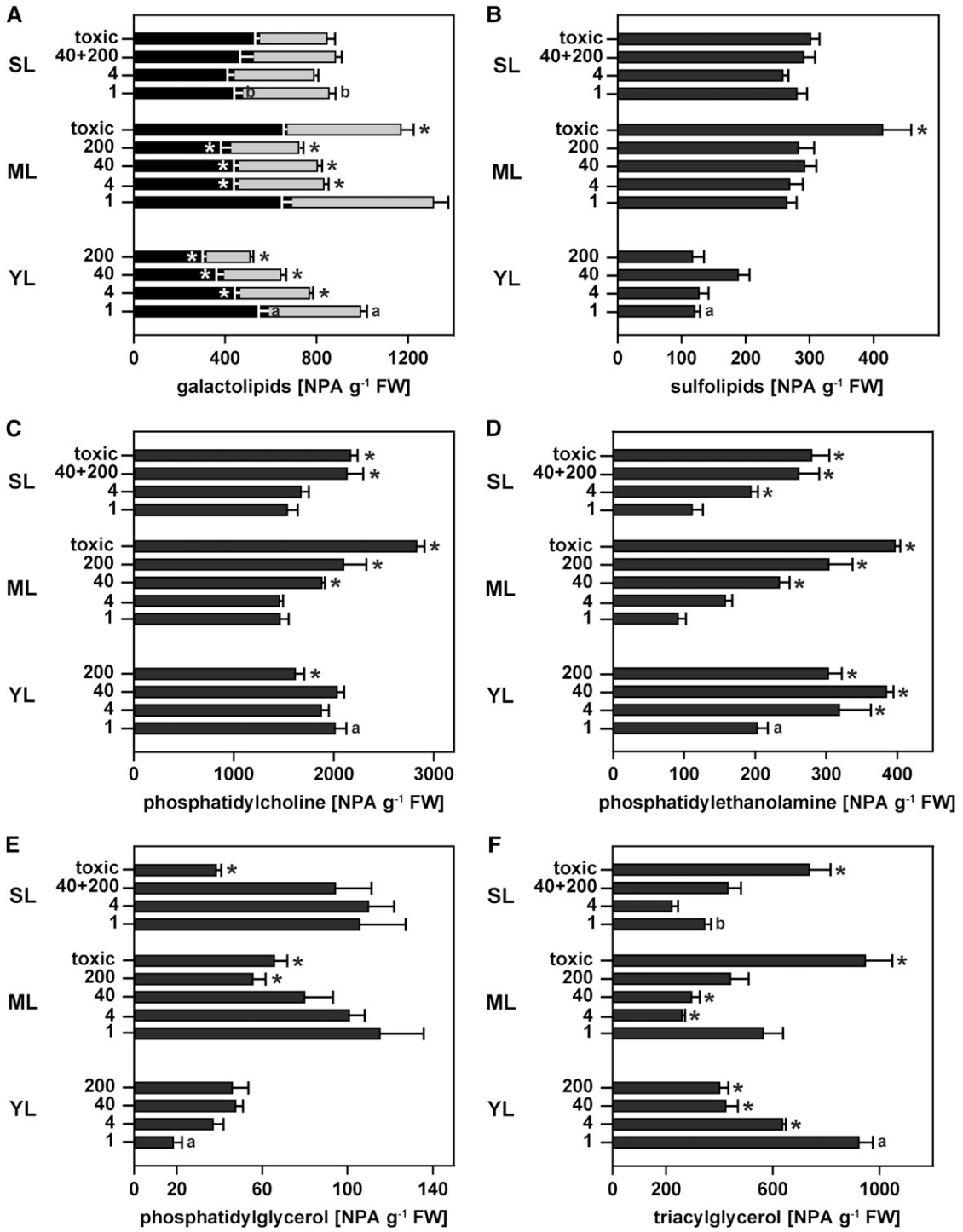

Figure 5. Lipid profiles across leaf developmental stages of *H. prostrata* plants grown at a range of Pi supplies. A, Galactolipids MGDG (black bars) and DGDG (gray bars). B, Sulfolipid SQDG. C, PC. D, PE. E, PG. F, TAG. Normalized signal intensities g^{-1} fresh weight (FW) are plotted against the leaf developmental stage (YL, young leaf; ML, mature leaf; SL, senescing leaf) at the indicated Pi application rates (1, 4, 40, and 200 μmol plant^{-1} d^{-1}) in experiment B. Data for healthy senescing leaves of plants grown at 40 and 200 μmol plant^{-1} d^{-1} were combined (40+200). The label toxic designates leaves with visible P-toxicity symptoms from plants grown at the two highest Pi application rates. Values shown are means ± SE; $n = 3$ to 6. Significant leaf stage-specific differences relative to leaves of plants receiving 1 μmol Pi plant^{-1} d^{-1} are indicated (*$P < 0.05$). Significant differences between young and mature leaves as well as between senescing and mature leaves of P-limited plants are also shown (a and b, respectively). Further details for individual lipid species are outlined in Supplemental Table S3.

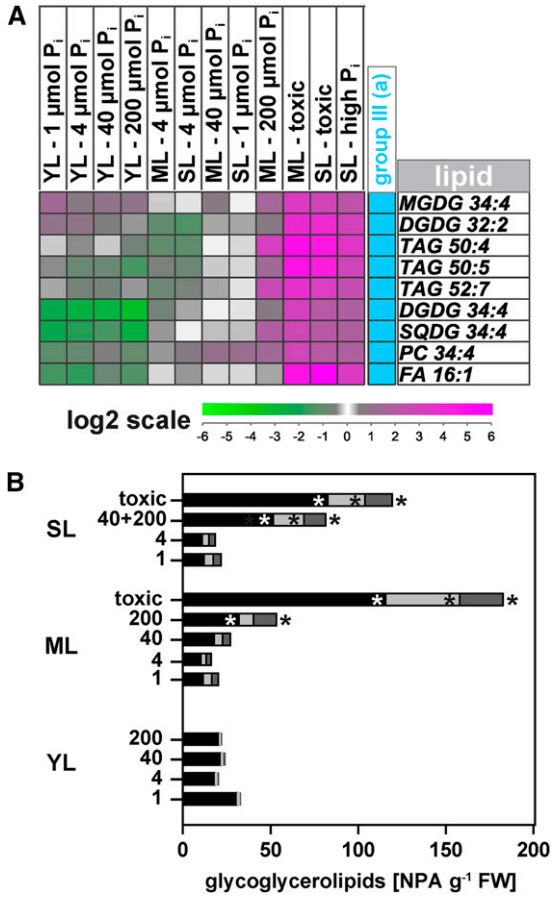

Figure 6. Profile of a subset of putative chloroplast-associated lipids that, unlike most glycoglycerolipids, are also highly abundant in leaves with P toxicity symptoms. A, Enlarged profile of group III (a) from the hierarchical cluster analysis shown in Supplemental Figure S2. Normalized mass spectral signal values are expressed relative to those in mature leaves of P-limited plants. B, Normalized signal intensities of glycoglycerolipids in group III (a) that are most likely synthesized via the prokaryotic pathway: MGDG 34:4 (black bars), DGDG 34:4 (light gray bars), and SQDG 34:4 (dark gray bars). Values are plotted against the leaf developmental stage (YL, young leaf; ML, mature leaf; SL, senescing leaf) at the indicated Pi application rates (1, 4, 40, and 200 μmol plant^{-1} d^{-1}) in experiment B. Data for healthy senescing leaves of plants grown at 40 and 200 μmol plant^{-1} d^{-1} were combined (40+200). The label toxic designates leaves with visible P-toxicity symptoms from plants grown at the two highest Pi application rates. Values shown are means; $n = 3$ to 6. Significant leaf stage-specific differences relative to leaves of P-limited plants are indicated (*$P < 0.05$). Further details on individual lipid species are outlined in Supplemental Table S3. FW, Fresh weight.

2012a). The total leaf P concentrations of plants in the field (Lambers et al., 2012b) resemble those in plants with the lowest Pi supply in our experimental system. While higher root and shoot biomass production are observed in the longer term when Pi supply is increased (Shane et al., 2004), plant biomass production was not altered significantly by a moderate to large increase in Pi supply over the experimental period in this study and even decreased slightly at the highest Pi supply. However, Pi supply resulted in a slight increase in the production of new leaves and major changes in leaf composition. Young *H. prostrata* leaves showed a large increase in protein concentration, increased chlorophyll levels and chlorophyll *a*/*b* ratios, lower pheophytin and anthocyanin concentrations, as well as an altered lipid profile. Mature leaves showed positive responses to moderate increases in Pi supply, such as increased protein and phospholipid levels and reduced starch, galactolipid, and TAG accumulation (Fig. 7). Surprisingly, *H. prostrata* did not show the higher shoot-to-root ratio that typically accompanies a higher Pi supply in other plants (Marschner et al., 1996). Two factors may explain why growth did not immediately increase with an increase in Pi supply. First, growth costs are higher at moderate levels of Pi than at low Pi because of greater investment in protein per unit fresh weight in both young and mature *H. prostrata* leaves. The assimilation of inorganic nitrogen and sulfur, amino acid synthesis, and protein synthesis are far more costly than the synthesis of polysaccharide cell walls, lipids, or secondary metabolites (Penning de Vries et al., 1974; Warner, 1999; Amthor, 2010). Second, *H. prostrata* has previously been shown to have a lower photosynthetic performance at higher leaf P concentrations (Shane et al., 2004). In agreement, high Pi supply resulted in lower chlorophyll and PG levels in this study. A decrease in carbon gain may contribute to the lower than expected biomass gain at elevated Pi supply.

Young *H. prostrata* Leaves Are Shielded against the Consequences of Low Pi Availability

Organ growth depends heavily on P-containing organic compounds such as RNA, DNA, phospholipids, ATP, and phosphorylated sugars; P-efficient species and cultivars tend to allocate a greater proportion of their limiting P resources to growing and differentiating tissues (Kavanová et al., 2006; Aziz et al., 2014; Sulpice et al., 2014). Proteaceae spp. in their natural habitat have relatively high levels of phospholipids in their young expanding leaves compared with mature leaves (Lambers et al., 2012b), suggesting that phospholipids are indispensable, for example in processes such as cell division or expansion (Jackowski, 1996; Cruz-Ramírez et al., 2004; Gagne and Clark, 2010). Here, we show that young *H. prostrata* leaves also had a more than 3-fold larger organic P fraction than mature leaves. This is a much more significant difference than their 1.5-fold greater phospholipid fraction, given that *H. prostrata* also economizes on other organic P pools in mature leaves, especially the rRNA pool (Sulpice et al., 2014).

Interestingly, young leaves also appeared to be shielded from P toxicity, as they had much lower Pi concentrations than mature leaves under high-P conditions. Two factors might contribute to this. First, they may be able to utilize the P more rapidly for growth and are metabolically more active, as suggested by the high levels of Po. Second, as their gas-exchange activity is fairly low (Lambers et al., 2012b), the evaporative

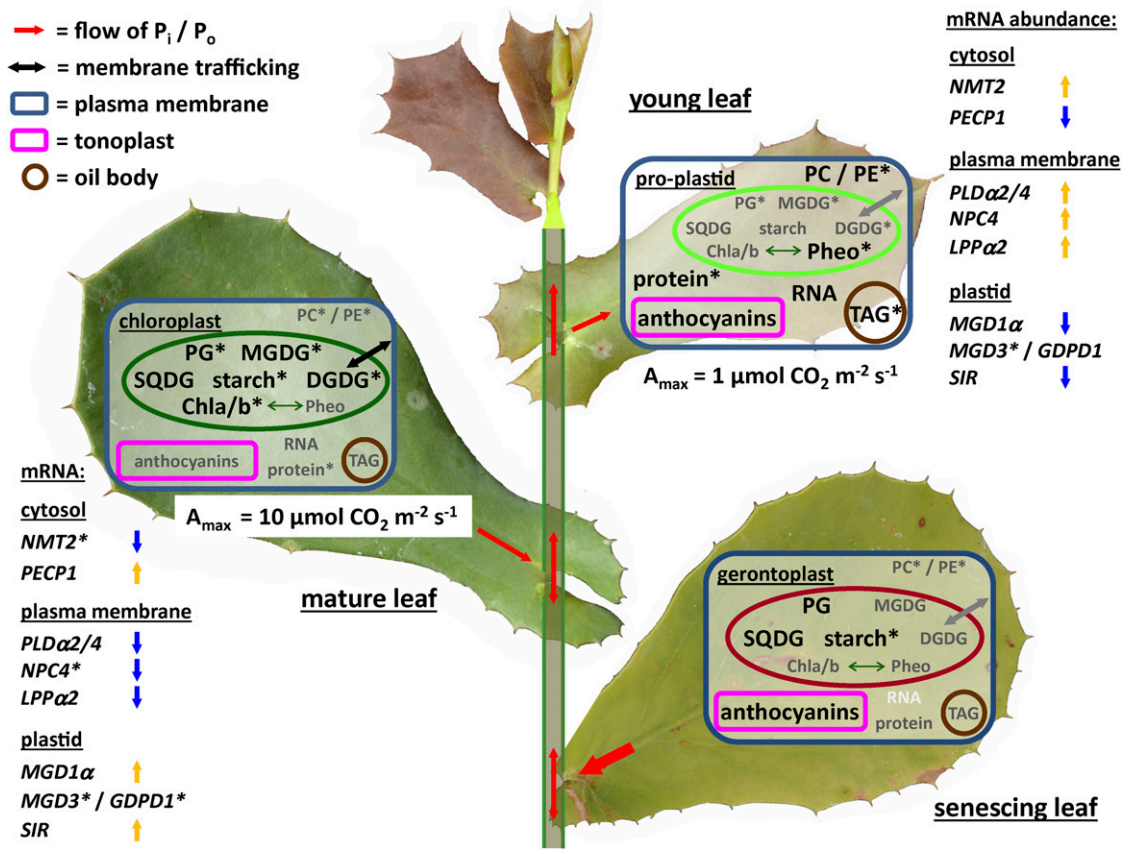

Figure 7. Summary of the changes in metabolite and transcript abundance during leaf development in *H. prostrata*. Young leaves are able to develop on plants with very low P status due to delayed chloroplast development that allows them to allocate limited P resources to growth. As leaves mature, P reserves are mobilized by phospholipid degradation, and thylakoid membranes develop fully. Leaf senescence progresses slowly, beginning with the degradation of rRNA pools, while plasma and chloroplast membranes are maintained. Ultimately, P remobilization from fully senescent leaves can be as high as 80% (Denton et al., 2007). To reduce the complexity of the figure, protein and RNA pools are shown only in the cytosol, representing their overall concentration across compartments. In higher plants, PC and PE are mostly found in the plasma membrane and endomembranes, while PGs, MGDGs, and SQDGs are predominantly found in chloroplasts. DGDGs are also found in extraplastidic membranes of P-limited plants, but their distribution is unclear in *H. prostrata*. Metabolite levels are indicated (black type, high abundance; gray type, low abundance; white type, absent). Changes in transcript abundance between young and mature leaves are indicated by blue (down-regulated) and yellow (up-regulated) arrows. Genes are grouped according to the predicted subcellular localization of the encoded proteins. Asterisks indicate Pi-dependent changes in abundance. A_{max}, Photosynthetic capacity; Chla/b, chlorophylls; Pheo, pheophytin; Pi, phosphate; Po, phloem-mobile organophosphates (e.g. phosphocholine, sugar phosphates, ATP, etc.).

water loss via stomata would be less than in mature leaves, resulting in less import of Pi via the transpiration stream. The observation that total P per unit fresh weight was only slightly lower in young than in mature leaves of plants supplied with high Pi, however, indicates that the key factor may be their ability to utilize P.

Young *H. prostrata* Leaves Show Delayed Development of Functional Chloroplasts, Including Changes in Pigment and Lipid Metabolite Concentrations, as Well as Altered Profiles of Transcripts from Lipid-Remodeling Genes

Consistent with higher phospholipid levels, *HpNMT2* transcripts encoding a putative phosphocholine-producing enzyme involved in de novo phospholipid biosynthesis (Bolognese and McGraw, 2000; Jost et al., 2009; BeGora et al., 2010) were more abundant in young than in mature leaves of P-limited *H. prostrata*. Interestingly, higher Po concentrations and NMT transcript abundance in young leaf tissues are also striking features of a highly P-efficient wheat (*Triticum aestivum*) cultivar (Aziz et al., 2014). Just as in *H. prostrata*, early wheat leaf developmental stages (e.g. leaf primordia, elongating blades, and mature leaf bases) are white and heterotrophic, as in many monocotyledons, while the activation of chloroplast transcription and translation occurs at later stages of development, in parallel with the greening of the leaf blade (Masle, 2000; Kusumi et al., 2010; Li et al., 2010). Lipid profiles in developing maize (*Zea mays*) leaves show some similarities to

those in *H. prostrata*. Concentrations of MGDG species increased almost 4-fold, those of DGDG and SQDG species increased 3-fold, and those of PG species increased 2-fold, while concentrations of both PC and PE species decreased by more than 3-fold in sections farther up the maize leaf blade compared with the leaf base (Leech et al., 1973). These results in maize are consistent with chloroplast maturation and thylakoid development in mesophyll and bundle sheath cells in those segments. In P-limited *H. prostrata* plants, mature leaf lipid profiles were matched by the higher abundance of transcripts encoding the putative plastid-localized type A MGDG synthase HpMGD1 and the sulfite reductase HpSIR compared with young leaves. Transcripts for the putative phosphatase HpPECP1 showed a similar expression pattern. This phosphatase, therefore, could play a central role in the replacement of phospholipids in extraplastid membranes by galactolipids and the release of Pi (May et al., 2012) to support metabolic processes associated with chloroplast development.

The control of chloroplast RNA metabolism is critical for the efficient use of P in plants (Marchive et al., 2009). We reported earlier that delayed chloroplast maturation during leaf development in Proteaceae spp. may allow for sequential P allocation, first to cytosolic rRNA and later to plastidic rRNA pools, as a means of making the most of this sparingly available resource (Sulpice et al., 2014). Here, we present further evidence of this smart P-allocation strategy, which is summarized in Figure 7. In young *H. prostrata* leaves, pheophytin was highly abundant while chlorophylls only accumulated as the leaves matured, a situation similar to that found in maize, where pheophytin accumulates in etiolated leaves prior to light-regulated conversion into chlorophyll (Ignatov and Litvin, 1995). The chlorophyll *a*/*b* ratio was also very low in young *H. prostrata* leaves (Sulpice et al., 2014) and resembled ratios found in shade leaves, with 2-fold lower CO_2 assimilation rates (Lichtenthaler et al., 2007). In young leaves of *H. prostrata*, the low total chlorophyll and low chlorophyll *a*/*b* ratio are accompanied by a 10-fold lower photosynthetic capacity compared with maximum Rubisco activity in mature leaves (Lambers et al., 2012b; Sulpice et al., 2014). Delayed greening, therefore, could involve the accumulation of chlorophyll intermediates, such as pheophytin or protochlorophyllide, in young leaf mesophyll cells (Matile et al., 1999).

Young *H. prostrata* leaves contain high anthocyanin concentrations. These may compensate for low levels of photosynthetic pigments and protect these leaves against photodamage and reactive oxygen species (Lee and Collins, 2001; Hatier and Gould, 2008) as well as provide protection against herbivory (Lev-Yadun et al., 2004). The pigment distribution in *H. prostrata* leaves is very different from that found in Arabidopsis, where anthocyanins accumulate in older leaves (Diaz et al., 2006) and chloroplast development takes place early during leaf development (Breeze et al., 2011), with high chlorophyll and Rubisco concentrations in young and expanding leaves. Another protective mechanism for young leaf plastids in *H. prostrata* could be the higher concentrations of more saturated 34:x PE and galactolipid species; a recent study in cotton (*Gossypium hirsutum*) found that young leaves have a higher proportion of saturated fatty acid side chains in chloroplast membrane lipids, and this is subject to seasonal variation and correlates with changes in their photosynthetic thermotolerance (Hall et al., 2014).

Further evidence for the delayed development of functional chloroplasts in *H. prostrata* includes (1) the sharp increase in starch accumulation, which can only occur in photosynthetically active chloroplasts, in mature leaves compared with young leaves of P-limited plants, and (2) the extremely low levels of SQDG and PG in young leaves, both of which are lipids essential for and found primarily in functional photosynthetic membranes (Marechal et al., 1997; Babiychuk et al., 2003; Shimojima et al., 2009). In Arabidopsis, lipid species found primarily in the chloroplast account for 75% of polar leaf lipids (Devaiah et al., 2006), while in mature *H. prostrata* leaves, this fraction was only about 37% (Supplemental Table S3). The smaller proportions of preferentially plastid-localized lipids suggest that *H. prostrata* leaves contain fewer or smaller chloroplasts on a fresh weight basis. This is especially true if we assume that some of the galactolipids are in fact localized in extraplastid membranes of mature leaves of P-limited plants. This assessment is consistent with the observations by Sulpice et al. (2014) and suggests a very high efficiency of the photosynthetic machinery, given that overall leaf area-based rates of photosynthesis in Proteaceae spp. leaves are similar to those of other plants (Denton et al., 2007; Lambers et al., 2012b).

Delayed Greening Is Only Partially Reversed by Increased Pi Supply

The delayed greening of young leaves is partially reversed at higher Pi supply. With increasing Pi availability, young leaves had higher chlorophyll *a*/*b* ratios, lower pheophytin, anthocyanin, and group II (a) and III sulfolipid levels (Supplemental Fig. S2), as well as higher RNA, protein, and in particular PG concentrations than analogous leaves from P-limited plants. However, the decrease in concentrations of pheophytin and anthocyanins was not matched by similar increases in chlorophyll *a* and *b*, which indicates that this reversal is incomplete.

In *H. prostrata*, transcripts encoding putative phospholipases HpNPC2, HpNPC4, HpPLDα2, HpPLDα4, and HpLPPα2 were more abundant in young leaves irrespective of Pi supply. This suggests that these enzymes are not involved in the release of Pi from membrane lipids but rather in developmentally controlled lipid remodeling. In Arabidopsis, PLDα activity is very prominent in metabolically active organs (Fan et al., 1999). These lipases, therefore, might be important for the turnover of phospholipids and associated signaling processes during organ growth (Dhonukshe et al., 2003; Gardiner et al., 2003; Potocký et al., 2003; Wang, 2005). Reduced PLDα activity in mature leaves, on the other hand, could delay leaf

senescence (Fan et al., 1997; Lee et al., 2012), promoting leaf longevity, which is characteristic of Proteaceae spp. like *H. prostrata* (Wright et al., 2002; Denton et al., 2007). The facts that NPC4 is only involved in lipid remodeling in the roots of P-limited Arabidopsis (Nakamura et al., 2005) and that *HpNPC4* transcripts are more abundant in young *H. prostrata* leaves without functional chloroplasts suggest that this phospholipase is important for lipid turnover in sink tissues. Consistent with extensive lipid-remodeling activity during *H. prostrata* leaf development, TAG levels were very high in young leaves of P-limited *H. prostrata*. It has been proposed that the transient accumulation of TAG could act as a buffer against the toxic effects of free fatty acids released from membranes (Fan et al., 2013; Troncoso-Ponce et al., 2013) and as a transient, readily accessible carbon storage pool (Zhang et al., 2009). High TAG accumulation, therefore, may partially compensate for the lower starch levels found in these organs.

H. prostrata Leaves Senesce Slowly with Early Remobilization of P from RNA

Southwestern Australian Proteaceae spp. remobilize scarce nutrients, such as P, very efficiently (Denton et al., 2007), perhaps through a process that extends cell viability (Hörtensteiner and Feller, 2002). Plants grown at the lowest Pi supply in this study showed similar leaf P concentrations compared with plants sampled in the field (Lambers et al., 2012b). In the oldest leaves of these P-limited plants, the photosynthetic pigment fraction declined by 30% compared with that in mature leaves. Anthocyanin concentrations also increased in these older leaves, which has been suggested to facilitate nutrient resorption by protecting against high radiation levels when chlorophyll levels decline (Hoch et al., 2003; Ougham et al., 2005; Zhang et al., 2013). Given that we were unable to isolate significant amounts of RNA from leaves in these very early stages of senescence, P resorption appears to start with the degradation of rRNA pools, which make up to about 50% of the total organic P fraction in photoautotrophic organisms (Veneklaas et al., 2012; Raven, 2013). In P-limited plants, protein concentrations in senescing leaves were similar to those in mature leaves, despite the large decline in the total RNA pool, indicating slow protein turnover. However, a clear decrease in protein concentration between mature and senescing leaves was seen for plants growing with an intermediate or high P supply. The overall normalized signal intensity for polar lipids in senescing leaves was about 80% of that at the other leaf developmental stages (Supplemental Table S3). Proportions of galactolipids and TAG, in particular, were much smaller than those in mature leaves. Interestingly, overall Pi and Po pools, and phospholipid levels in particular, did not decrease to the same extent during this early stage of leaf senescence as they do in other plants (Thompson et al., 1998).

The contrast between lower chlorophyll and galactolipid concentrations and relatively constant levels of PG and SQDG in senescing leaves suggests that the degradation of thylakoid membranes in the developing gerontoplast has not started (Koiwai et al., 1981; Matile et al., 1999; Kaup et al., 2002; Kolodziejek et al., 2003). A decline in phospholipid concentration during leaf senescence in other plant species increases membrane leakiness (Thompson et al., 1998). An extended lifetime for cellular membranes appears to be crucial for prolonging transport functions (Gniazdowska et al., 1999; Rilfors and Lindblom, 2002; Tjellström et al., 2010). This would give Proteaceae spp. like *H. prostrata* the time necessary to proficiently remobilize P from senescing leaves (Denton et al., 2007). Nutrient resorption in these long-lived leaves, therefore, seems to proceed relatively slowly, most likely leading to the transient accumulation of low-M_r Po catabolites prior to their export (Zhang et al., 2013).

The P Responsiveness of Regulatory Networks Is Reduced in *H. prostrata* Compared with That in Arabidopsis

Mature leaves of P-limited *H. prostrata* contained similar levels of starch, Pi, and Po as leaves of 3-week-old P-limited Arabidopsis seedlings (Nilsson et al., 2007). Lipid remodeling similar to that observed in P-limited Arabidopsis shoots (Kobayashi et al., 2009a) also occurred in *H. prostrata* leaves; galactolipids showed a negative correlation with Pi supply in all except senescing leaves, and phospholipids showed a positive correlation with Pi supply in all except young *H. prostrata* leaves. The decrease in galactolipid concentrations in young and mature leaves with Pi supply could be attributed to the P-dependent 4- to 5-fold decreases in transcripts encoding putative *H. prostrata* orthologs of the phosphodiesterase GDPD1 and the type B MGDG synthase MGD3 (Dubots et al., 2010; Cheng et al., 2011). The concomitant increase in phospholipids was accompanied by a modest 2-fold decrease in *HpNPC4* and a 4-fold increase in *HpNMT2* expression. While these changes in transcript abundance were moderate and affected very few of the lipid-remodeling genes examined in *H. prostrata*, leaves of P-limited Arabidopsis seedlings rapidly accumulated 4- to 60-fold more transcripts for a whole suite of genes encoding lipid-remodeling enzymes (Misson et al., 2005; Morcuende et al., 2007; Müller et al., 2007; Lan et al., 2012). The moderate transcriptional response in *H. prostrata* shows that while this species has not entirely lost the capacity to regulate P use for lipid metabolism through changes in gene expression, the magnitude of the response is greatly reduced compared with that in other plants. While profiles of Arabidopsis transcripts correlate well with changes in lipid profiles in response to environment (Szymanski et al., 2014), lipid metabolism in *H. prostrata* appears to be mostly regulated at the posttranscriptional level, perhaps through other mechanisms that are known to operate in other species like selective RNA translation and protein turnover (Galland et al., 2014) or allosteric enzyme regulation by PA and other

lipid metabolites (Ohlrogge and Browse, 1995; Dubots et al., 2012). *H. prostrata* is restricted to low-P environments. It is thus likely that the P-starvation response that is well documented in other plants would never be triggered under natural low-P conditions in *H. prostrata* and that regulatory components found in plants such as Arabidopsis and rice (*Oryza sativa*; Rouached et al., 2010; Lin et al., 2014) have been attenuated or lost. This possibility is supported by the absence of orthologs in the *H. prostrata* transcriptome database for many lipid genes that are induced by the myeloblastosis transcription factor PHOSPHATE STARVATION RESPONSE1 in response to Pi limitation in Arabidopsis (Bustos et al., 2010; Acevedo-Hernández et al., 2012). Instead, transcript profiles suggested that rate-limiting steps in the pathway, such as increased flux through the sulfate assimilation pathway mediated by SIR (Khan et al., 2010), MGDG synthesis by the type A MGDG synthase MGD1 in the chloroplast (Dubots et al., 2010), and the release of Pi from the phosphocholine head group (May et al., 2012), are mostly developmentally controlled.

Chloroplast Lipid Metabolism Is Perturbed at Higher Leaf Pi Concentrations

The most remarkable and unusual response to increasing Pi supply was the decrease in PG levels in mature *H. prostrata* leaves. In higher plants, there are two pathways for glycoglycerolipid synthesis: the prokaryotic pathway of the chloroplast and the eukaryotic pathway that involves PA synthesis in the ER (Roughan and Slack, 1982; Ohlrogge and Browse, 1995). PG production via the prokaryotic pathway is essential for chloroplast function and growth in Arabidopsis (Babiychuk et al., 2003; Xu et al., 2006). Lower PG levels in mature *H. prostrata* leaves appear to be accompanied by a compensatory increase in sulfolipid species of the 34:x acyl chain configuration that were mainly found in groups II (a) and III of the hierarchical cluster analysis. This differs from the situation in Arabidopsis, where levels of all phospholipids show a positive correlation and all glycoglycerolipids show a negative correlation with Pi supply (Dörmann and Benning, 2002). By contrast, 36:x SQDG species, which are synthesized from the ER-derived secondary DAG pool in the chloroplast (Fritz et al., 2007; Li-Beisson et al., 2010; Boudière et al., 2014), clustered in group II (b) and showed the typically documented decrease with increasing Pi supply in *H. prostrata*. These contrasting responses resulted in an overall sulfolipid profile that responded very little to altered Pi availability. While the majority of galactolipids in mature leaves decreased with increasing Pi supply, some 34:x galactolipid species also showed an unexpected positive response to Pi supply. In Arabidopsis leaves, 34:x lipid species show mixed acyl chain configurations suggesting biosynthesis from both ER-derived and chloroplast DAG pools, making it hard to determine their precise origin. In Arabidopsis leaves, PG 34:4, SQDG 34:3/34:4, MGDG 34:4 to 34:6, and DGDG 34:1 species almost exclusively carried the C-16 fatty acid in the *sn*-2 position (DGDG 34:4 species were not detected; Devaiah et al., 2006; Maatta et al., 2012), which would suggest that these lipid species are synthesized from the primary DAG pool in the chloroplast inner envelope membrane via the so-called prokaryotic pathway (Fritz et al., 2007; Li-Beisson et al., 2010; Boudière et al., 2014). In Arabidopsis, both PG and sulfolipids are essential for chloroplast structure and function, but only about 30% of PG species are actually produced in the chloroplast (Xu et al., 2002; Yu and Benning, 2003). PG and SQDG production in the chloroplast are tightly regulated in an antagonist fashion (Essigmann et al., 1998). The fact that *H. prostrata* appears to produce most of its PG in the chloroplast might account for the fact that only the sulfolipid (and galactolipid) species connected to the primary DAG pool of the chloroplast show the typical compensatory response to lower PG levels in mature leaves (Fritz et al., 2007; Shimojima et al., 2009; Boudière et al., 2014), despite their overall higher P status. The inferred contribution of the prokaryotic pathway for MGDG synthesis rose to as much as 20% in leaves with toxicity symptoms compared with about 2% in leaves of P-limited plants (Supplemental Table S3). The glycoglycerolipids found in group II (b), on the other hand, seem to be synthesized from the secondary DAG pool derived from the ER. Their metabolite profiles suggest that they are more responsive to the plants' P status and are involved in P-dependent lipid remodeling in extraplastid membranes. The disparity in the regulation of these two groups in response to Pi supply might provide an excellent tool to further dissect the regulation of prokaryotic and eukaryotic pathways (Roughan and Slack, 1982; Shimojima and Ohta, 2011) using *H. prostrata*. They also could be indicative of a strong adaptive mechanism in *H. prostrata* that enables it to maintain high PG levels in mature leaf chloroplasts under low Pi availability, ensuring optimal photosynthetic performance (Lambers et al., 2012b).

This specialized adaptation of lipid metabolism might provide a further reason why *H. prostrata* does not show a positive growth response with highest Pi supply: in mature and senescing leaves with P-toxicity symptoms, free fatty acid, lyso-PC, and TAG levels almost doubled. Most notably, the concentrations of PG species and starch were significantly lower than those in healthy leaves, while PC, PE, galactolipid, and sulfolipid concentrations were higher. These metabolite profiles would indicate a severe perturbation of chloroplast function. Given that *HpNPC4* transcript abundance was lowest in mature leaves with P-toxicity symptoms, overall lipid turnover at the plasma membrane (Nakamura et al., 2005) also appears to be greatly reduced under conditions of excessive Pi accumulation in leaves. It will be interesting to further explore if the observed swelling of *H. prostrata* palisade mesophyll cells that accumulate the highest concentrations of Pi (Shane et al., 2004) is indicative of a loss of membrane integrity and function caused by the imbalance in lipid biosynthesis between the ER and chloroplast (Sakurai et al., 2003; Eastmond et al., 2010).

CONCLUSION

Phospholipids are the major lipid class in plant cell membranes and essential for cell division and expansion. Higher plants characterized to date exhibit some degree of lipid remodeling during P starvation. *H. prostrata* has adapted to its low-P environment partly through delayed greening and reduced chloroplast size or chloroplast numbers in mature leaves and extensive lipid remodeling during leaf development. This is accompanied by the developmental regulation of PG levels and key lipid-remodeling genes and their reduced P responsiveness. Other aspects of leaf metabolism, on the other hand, are highly responsive to Pi: protein synthesis in particular is tightly linked to Pi supply without being coupled to the developmental programs controlling leaf expansion and leaf size. Future work will further explore the metabolic adjustments, enzymatic properties, and regulatory networks that define *H. prostrata*'s high photosynthetic P-use efficiency but may have rendered its lipid metabolism and associated regulatory networks sensitive to high soil P.

MATERIALS AND METHODS

Plant Growth

Washed roots of 4-month-old soil-grown *Hakea prostrata* seedlings (Men of the Trees Nursery) were treated with 5% (v/v) sodium hypochlorite for 15 min to avoid fungal contamination. Individual seedlings were transferred to 5-L black plastic pots covered with black panels and gray foam plugs to support the shoots. The seedlings were grown in a temperature-controlled glasshouse at the University of Western Australia in cooling tanks that kept the root temperature at 19°C. Each pot contained 4 L of a continuously aerated reduced-strength Hoagland solution [40 μM $Ca(NO_3)_2$, 20 μM K_2SO_4, 10 μM KH_2PO_4, 11 μM $MgSO_4$, 2 μM FeNaEDTA, 4 μM KCl, 0.05 μM $MnCl_2$, 0.02 μM $ZnSO_4$, 0.004 μM $CuSO_4$, 0.5 μM H_3BO_3, 0.003 μM $CoCl_2$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 10 μM Na_2O_3Si, pH 5.8] that was changed weekly.

During experiment A, the average minimum and maximum temperatures were 14°C at night and 22°C during the day, and the average maximum light intensity between 12 noon and 2 PM was 820 μmol m^{-2} s^{-1} (70% transmission), with sunrise at 7.30 AM and sunset at 5:30 PM. Relative humidity varied from 47% (day) to 66% (night). After 12 weeks, similarly sized seedlings were selected for growth in nutrient solution containing different Pi concentrations: 0, 5, 20, and 150 μM KH_2PO_4. The plants were randomly assigned to each treatment, and the nutrient solution was changed twice per week, resulting in average application rates of 0, 6, 23, and 171 μmol Pi $plant^{-1}$ d^{-1}. After 21 d of treatment, leaves (young, mature [i.e. the most recently fully expanded leaves on the main stem], and senescing [i.e. the oldest, chlorotic leaves on the main stem]) were harvested around midday, between 11 AM and 4 PM, weighed, frozen in liquid N_2, and stored at −80°C. Remaining roots, stems, and leaves were weighed separately and added to the mass of the collected samples to give the total fresh weight of the seedlings. This material was dried at 60°C for 4 d to determine plant dry weight.

For the polar lipid profiling work (experiment B), 6-month-old cuttings propagated from a mature *H. prostrata* plant (Nuts about Natives) were established as described above, but with a 4-week acclimation period and an experimental period of 12 weeks in the autumn/winter of 2011 (March to July 2011), with sunrise at 7:30 AM and sunset at 5:30 PM, average temperatures of 27°C (day) and 17°C (night), average relative humidity of 49% (day) and 72% (night), and average midday maxima of around 810 μmol photons m^{-2} s^{-1}, 37% relative humidity, and 35°C. The experimental period started with application rates of 0, 0.8, 40, and 100 μmol KH_2PO_4 $plant^{-1}$ d^{-1} for 4 weeks. Due to signs of severe P limitation at the lowest Pi supply and the absence of P-toxicity symptoms at the highest Pi supply, application rates were adjusted to 0.8, 4, 40, and 200 μmol KH_2PO_4 $plant^{-1}$ d^{-1} for the remaining 8 weeks. Plants were harvested as described above, except that mature leaves from plants exposed to the two highest Pi supplies that displayed signs of P toxicity such as chlorosis and blotchiness (most likely due to localized cell death) were harvested separately from leaves that showed no visible symptoms.

H. prostrata is an outcrossing species, and seed material used in nurseries is collected from the wild. To ensure that our chosen treatments triggered the expected Pi responses, phenotypic data were recorded for all plants. Plants that did not show the typical responses were excluded from the downstream analyses.

Phosphate and Total P Analyses

To determine Pi uptake rates, samples were taken from the spent nutrient solution from three pots per treatment when the solution was replaced. To determine the amount of Pi within plant organs, 50 mg of ground and frozen plant material was combined with 700 μL of 1% (v/v) acetic acid and homogenized (Precellys 24 Tissue Disruptor; Bertin Technologies) for three cycles of 30 s at 4,500 rpm. The cleared supernatant was stored at −20°C. For total P analysis, 50 mg of oven-dried leaf tissue was digested to convert esterified P to Pi (Zasoski and Burau, 1977), and the ash was resuspended in 5 mL of deionized water. The Pi concentration of all samples was determined using an ammonium molybdate/ascorbic acid-based assay (Ames, 1966).

Sequence Identification and Primer Design

Unique *H. prostrata* orthologs of 26 Arabidopsis (*Arabidopsis thaliana*) genes of interest associated with membrane-lipid remodeling were identified in a database (InParanoid; Remm et al., 2001) generated using open-reading frame translations of *H. prostrata* young leaf and root transcriptomes (http://www.onekp.com/). Unique primer pairs for the selected *H. prostrata* transcripts were designed in QuantPrime (Supplemental Table S1; Arvidsson et al., 2008). The specificity of the primer amplicons was confirmed by melting-curve analysis. Primer efficiencies were determined across cDNA samples in independent quantitative PCR assays using the LinReg algorithm (Ruijter et al., 2009).

RNA Extraction, Reverse Transcription, and Quantitative PCR

Ground frozen leaf powder (100 mg) was mixed with 700 μL of 100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% (w/v) lithium dodecyl sulfate, 5 mM dithiothreitol, 0.5% (w/v) polyvinylpyrrolidone, and 0.5% (w/v) polyvinylpolypyrrolidone. Following the addition of 350 μL of Fruit-mate (Takara Biotechnology), samples were homogenized as described above. After several chloroform:isoamyl alcohol (24:1) extractions, RNA was concentrated by isopropanol precipitation, followed by another precipitation of the resuspended RNA in 2 M LiCl. The RNA pellet was washed with 2 M LiCl followed by two washes with 70% (v/v) ethanol, air dried, and resuspended in 20 μL of RNase-free water. After removal of contaminating DNA (RQ1 DNase; Promega), the RNA concentration was determined (NanoDrop 1000 Spectrophotometer; Thermo Scientific). The average residual genomic DNA contamination was less than 0.01%. About 1 μg of total RNA was reverse transcribed according to the manufacturer's instructions (Tetro cDNA synthesis kit; Bioline). Quantitative PCR and threshold cycle determination were performed (7500 FAST Real-Time PCR System; Applied Biosystems) in reactions that contained 2.5 μL of cDNA (synthesized from approximately 25 ng of total RNA), 2.5 μL of gene-specific primer mix (1.2 μM each), and 5 μL of PCR master mix (Power SYBR Green; Applied Biosystems). The normalized threshold fluorescence level was set to 0.2. The relative expression levels of transcripts of interest were normalized to orthologs of the reference genes *ACTIN7* (Hong et al., 2008) and *YLS8* (Czechowski et al., 2005) after validation using the geNORM algorithm (Vandesompele et al., 2002).

Polar Lipid and Photosynthetic Pigment Profiling

Frozen leaf powder (50 mg) was extracted as described by Hummel et al. (2011). Pellets of the final organic phase were stored at −80°C prior to analysis by ultra-performance liquid chromatography (UPLC) quadrupole time-of-flight mass spectrometry (Burgos et al., 2011). The quantity of each lipid or pigment was derived as the normalized mass spectral signal relative to the internal standard 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (PC 34:0; 17:0/17:0). These values were then divided by the fresh weight of the extracted sample to give the normalized mass spectral signal per gram fresh weight. Alternatively, the values were divided by the total normalized signal

to express them as percentages of the normalized mass spectral signal. All chemicals used were UPLC/mass spectrometry grade (BioSolve).

To account for possible chlorophyll degradation during extraction, chlorophyll *a*/*b* concentrations were also determined spectrophotometrically using methanol extraction of freshly harvested mature leaf discs (Supplemental Fig. S1). Profiles across treatments were found to be comparable. We were not able to confidently annotate phosphatidylserine, PA, or DAG-derived peaks in the mass spectra of any of our *H. prostrata* leaf samples. At this stage, it is unclear whether this is due to low levels of these lipids, the lack of the entire phosphatidylserine pathway, or a very rapid turnover of DAG. Our UPLC-mass spectrometry-based analytical approach did not allow the resolution of PA lipid species.

Starch and Total Protein Determination

The first pellet from the lipid extraction (see above) was resuspended in 400 μL of 0.1 M NaOH and heated for 30 min at 120°C with vigorous shaking. The cooled supernatant was assayed for protein at 595 nm against a bovine serum albumin standard (Bradford, 1976). The starch pellet was suspended in 80 μL of 0.5 M HCl and 0.1 M acetate, pH 4.9, buffer. The starch was degraded for 16 h at 37°C using an amyloglucosidase/α-amylase enzyme mixture, and the Glc end-product concentration was determined via NADPH production by Glc-6-P dehydrogenase (Hendriks et al., 2003).

Statistics

Statistical significance between treatments was determined using ANOVA in SigmaStat version 12.3 and defined as $P \leq 0.05$. Unless stated otherwise, two-way ANOVA followed by Tukey's posthoc test was used to separate means. Hierarchical clustering analysis was performed using J-Express 2012 (Norwegian Bioinformatics Platform and Norwegian Microarray Consortium [http://www.molmine.com]; Dysvik and Jonassen, 2001).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Accumulation of photosynthetic pigments and anthocyanins in leaves of *H. prostrata* plants grown at a range of Pi supplies.

Supplemental Figure S2. Hierarchical cluster analysis of changes in lipid profiles during leaf development and in response to Pi supply.

Supplemental Table S1. Sequence information on *H. prostrata* transcripts encoding orthologs of known lipid-remodeling genes in Arabidopsis, including quantitative reverse transcription-PCR primer sequences used in this study.

Supplemental Table S2. Detailed information on the relative abundance of individual transcripts in young and mature *H. prostrata* leaves in response to phosphate supply.

Supplemental Table S3. Detailed information on the relative abundance of individual lipid species along the leaf developmental gradient and in response to phosphate supply in *H. prostrata*.

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