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Early response mechanisms of perennial ryegrass (*Lolium perenne*) to phosphorus deficiency

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• *Background and Aims* Improving phosphorus (P) nutrient efficiency in *Lolium perenne* (perennial ryegrass) is likely to result in considerable economic and ecological benefits. To date, research into the molecular and biochemical response of perennial ryegrass to P deficiency has been limited, particularly in relation to the early response mechanisms. This study aimed to identify molecular mechanisms activated in response to the initial stages of P deficiency.
• *Methods* A barley microarray was successfully used to study gene expression in perennial ryegrass and this was complemented with gas chromatography-mass spectrometry metabolic profiling to obtain an overview of the plant response to early stages of P deficiency.
• *Key Results* After 24 h of P deficiency, internal phosphate concentrations were reduced and significant alterations were detected in the metabolome and transcriptome of two perennial ryegrass genotypes. Results indicated a replacement of phospholipids with sulfolipids and the utilization of glycolytic bypasses in response to P deficiency in perennial ryegrass.
• *Conclusions* The transcriptome and metabolome of perennial ryegrass undergo changes in response to reductions in P supply after 24 h.

Key words: *Lolium perenne*, perennial ryegrass, phosphorus deficiency, metabolic profiling, transcript profiling, cross species hybridization.

INTRODUCTION

Depleting phosphorus (P) reserves has been described as one of the 'most significant sustainability issues of our time' (Vaccari, 2009). Continually increasing demand will deplete global supplies by the end of the century (Vaccari, 2009), leading to price volatility that threatens the economic sustainability of agriculture. Overuse of fertilizers has increasingly been shown to exert a detrimental impact on the environment largely through run-off (Ferrier and Edwards, 2002) and associated environmental degradation (Tilman, 1998; Gyaneshwar *et al.*, 2002). However, predictions suggest that worldwide fertilizer usage is projected to increase as the population increases (by 46 % to 8·9 billion people by 2050; Anon, 2004) and exerts an associated requirement for food. In the past, significant attention has been focused on the negative impacts of nitrogen but more recently there has been an increased focus on the environmental impacts of P. It was revealed in one Canadian study that decreasing inputs of P was more critical in reducing eutrophication than controlling nitrogen inputs (Schindler *et al.*, 2008). Furthermore, there is also evidence that excess P is likely to result in a greater loss of plant species than that associated with enhanced

nitrogen, therefore having a greater impact on biodiversity (Wassen *et al.*, 2005).

Perennial ryegrass, *Lolium perenne*, is the principal forage used in temperate regions (Humphreys, 2005). Improving P nutrient efficiency in this species is likely to result in considerable economic and ecological benefits. To date, no studies have investigated the (bio)molecular response of perennial ryegrass to P deficiency. Publicly available genomic resources, including available microarrays, for the study of gene expression are limiting in perennial ryegrass (Ciannamea *et al.*, 2006). To overcome this limitation, microarrays developed for related species can be employed to study gene expression in the target organism (Hammond *et al.*, 2005; Bar-Or *et al.*, 2007). In other species, P deficiency was reported to result in a variety of responses both at the gene expression (Hammond *et al.*, 2003, 2005; Misson *et al.*, 2005; Wasaki *et al.*, 2006; Guo *et al.*, 2008) and metabolite (Hernandez *et al.*, 2007; Huang *et al.*, 2008) levels.

Improving a plant's ability to acquire and efficiently utilize P is one approach to both alleviating the scarcity of the resources and reducing environmental impacts. Efforts towards this have been accelerated by legislative measures to reduce fertilizer usage and pollution (Anon, 2000). The

adaptation of plants to limited P can be optimized by allocating a greater proportion of their biomass to the root system (Hermans *et al.*, 2006), remobilization of internal P and modifications in the carbon metabolism (Raghothama, 1999; Ramirez-Rodriguez *et al.*, 2005). Plants generally adjust their metabolism to optimize internal P utilization and improve P acquisition (Theodorou and Plaxton, 1993). In barley, carbohydrate metabolism is initially readjusted to reduce P consumption, and when stress becomes more severe, P is salvaged from small P-containing metabolites such as glucose 6-P, fructose 6-P, inositol 1-P and glycerol 3-P (Huang *et al.*, 2008).

P uptake can be enhanced by secretion of phosphatases and organic acids, modified root growth, and enhanced expression of P transporters (Ramirez-Rodriguez *et al.*, 2005). Organic P generally constitutes the majority of P in soils; however, it is unavailable for plant uptake (Wang *et al.*, 2004; Richardson *et al.*, 2009) but can be converted to a more available form with the excretion of phosphatases (Plaxton, 2004). Transgenic studies aimed at improving P use efficiency have generally focused on developing plants with better ability to make use of organic P in the soil. The expression of phytase from *Aspergillus* in *Arabidopsis* resulted in increase root phytase activity and subsequently enabled plants to meet their P requirements from phytate (Richardson *et al.*, 2001). An alternative route to increased P uptake was taken by Wang *et al.* (2004), who over- expressed an *Arabidopsis thaliana* purple phosphatase gene (*AtPAP15*) containing a carrot (*Daucus carota*) extracellular targeting peptide in soybean to obtain inorganic P from organic P sources. This resulted in a 60–120 % increase in plant weight and an associated 60–90 % increase on plant P content (Wang *et al.*, 2004).

The increase in the expression of genes related to organic acid metabolism and their secretion have also been reported under P deficiency, which suggests that organic acids play an important role in the adaptation to P-limiting conditions (Uhde-Stone *et al.*, 2003*a*, *b*). A citrate synthase gene over-expressed in *Arabidopsis* was shown to improve growth on P-limited soils due to enhanced citrate excretion from roots (Koyama *et al.*, 2000). The majority of the inorganic P present in soil is in precipitated form or adsorbed to soil constituents such as organic matter and clays (Richardson *et al.*, 2009). It is thought that an increase in the levels of organic acids in the rhizosphere will chelate ions which are usually associated with phosphate groups, such as Ca^{2+}, Fe^{2+} or Al^{3+}, thus allowing the inorganic P to be released for plant uptake (Vance *et al.*, 2003).

The objective of the present study was to determine the early response mechanisms of perennial ryegrass to P deficiency. We used a 44k barley microarray to study changes in gene expression whilst a complementary gas chromatography-mass spectrometry (GC-MS) approach was taken to study changes in primary metabolism.

MATERIALS AND METHODS

Selection of genotypes

A screen was carried out on five seedlings from each of 34 ecotypes and two cultivars of *Lolium perenne* (Supplementary Data 1, available online) with the aim of identifying material with a high capacity to remove P from solution. After 5 d, seedlings were weighed, transferred to 50-mL glass test tubes containing 40 mL nutrient solution [$CaCl_2.2H_2O$, 0·75 mM; $MgSO_4.7H_2O$, 0·38 mM; MS Micro Salts (Duchefa, Haarlem, The Netherlands), 0·146 g L^{-1}; NH_4NO_3, 5 mM; $Ca(NO_3)_2.4H_2O$, 2·33 mM; KH_2PO_4, 0·31 mM] and placed in a growth room maintained at 23 °C with a 16-h daylight regime [photosynthetically active radiation (PAR) = 360 μmol m^{-2} s^{-1}]. After 3 d, samples were taken from each solution, the P content was determined using the molybdenum blue assay as described by He and Honeycutt (2005), and a standard curve was generated ($R^2 = 0.99$) using dilutions of initial nutrient solution. Results were analysed in GenStat V10 (VSNi, Hemel Hempstead, UK) using a one-way ANOVA (without blocking). The fresh weights of seedlings at the start of the experiment were used as covariates in the analysis. Two genotypes (IRL-OP-02538-P and Cashel-P) were selected from this screen and propagated to provide adequate clonal replicates for the experiments described below.

Experimental conditions

Seedlings of IRL-OP-02538-P and Cashel-P were cleaned of soil and transferred to perlite medium held inside plastic plug trays (P84 Plug Trays, Carley's Bridge Potteries, Enniscorthy, Ireland) floated on the plant nutrient solution (see above). Solutions were placed in two 25-L tanks and aerated with an aquarium pump (Rena Air 300, RENA, Chalfont, PA, USA). Experiments were performed in a controlled glasshouse with a mean daily temperature of 22 °C and supplemented with lighting (PAR = 650 μmol m^{-2} s^{-1}) for 16 h. The plants were allowed to acclimate for 1 week to the hydroponics growth conditions before applying treatments. At the start of the treatment the solutions in both tanks were replaced with either solution A, identical to that above, or solution B, identical to that above except that the KH_2PO_4 content was reduced to 0·016 mM. After 24 h (noon) root and leaf tissues were separated and flash frozen in liquid nitrogen. Two separate repeats of the experiment were performed to obtain samples for array hybridizations and metabolite profiling, respectively, and independent biological replicates (four in total) were sampled for each.

Microarray processing: genomic DNA hybridizations

Genomic DNA (gDNA) was isolated according to the method of Doyle and Doyle (1987) with minor modifications. Throughout this study, a custom microarray SCRI-Hv35-44k-v1 (Agilent design 020599) representing approx. 42 000 barley unigene sequences from the public HarvEST database (assembly 35; http://www.harvest-web.org/) was used (ArrayExpress accession A-MEXP-1728). This microarray along with a smaller barley array design (Chen *et al.*, 2010) have been successfully used for gene expression analysis in our laboratory. Array procedures followed MIAME guidelines (Brazma *et al.*, 2001). The design of the microarray experiment and the data derived from it are detailed in the public database ArrayExpress (http://www.

ebi.ac.uk/microarray-as/ae/; accession E-TABM-950). For gDNA hybridizations, fluorescent labelling and purification was carried out according to the protocol detailed by Ducreux *et al.* (2008).

Microarray processing: cDNA hybridizations

Total RNA was isolated independently from four biological replicates of leaf and root tissues using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with on-column DNase I digestion according to the manufacturer's protocol. Samples were run on Bioanalyzer RNA 6000 Nano Chips (Agilent Technologies, Santa Clara, CA, USA) to assess quality and integrity.

The design of the microarray experiment and the data derived from it are detailed in the public database ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/; accession E-TABM-943). In total, four biological replicates of each sample were processed (two genotypes, two treatments, two tissues, four replicates; 32 samples; 16 arrays). The experimental design was balanced with respect to fluorescent dyes, with two replicates of each sample labelled with either Cy3 or Cy5 to minimize dye bias. RNA samples were labelled as cDNA and purified using the Quick Amp Labelling Kit (Agilent Technologies) as recommended using 1 μg total RNA per sample.

Microarray processing: data extraction and analysis

For data extraction, microarray images were imported into Agilent Feature Extraction (FE) (v·10·5·1·1) software and aligned with the array grid template (020599-D-F-20080612). Intensity data for each spot were extracted using a defined FE protocol (GE2-v5-95-Feb07) and data from each array were normalized using the LOWESS (locally weighted polynomial regression) algorithm (Yang *et al.*, 2002). Normalized datasets for each array were subsequently loaded into GeneSpring software (v.7·3·1; Agilent Technologies) for analysis.

For the RNA-based microarray study, specific samples were subjected to dye-swap according to the experimental design, and data with consistently low probe intensity level – flagged as absent in all replicate samples – were discarded. Comparisons were made between the two P treatments for each tissue and genotype independently using volcano plots (Cui and Churchill, 2003), with thresholds of >two-fold change and a Student's *t*-test *P* value <0·05 applied, to identify significantly regulated genes. Unique and overlapping genes between the lists were selected using Venn diagrams.

Quantitative RT-PCR

Total RNA was isolated as described above and 200 ng was converted to cDNA using SuperScript III and Oligo(dt)$_{20}$ primer (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. Real-time assays and melting curve analysis were performed with SYBR Green I Master (Roche) on the LightCycler 480 according to the pack insert (http://www.roche-applied-science.com/pack-insert/4707516a.pdf). Analysis was carried out on three biological replicates (each in technical triplicates). Reaction efficiency values were calculated by running each primer set on serial dilutions of a cDNA mixture comprising leaf and root material. Normalization was performed using the housekeeping gene LpGAPDH (Petersen *et al.*, 2004). Primers were designed from the barley sequences used for microarray probe design (Supplementary Data 2, available online). Statistical analyses were performed in REST 2008 (Pfaffl *et al.*, 2002).

Sample preparation for metabolite profiling

Frozen tissues of roots and leaves were freeze dried and homogenized using a mortar and pestle. Frozen tissue powder (approx. 50 mg) was extracted as described by Foito *et al.* (2009). In summary, samples were extracted sequentially with methanol (3 mL), water (0·75 mL), chloroform (6 mL) and an additional volume of water (1·5 mL) resulting in a biphasic extract in order. Aliquots from the resulting polar and non-polar fractions (750 μL and 4 mL, respectively) were derivatized as described by Foito *et al.* (2009).

Analysis of metabolites by GC-MS

The polar and non-polar samples were analysed similarly using a GC-DSQ-MS system (Thermo Finnigan, Manchester, UK). Samples (1 μL) were injected into a programmable temperature vaporizing (PTV) injector with a split of 40 : 1. The PTV and chromatography conditions used are described by Foito *et al.* (2009). Mass spectra were acquired under electron impact ionization conditions at 70 eV and 100 mA of emission current over the mass range 35–900 a.m.u at 6 scans s^{-1} with a source temperature of 200 °C and a solvent delay of 1·3 min. Acquisition rates were set to give approximately ten data points across a chromatographic peak. Data were acquired using the Xcalibur^{TM} software package v. 1·4. Acquired total ion chromatograms and mass spectra were analysed using Xcalibur^{TM} software package v2·0·7 (Thermo Finnigan) as described by Foito *et al.* (2009). Analysis of variance was used to compare across P treatments and identify significantly regulated metabolites ($P < 0.05$). The complete profiles were initially compared by principal components analysis (PCA) and the results for metabolite analysis with respect to data acquisition controls are described in Supplementary Data 3 (Figs S1 and S2, available online). All statistical analysis was performed using GenStat version 12·1·0·3338 (VSNi).

RESULTS

Genotype selection

The purpose of the pre-screen was to identify seedlings with increased rates of Pi removal from solution. The results showed that ecotype had an effect on P removal ($F_{35,142} = 3·07$, $P < 0.001$). The ecotype with the highest removal of P from solution was IRL-OP-02538 (Supplementary Data 1). The seedling with the highest P removal (63·56) was propagated together with a seedling from the cultivar 'Cashel' (41·51), which displayed an average removal of P from solution. We chose to test two genotypes as opposed to a single

genotype in order to identify more general response mechanisms of perennial ryegrass to 24 h of P deficiency and avoid making assumptions based on the response of a single genotype.

Array hybridization across species

Initial experimentation used genomic DNA to determine the overall level of hybridization of perennial ryegrass genome to probes on the barley microarray. This took advantage of the extreme sensitivity of the Agilent microarray platform. Approximately 40 % of probes (16000 genes) hybridized with measurable signal to perennial ryegrass genomic DNA. This level of hybridization reflects the levels of sequence diversity between the two species, although the 60-mer probes utilized in the Agilent microarray allow for a certain degree of mismatches, as reported previously for analysis of diverse potato genotypes (Ducreux *et al.*, 2008). After hybridization with cDNA derived from leaf and root RNA, approx. 6800 and approx. 6300 probes, respectively, produced an acceptable signal amongst replicates.

The validity of the array results was verified by real-time RT-PCR of seven selected genes (Table 1) identified as significantly regulated by using the barley arrays. The results were in general agreement with the array results with the exception of U35-44k-v1-8347, a putative MAP3K-like protein kinase, which was not identified as being significantly up-regulated by real-time RT-PCR (Table 1). Indeed, the strong agreement confirms the efficacy of the barley arrays for analysing gene expression in perennial ryegrass.

Transcriptomic changes in leaf tissue under P deficiency

Using the barley arrays the expression profiles of two perennial ryegrass genotypes changed in response to 24 h of P deficiency (Fig. 1; Supplementary Data 4 and 5, available online). The genes significantly regulated ($\geq$two-fold and $P < 0.05$) in each genotype are shown in Supplementary Data 4. The greatest number of significantly regulated genes (222) was discovered in the leaf tissue of Cashel-P under P deficiency, with 95 % of these genes being up-regulated. In

contrast, a much smaller number of genes (35) were significantly regulated in the leaf tissue of IRL-OP-02538-P (Fig. 1A).

Only 21 genes were significantly regulated in a similar fashion in both genotypes, some of which have an unknown function (Table 2). No down-regulated genes were common between genotypes. The gene with the greatest induction in both genotypes encodes a putative glycerol 3-phosphate permease (G3PP).

In all cases there were a number (three) of genes encoding phosphatases up-regulated under P deficiency in leaf tissue (Supplementary Data 4). In addition, one gene encoding a phosphate transporter was also up-regulated but to a lesser extent. One group of genes was notably up-regulated, particularly in the Cashel-P genotype, and these were those involved in cell-wall synthesis with five cellulose synthase genes up-regulated under P deficiency (CESA4, CESA9, CESA1, CESA2 and CESA8). Other genes involved in cell-wall modification were also identified with the gene encoding ENDO-1, 4-beta-xylanase, which catalyses the hydrolysis of the major plant hemicellulose, xylan, showing the greatest increase in expression in Cashel-P under P deficiency.

Metabolite changes in leaf tissue under P deficiency

Metabolite profiling was carried out on genotypes from IRL-OP-02538 and 'Cashel' when grown under normal and restricted P supply. The profiles included the levels of 206 metabolites (140 identified), which included 116 polar metabolites and 90 non-polar metabolites. PCA revealed distinguishing differences between the profiles of leaves from different genotypes (Supplementary Data 3, Fig. S3, available online) and, to a lesser extent, between treatments. An ANOVA identified a number of significantly different ($P < 0.05$) metabolites in the different tissues and genotypes (Fig. 1B). In contrast to the transcriptomic data, no major difference in the number of up-regulated metabolites between genotypes was found, although the majority of the significantly regulated metabolites were different. In the leaf tissue of IRL-OP-02538-P there were 27 regulated metabolites (including nine unidentified). One unidentified metabolite experienced an increase under P

TABLE 1. *Validation of microarray leaf and root data by real-time RT-PCR of seven genes identified as significantly regulated from leaf or root hybridizations*

Description	Rice match	Putative function	Array		RT-PCR	
			Cashel-P	IRL-OP-02538-P	Cashel-P	IRL-OP-02538-P
Leaf						
U35-44k-v1-12109	LOC-Os02g33710·1	Histidine decarboxylase	**5·9 (0·001)**	**5·8 (0·000)**	**8·2 (0·000)**	**7·4 (0·000)**
U35-44k-v1-37470	LOC-Os12g37600·1	Glycerol-3-phosphate acyltransferase 1	0·9 (0·951)	**5·8 (0·005)**	0·9 (0·904)	**2·7 (0·029)**
U35-44k-v1-8347	LOC-Os03g30130·2	Phospholipase C	**2·8 (0·007)**	**4·3 (0·006)**	1·4 (0·374)	1·6 (0·178)
U35-44k-v1-7335	LOC-Os01g54620·1	CESA4 – cellulose synthase	1·4 (0·702)	**7·0 (0·038)**	1·2 (0·626)	**4·0 (0·013)**
Root						
U35-44k-v1-22715	LOC-Os01g67126·1	60S ribosomal protein L5-2	3·3 (0·585)	**3·6 (0·032)**	0·6 (0·714)	**8·2 (0·000)**
U35-44k-v1-49610	LOC-Os08g43870·1	Hypothetical protein	1·0 (0·939)	**2·6 (0·024)**	0·9 (0·543)	**2·8 (0·003)**
U35-44k-v1-43490	LOC-Os08g39300·1	Serine-glyoxylate aminotransferase	1·2 (0·718)	**0·3 (0·021)**	0·7 (0·487)	**0·3 (0·000)**

The fold change for both array and RT-PCR data are shown with associated significance values in parentheses; significant differences in bold. Array data were analysed in GeneSpring and RT-PCR data in REST.

deficiency, while levels of the remaining 26 (Table 3) declined. This is in contrast to the genotype Cashel-P in which levels of 11 metabolites increased and levels of ten decreased (Table 4). From the 21 significantly regulated metabolites in Cashel-P in leaf tissue, four remain unidentified. These differences in trends between genotypes contributed significantly to the segregation seen in the PCA of the leaf tissue. In Cashel-P, there was a significant decrease in amino acids (asparagine, glutamine, homoserine and histidine)

while the levels of putative phytol-derived metabolites appeared to be up-regulated (phytol A-C and phytil methyl ether). Despite the overall differences in the metabolic response to P limitation, both genotypes experienced a significant decrease in levels of Pi.

Trancriptomic changes in root tissue under P deficiency

There were a much lower number of significantly regulated genes under P deficiency in the root tissue in comparison with leaf tissue. Furthermore, the majority of the significantly regulated genes were down-regulated in both genotypes (Supplementary Data 5). In Cashel-P the down-regulated genes included those which encode a pair of ribosomal proteins, an asparagine synthetase (EC 6·3·5·4), a serine-glyoxylate aminotransferase (EC _2·6·1·45), a fructose-1, 6-bisphosphatase (EC 3·1·3·11) and a sedoheptulose-1, 7-bisphosphatase (EC 3·1·3·37; full list in Supplementary Data 5). Only one gene was commonly regulated in both genotypes and this encoded a 1,4-beta-xylanase (EC 3·2·1·8), which was up-regulated.

Metabolite changes in root tissue under P deficiency

In the PCA of the metabolite profiles from root tissue (Supplementary Data 3, Fig. S4) there was no segregation between genotypes as observed in the leaf tissue. However, the ANOVA highlighted differences which could not be visualized using PCA of the root tissue. A greater difference in the number of significantly regulated metabolites was found between genotypes in the root tissue: 14 metabolites (eight unidentified) were regulated in Cashel-P (Table 4) whereas 36 (ten unidentified) were regulated in IRL-OP-02538-P under P
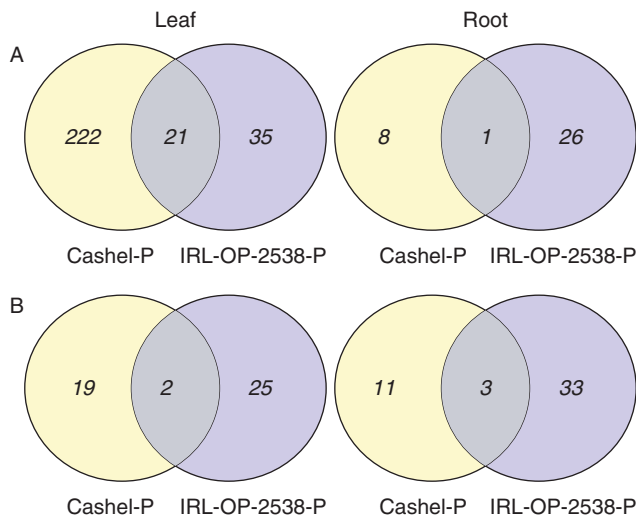

Fig. 1. (A) Number of genes from barley array hybridizations with ≥ two-fold change in expression ($P < 0.05$) under limited phosphorus for each genotype. (B) Number of metabolites with significant fold change ($P < 0.05$) under limited phosphorus for each genotype. Leaf tissue on left and root tissue on right.

TABLE 2. *Genes significantly ($P < 0.05$) induced ≥ two-fold after 24 h of P deficiency in the leaf tissue of both Cashel-P and IRL-OP-02538-P*

| Array ID | Best hit rice pp5 | E value | IRL-OP-02538-P | | Cashel-P | |
			Fold change	P	Fold change	P
U35-44k-v1-27526	Glycerol 3-phosphate permease	1×10^{-45}	7·6	<0·01	17·1	<0·01
U35-44k-v1-31414	Glycerol 3-phosphate permease	7×10^{-70}	7·0	<0·01	12·3	<0·01
U35-44k-v1-12109	Histidine decarboxylase	1×10^{-121}	5·9	<0·01	5·8	<0·01
U35-44k-v1-7536	Purple acid phosphatase precursor	1×10^{-166}	5·5	<0·01	5·3	<0·01
U35-44k-v1-26073	Nucleotide pyrophosphatase/phosphodiesterase	1×10^{-130}	4·5	<0·01	5·4	<0·01
U35-44k-v1-31178	No hits found		3·7	<0·01	2·9	0·01
U35-44k-v1-7801	UDP-sulfoquinovose synthase chloroplast precursor	1×10^{-68}	3·4	0·01	4·2	<0·01
U35-44k-v1-27915	ids4-like protein	4×10^{-18}	3·4	<0·01	5·3	<0·01
U35-44k-v1-48069	No hits found		3·1	<0·01	2·4	0·02
U35-44k-v1-24642	Expressed protein	2×10^{-15}	2·9	0·04	5·7	<0·01
U35-44k-v1-8347	Phospholipase C	1×10^{-159}	2·8	0·01	4·3	0·01
U35-44k-v1-30444	Expressed protein	4×10^{-43}	2·8	<0·01	2·0	<0·01
U35-44k-v1-36750	Expressed protein	4×10^{-85}	2·7	<0·01	2·4	0·04
U35-44k-v1-28139	Phosphate transporter 1	1×10^{-82}	2·7	0·04	10·5	0·01
U35-44k-v1-27385	Acid phosphatase/vanadium-dependent haloperoxidase-related	4×10^{-5}	2·5	0·01	2·4	<0·01
U35-44k-v1-28600	Expressed protein	4×10^{-65}	2·4	0·01	2·5	<0·01
U35-44k-v1-2801	Expressed protein	3×10^{-21}	2·3	0·05	2·6	<0·01
U35-44k-v1-9242	Diacylglycerol O-acyltransferase 1 putative expressed	1×10^{-97}	2·1	0·05	6·3	0·01
U35-44k-v1-11282	Xyloglucan endotransglucosylase/hydrolase protein 30 precursor	8×10^{-47}	2·1	0·05	2·3	0·04
U35-44k-v1-1867	Pyrophosphate−fructose 6-phosphate 1-phosphotransferase alpha subunit	0·0	2·1	0·01	2·6	0·01
U35-44k-v1-6244	Glycerophosphoryl diester phosphodiesterase precursor	3×10^{-93}	2·1	0·03	2·4	<0·01

TABLE 3. *Comparison of identified significantly different (P < 0·05) metabolites in the leaves and roots of IRL-OP-2538-P under P-sufficient and P-deficient phosphorous*

	Average response ratio of IRL-OP-2538-P under sufficient P supply	Average response ratio of IRL-OP-2538-P under limited P supply	Low/high	P	Log(ratio)
Leaf					
Unknown	$1·83 \times 10^{-4}$	$3·10 \times 10^{-4}$	1·69	0·007	0·23
Phytil methyl ether 2nd peak	$4·47 \times 10^{-1}$	$4·01 \times 10^{-1}$	0·90	0·028	−0·05
Unknown	$5·17 \times 10^{-1}$	$4·49 \times 10^{-1}$	0·87	0·045	−0·06
Unknown	$1·10 \times 10^{-2}$	$9·22 \times 10^{-3}$	0·84	0·037	−0·08
Phenylalanine	$5·64 \times 10^{-4}$	$4·71 \times 10^{-4}$	0·84	0·015	−0·08
n-Tetracosanol	$1·11 \times 10^{-2}$	$9·01 \times 10^{-3}$	0·81	0·010	−0·09
Octadecanol	$5·25 \times 10^{-3}$	$4·02 \times 10^{-3}$	0·76	0·044	−0·12
Isoleucine	$3·09 \times 10^{-4}$	$2·13 \times 10^{-4}$	0·69	0·002	−0·16
Unknown	$3·71 \times 10^{-4}$	$2·53 \times 10^{-4}$	0·68	0·001	−0·17
Unknown	$5·13 \times 10^{-4}$	$3·48 \times 10^{-4}$	0·68	0·007	−0·17
Citric acid	$5·48 \times 10^{-3}$	$3·50 \times 10^{-3}$	0·64	0·032	−0·19
Pentadecenoic acid	$5·43 \times 10^{-3}$	$3·37 \times 10^{-3}$	0·62	0·002	−0·21
β-Alanine	$4·94 \times 10^{-5}$	$3·01 \times 10^{-5}$	0·61	0·016	−0·22
Leucine	$4·13 \times 10^{-4}$	$2·49 \times 10^{-4}$	0·60	0·030	−0·22
Fructose*	$1·19 \times 10^{-2}$	$7·15 \times 10^{-3}$	0·60	0·005	−0·22
Tyrosine	$2·40 \times 10^{-4}$	$1·37 \times 10^{-4}$	0·57	0·013	−0·24
Unknown	$4·19 \times 10^{-4}$	$2·39 \times 10^{-4}$	0·57	0·049	−0·24
Malic acid	$1·22 \times 10^{-2}$	$6·73 \times 10^{-3}$	0·55	<0·001	−0·26
Unknown	$5·80 \times 10^{-5}$	$2·58 \times 10^{-5}$	0·44	<0·001	−0·35
n-Pentadecanoic acid	$8·70 \times 10^{-4}$	$3·82 \times 10^{-4}$	0·44	0·029	−0·36
Lysine	$3·61 \times 10^{-5}$	$1·56 \times 10^{-5}$	0·43	0·017	−0·36
Fucosterol	$1·84 \times 10^{-3}$	$7·44 \times 10^{-4}$	0·41	0·020	−0·39
Phosphate	$3·06 \times 10^{-3}$	$1·20 \times 10^{-3}$	0·39	0·004	−0·41
2-Piperidinecarboxylic acid	$2·27 \times 10^{-5}$	$8·58 \times 10^{-6}$	0·38	0·015	−0·42
Unknown	$2·26 \times 10^{-3}$	$8·37 \times 10^{-4}$	0·37	0·023	−0·43
Unknown	$2·12 \times 10^{-3}$	$7·73 \times 10^{-4}$	0·36	<0·001	−0·44
Proline	$2·20 \times 10^{-3}$	$5·26 \times 10^{-4}$	0·24	0·043	−0·62
Root					
Unknown	$5·63 \times 10^{-4}$	0·00	*	<0·001	*
Stigmastadienol	$6·68 \times 10^{-5}$	$6·03 \times 10^{-4}$	9·03	0·032	0·96
Diamino-1,3-propane	$3·25 \times 10^{-6}$	$1·93 \times 10^{-5}$	5·94	0·009	0·77
Unknown	$8·96 \times 10^{-4}$	$3·65 \times 10^{-3}$	4·08	0·006	0·61
Unknown	$1·72 \times 10^{-3}$	$5·29 \times 10^{-3}$	3·07	0·003	0·49
Unknown	$4·07 \times 10^{-5}$	$9·54 \times 10^{-5}$	2·34	0·002	0·37
D-5-Avenasterol	$1·63 \times 10^{-3}$	$3·63 \times 10^{-3}$	2·23	0·010	0·35
Putrescine	$7·52 \times 10^{-5}$	$1·62 \times 10^{-4}$	2·15	<0·001	0·33
Galactose	$2·89 \times 10^{-5}$	$5·78 \times 10^{-5}$	2·00	0·046	0·30
Fructose*	$1·22 \times 10^{-3}$	$2·35 \times 10^{-3}$	1·93	<0·001	0·29
Glucose*	$1·23 \times 10^{-3}$	$2·27 \times 10^{-3}$	1·85	<0·001	0·27
Unknown	$5·51 \times 10^{-5}$	$9·49 \times 10^{-5}$	1·72	0·008	0·24
Shikimic acid	$2·66 \times 10^{-5}$	$4·00 \times 10^{-5}$	1·50	0·001	0·18
Unknown	$2·07 \times 10^{-4}$	$3·09 \times 10^{-4}$	1·49	0·011	0·17
Sucrose	$7·85 \times 10^{-2}$	$1·16 \times 10^{-1}$	1·48	0·027	0·17
n-Octadecanoic acid	$7·44 \times 10^{-3}$	$9·96 \times 10^{-3}$	1·34	0·020	0·13
Linoleic acid	$2·06 \times 10^{-1}$	$2·62 \times 10^{-1}$	1·27	0·001	0·10
α-Linolenic acid	$5·63 \times 10^{-2}$	$6·82 \times 10^{-2}$	1·21	0·016	0·08
β-Sitosterol	$1·55 \times 10^{-1}$	$1·86 \times 10^{-1}$	1·20	0·017	0·08
n-Hexadecanoic acid	$2·05 \times 10^{-1}$	$2·47 \times 10^{-1}$	1·20	0·017	0·08
Valine	$1·61 \times 10^{-3}$	$1·27 \times 10^{-3}$	0·79	0·031	−0·10
Eicosanol	$1·08 \times 10^{-2}$	$7·60 \times 10^{-3}$	0·70	0·026	−0·15
Leucine	$9·10 \times 10^{-4}$	$6·26 \times 10^{-4}$	0·69	0·015	−0·16
Unknown	$4·80 \times 10^{-4}$	$3·02 \times 10^{-4}$	0·63	0·041	−0·20
Lysine	$2·29 \times 10^{-4}$	$1·42 \times 10^{-4}$	0·62	0·006	−0·21
Phosphate	$1·24 \times 10^{-2}$	$7·33 \times 10^{-3}$	0·59	<0·001	−0·23
Cinnamic acid	$4·50 \times 10^{-2}$	$2·60 \times 10^{-2}$	0·58	0·045	−0·24
4- or 3-Hydroxycinnamic acid	$2·23 \times 10^{-2}$	$1·23 \times 10^{-2}$	0·55	<0·001	−0·26
Alanine	$1·83 \times 10^{-3}$	$9·89 \times 10^{-4}$	0·54	0·001	−0·27
Unknown	$5·29 \times 10^{-4}$	$2·75 \times 10^{-4}$	0·52	0·006	−0·28
Allantoin	$8·70 \times 10^{-4}$	$4·16 \times 10^{-4}$	0·48	<0·001	−0·32
Unknown	$1·25 \times 10^{-3}$	$5·05 \times 10^{-4}$	0·40	<0·001	−0·39
Unknown	$1·36 \times 10^{-4}$	$4·74 \times 10^{-5}$	0·35	<0·001	−0·46
n-Docosanol	$8·50 \times 10^{-4}$	$2·97 \times 10^{-4}$	0·35	0·007	−0·46
Heneicosanol	$9·84 \times 10^{-4}$	$3·15 \times 10^{-4}$	0·32	0·002	−0·49
Mannitol	$3·14 \times 10^{-4}$	$3·64 \times 10^{-5}$	0·12	<0·001	−0·94

* Both glucose and fructose result in two peaks corresponding to their isomers. The levels of both isomers were combined to calculate the total value of glucose and fructose.

TABLE 4. *Comparison of identified significantly different (P < 0·05) metabolites in the leaves and roots of Cashel-P under P-sufficient and P-deficient conditions*

	Average response ratio of Cashel-P under sufficient P supply	Average response ratio of Cashel-P under limited P supply	Low/high	P	Log(ratio)
Leaf					
γ-Aminobutyric acid	3.41×10^{-4}	6.86×10^{-4}	2·01	0·006	0·30
Unknown	3.56×10^{-5}	5.65×10^{-5}	1·59	0·029	0·20
Galactose/glycerol conjugate	8.19×10^{-5}	1.25×10^{-4}	1·53	0·042	0·18
Threonic acid	8.27×10^{-4}	1.21×10^{-3}	1·46	0·034	0·16
Unknown	2.60×10^{-1}	3.62×10^{-1}	1·4	<0·001	0·14
Phytol B	2.12×10^{-2}	2.85×10^{-2}	1·34	0·002	0·13
Phytol A	1.27×10^{-2}	1.66×10^{-2}	1·31	<0·001	0·12
Sucrose	4.01×10^{-1}	5.04×10^{-1}	1·26	0·038	0·10
Phytol C	6.33×10^{-3}	7.88×10^{-3}	1·25	0·025	0·10
n-Hexadecenoic acid	4.43×10^{-2}	5.54×10^{-2}	1·25	0·014	0·10
Phytil methyl ether	1.49×10^{-1}	1.79×10^{-1}	1·2	0·004	0·08
Homoserine	2.16×10^{-4}	1.17×10^{-4}	0·54	0·047	−0·27
2-Piperidinecarboxylic acid	3.95×10^{-5}	2.03×10^{-5}	0·51	0·048	−0·29
Unknown	7.45×10^{-5}	3.18×10^{-5}	0·43	0·010	−0·37
5-Oxoproline	5.29×10^{-2}	2.27×10^{-2}	0·43	0·049	−0·37
Histidine	7.89×10^{-6}	2.04×10^{-6}	0·26	0·024	−0·59
Spermidine	5.38×10^{-6}	1.18×10^{-6}	0·22	0·048	−0·66
Glutamine*	2.66×10^{-3}	5.3×10^{-4}	0·20	0·026	−0·70
Phosphate	7.64×10^{-3}	1.17×10^{-3}	0·15	0·002	−0·82
Asparagine*	1.11×10^{-3}	1.5×10^{-4}	0·14	0·009	−0·87
Unknown	3.48×10^{-4}	3.10×10^{-5}	0·09	0·046	−1·05
Root					
Unknown	2.40×10^{-4}	5.71×10^{-4}	2·38	0·045	0·38
Unknown	1.08×10^{-4}	2.28×10^{-4}	2·11	0·019	0·32
Unknown	3.69×10^{-4}	7.56×10^{-4}	2·05	0·046	0·31
Methionine	3.62×10^{-5}	7.12×10^{-5}	1·97	0·027	0·29
Unknown	5.11×10^{-5}	9.36×10^{-5}	1·83	0·046	0·26
Sucrose	5.50×10^{-2}	9.70×10^{-2}	1·76	0·022	0·25
Aspartic acid	5.14×10^{-3}	8.28×10^{-3}	1·61	0·002	0·21
Glutamic acid	6.61×10^{-3}	9.84×10^{-3}	1·49	0·034	0·17
n-Tetracosanoic	1.35×10^{-2}	9.20×10^{-3}	0·68	0·043	−0·17
Unknown	1.21×10^{-4}	7.60×10^{-5}	0·63	0·028	−0·20
Octadecenoic acid	4.61×10^{-3}	2.53×10^{-3}	0·55	0·005	−0·26
Unknown	2.09×10^{-3}	1.05×10^{-3}	0·50	0·044	−0·30
Unknown	6.10×10^{-4}	2.69×10^{-4}	0·44	0·003	−0·36
Unknown	4.32×10^{-5}	1.39×10^{-5}	0·32	0·035	−0·49

* Both glutamine and asparagine generate two trimethylsilyl (TMS) derivatives which were combined to generate the total levels of asparagine and glutamine. For glutamine the TMS4 derivative was multiplied by a factor of 2 before being combined with its other derivative.

deficiency (Table 3). Furthermore, the latter genotype experienced a general increase in sugar levels (glucose, fructose, galactose and sucrose) and fatty acids ($C_{16:0}$, $C_{18:0}$, $C_{18:2}$ and $C_{18:3}$) whilst levels of some of the pyruvate-derived amino acids (alanine, leucine and valine) decreased. Among the metabolites significantly regulated in Cashel-P (14), only six were putatively identified. For Cashel-P, the imposition of limiting P conditions resulted in an increase in the levels of sucrose and some amino acids (methionine, aspartic acid and glutamic acid) whilst levels of some fatty acid ($C_{24:0}$ and $C_{18:1}$) decreased.

DISCUSSION

This study aimed to uncover changes in the transcriptome and metabolome of two perennial ryegrass genotypes during the initial stages of P deficiency. It is the first study looking at the global responses to low P availability in perennial ryegrass and provides an insight into the early-stage response mechanisms of the plant to P deficiency. Metabolic profiling demonstrated that P deficiency resulted in a Pi decrease in the leaves of both genotypes (Tables 3 and 4), thus confirming that the experimental conditions have successfully produced an effect on the levels of Pi and that the time frame (24 h) was sufficient to elicit this response. However, no significant differences in growth were observed (data not shown). Hammond *et al.* (2003) reported that in *A. thaliana* the concentration of P in the shoots decreases without causing an effect of plant growth between 24 and 72 h after P withdrawal, suggesting that 24 h of exposure to P withdrawal is a plausible time frame to expect a decrease in shoot P in perennial ryegrass. Interestingly, Hammond *et al.* (2003) used the promoter of the SDQ1 gene (UDP-sulfoquinovose synthase), which specifically responds to P limitation, to control the expression of the GUS marker gene in an attempt to develop smart plants allowing the detection of P-limitation before it affected plant growth. In this study we observed an increase in the expression of SDQ1 after 24 h (Table 2), which appears to confirm that the experimental conditions have resulted in a specific response from the plant to P limitation.

Furthermore, an increase in the expression of *SDQ1* in *A. thaliana* pho1 mutants (with low internal Pi concentration) and in wild-type plants exposed to P imitation has been reported (Essigmann *et al.*, 1998). This led the authors to suggest that this effect is caused by low shoot P (Essigmann *et al.*, 1998), which appears to be consistent with our results.

Lipid membrane remodelling

One mechanism of adaptation to P-deficient growth conditions is the remodelling of plant lipid membranes in order to liberate Pi bound in membrane phospholipids (Plaxton, 2004). The increase reported in the expression levels of genes encoding phospholipase A1 (EC 3·1·1·32) together with increases in glycerophosphoryl diester phosphodiesterase (EC 3·1·4·46, hydrolysis of deacylated phospholipids to glyceraldehyde 3-phosphate), acid phosphatase/vanadium-dependent hydrolase and diacylglycerol O-acyltransferase 1 (EC 2·3·1·20) also appears to suggest a certain degree of phospholipid remodelling in the response to P deficiency in perennial ryegrass. Photosynthetic membranes are characterized by a substantial fraction of non-phosphorus lipids, such as galactolipids or sulfolipids (e.g. sulfoquinovosyldiacylglycerol), which reflects the need to conserve P by plants (Benning, 2009). Here we observed a significant increase in expression of genes encoding UDP-sulfoquinovose synthase (EC 3·13·1·1), a key enzyme in the sulfoquinovosyldiacylglycerol (SQDG) synthesis pathway, during P deficiency in the leaf tissue of both genotypes. Hammond *et al.* (2003) demonstrated that, in *Arabidopsis*, the expression of a gene involved in sulfolipid biosynthesis (*SQD1*) is specifically increased in response to P limitation. Furthermore, Misson *et al.* (2005) demonstrated in *Arabidopsis* that under Pi deficiency SQDG increased, whereas phosphatidylglycerol (PG) decreased. These results are in agreement with elevated expression of genes involved in SQDG synthesis and phospholipid degradation.

We also observed an increase in gene expression levels of a sulphate transporter in both genotypes, although the increase was only significant in Cashel-P (Cashel-P: 5·2-fold, $P < 0.001$; IRL-OP-02538-P: 2·8-fold, $P = 0.054$). This has previously been observed in *Arabidopsis* and was proposed to support an increased demand for sulphur during P deficiency to meet the needs for increased sulfolipid synthesis (Misson *et al.*, 2005). An *Arabidopsis* mutant, *sqd2*, disrupted in a putative identified sulfolipid synthase (*SQD2*) gene, displayed no measurable sulfolipid content and reduced growth under P-deficient conditions (Yu *et al.*, 2002). The authors of that study hypothesized that sulfolipids may substitute for anionic phospholipids under P-deficient conditions and therefore allow photosynthesis to continue. Further support for lipid membrane remodelling in perennial ryegrass can be seen from the strong induction of a gene encoding G3PP under P deficiency (7·5- and 17·1-fold, $P < 0.001$ in IRL-OP-02538-P and Cashel-P, respectively). This suggests the enhanced mobilization of glycerol 3-phosphate from the cytosol into the chloroplast or endoplasmic reticulum where it could be utilized in lipid biosynthesis (Fig. 2).

Our results indicate that the replacement of phospholipids with sulfolipids in the membranes of photosynthetic tissue is also occurring in perennial ryegrass under P deficiency and that this is a rapid response to changes in P status.

Carbon partitioning

At the metabolome level in the leaves of IRL-OP-02538-P, P deficiency was accompanied by an overall decrease in metabolite levels whilst there were no putatively identified up-regulated metabolites. The significantly regulated metabolites included a variety of metabolite classes such as sugars, amino acids, fatty acids and organic acids. The overall decrease in metabolite levels may be symptomatic of either a decrease of source activity due to decreases in photosynthetic activity, an increase in sink activity or both. P limitation has been reported to impact upon photosynthetic activity (Foyer and Spencer, 1986; Lauer *et al.*, 1989; Rodriguez *et al.*, 1998; Hammond and White, 2008) although increases in the root/shoot ratio are also considered typical effects of P limitation (Freedan *et al.*, 1989; Hermans *et al.*, 2006; Hammond and White, 2008) and may account for increased sink activity as carbohydrates are exported from the leaves to the roots. A study by Wissuwa *et al.* (2005) focused on determining whether root growth in rice is affected by either source or sink limitations when grown under P-deficient conditions and they did this by comparing rice genotypes under four P levels and two light treatments. The authors reported that net photosynthesis was 70 % greater in plants grown under high, compared with low, light but it was found that this did not translate into greater root growth under P limitation, leading them to suggest that assimilate supply from source leaves was not a limiting factor under P limitation. Conversely, low P supply appears to limit root growth directly. It was proposed that the most tolerant genotype used in the experiment of Wissuwa *et al.* (2005) preferably mobilized P to the roots, which would increase P concentrations and consequently promote growth. The levels of sugars in the roots of IRL-OP-02538-P increase in response to P limitation, which seem to suggest a decrease in sink activity, resulting from a reduction in root growth. Root growth inhibition in response to P limitation has been suggested to be mediated by putrescine in rice (Shih and Kao, 1996), and although no root/shoot ratios were measured, the significant up-regulation of putrescine levels found in this study suggests a limitation of root growth in IRL-OP-02538-P plants as an early response to limited P availability.

Glycolytic bypasses

The results showed evidence of glycolytic bypasses being utilized and these have previously been reported in other species as being valuable mechanisms to enable the efficient use of P during periods of deficiency (Theodorou and Plaxton, 1993). We observed a significant increase in the expression of a gene encoding an α-subunit of pyrophosphate : fructose 6-phosphate 1-phosphotransferase (PFP, EC 2·7·1·90) during P deficiency in the leaf tissue of both genotypes. The phosphorylation of fructose 6-phosphate to fructose 1-6 bisphosphate during glycolysis is efficiently catalysed by
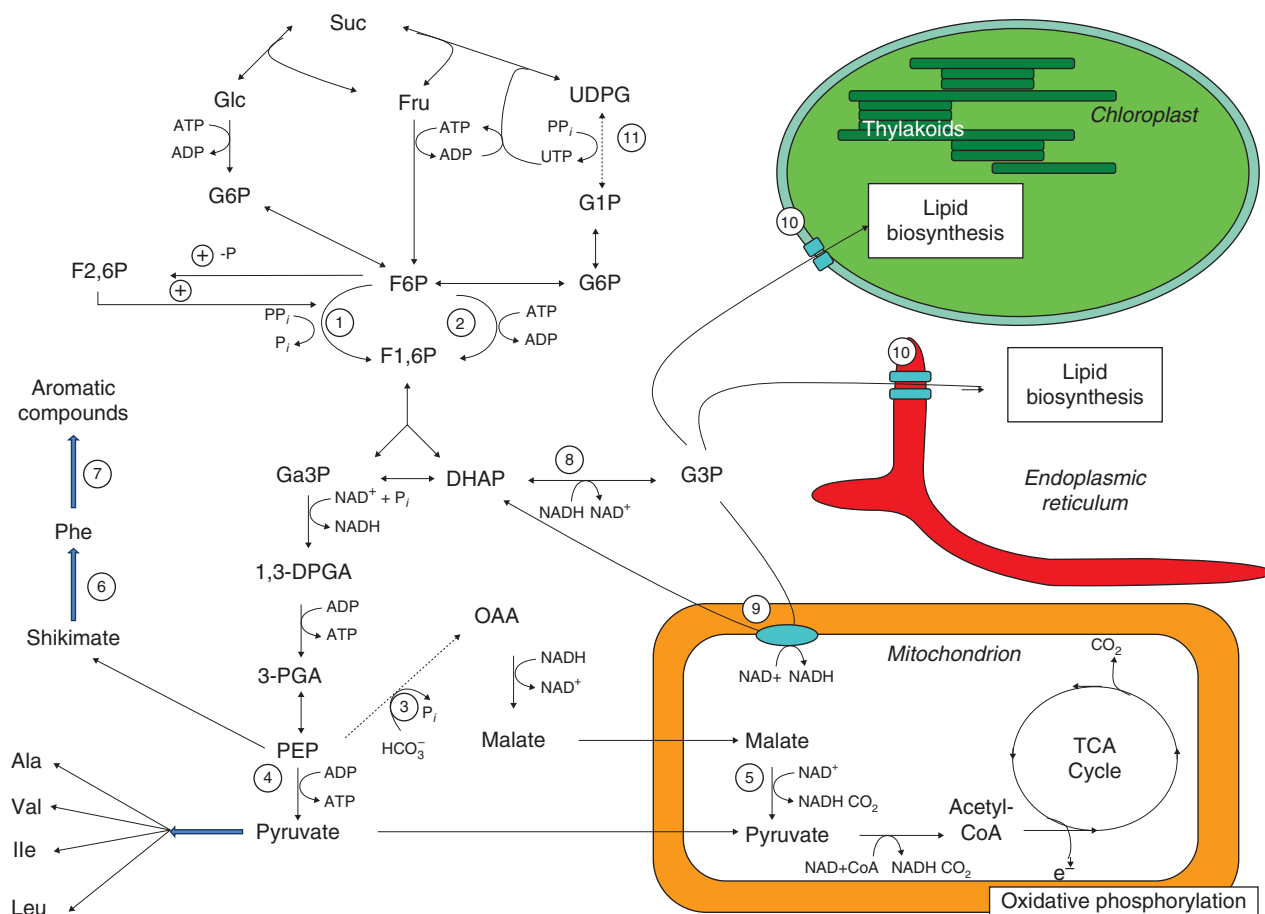
FIG. 2. Diagram depicting the primary metabolism in *Lolium perenne*, adapted from Hammond *et al.* (2004). Bold arrows represent a downstream set of reactions, not single reactions. Dashed arrows highlight alternative pathways for glycolysis to conserve Pi. Microarray results indicate an increase in expression of transcripts encoding genes involved in reactions 1, 6, 7, 8 and 10. Abbreviations: Suc, sucrose; Glc, glucose; Fru, fructose; UDPG, UDP-glucose; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F2,6P, fructose 2,6-bisphosphate; F1,6P, fructose 1,6-bisphosphate; G3P, glycerol 3-phosphate; DHAP, dihidroxyacetone phosphate; OAA, oxalo acetic acid; Ga3P, glyceraldehyde 3-phosphate; 1,3-DPGA, 1,3- diphosphoglyceraldehyde; 3-PGA, 3-phosphoglycerate; PEP, phosphoenol pyruvate; Phe, phenylalanine; Ala, alanine; Val, valine; Ile, isoleucine; Leu, leucine. Reactions: (1) Pyrophosphate fructose 6-phosphate 1-phosphotransferase, EC 2·7·1·90; (2) ATP : fructose 6-phosphate 1-phosphotransferase, EC 2·7·1·11; (3) phosphoenolpyruvate carboxykinase (ATP), EC 4·1·1·49; (4) pyruvate kinase, EC 2·7·1·40; (5) malate dehydrogenase (oxaloacetate-decarboxylating), EC 1·1·1·38; (6) involvement of anthranilate phosphoribosyltransferase, EC 2·4·2·18 (7) involvement of flavonol synthase/flavanone 3-hydroxylase; (8) glycerol 3-phosphate dehydrogenase, EC 1·1·5·3; (9) glycerol 3-phosphate dehydrogenase, EC 1·1·5·3; (10) glycerol 3-phosphate permease; (11) UDP-glucose pyrophosphorylase, EC 2·7·7·9.

ATP : fructose 6-phosphate 1-phosphotransferase (PFK, EC 2·7·1·11), using ATP (Stryer, 1995). PFP can also catalyse this reaction but employing PPi rather than ATP (Fig. 2). It has already been demonstrated in black mustard (*Brassica nigra*) suspension cells that PFP activity is increased under P deficiency and its activity falls to below the level of PFK as solute P levels increase (Duff *et al.*, 1989). Furthermore, it was shown that the α-subunit of PFP has a regulatory role over the β-subunit and that its activity is tightly regulated by intracellular Pi status (Theodorou *et al.*, 1992). Our results demonstrate that a switch to alternative pathways during glycolysis occurs at an early stage of P deficiency in perennial ryegrass.

Cell-wall metabolism

One class of genes stands out as being abundant within the list of transcripts significantly up-regulated in leaf tissue under P

deficiency, particularly in the Cashel-P genotype. These are genes involved in cell-wall synthesis and remodelling. Previous reports have also reported the up-regulation of genes involved in cell-wall synthesis under P-limiting conditions (Wasaki *et al.*, 2003). We identified the up-regulation of five genes encoding the catalytic subunit of cellulose synthase (CESA) between both genotypes: four in Cashel-P and one in IRL-OP-02538-P. Ten CESA genes are reportedly present in the *Arabidopsis* genome and the role of these in cell-wall synthesis has previously been described by Scheible and Pauly (2004). The significant up-regulation of this gene family points to an increased production of cellulose during the early stages of P deficiency. However, the gene with the highest increase in expression under P deficiency in Cashel-P leaves encodes an ENDO-1, 4-beta-xylanase (EC 3·2·1·8), a xylan hydrolase. Together, these results may suggest a remodelling of cell walls, with cellulose content increasing and xylan content decreasing, although direct evidence is still needed to

corroborate this. The exact role, if any, that this modification could have under P deficiency remains unknown. Interestingly, it has been recently demonstrated that the over-expression of a wall-bound purple acid phosphatase from tobacco (*NtPAP12*) resulted in an increased deposition of cellulose in transgenic cells (Kaida *et al.*, 2009). Transcripts coding for acid phosphatases (purple acid phosphatase precursor and acid phosphatase 1) are up-regulated in both genotypes in our study. The induction of acid phosphatases is regarded as a universal symptom/response of P limitation (Duff *et al.*, 1994) and their role in hydrolysing Pi from mono-esters makes them important in intracellular and extracellular Pi salvage systems (Plaxton, 2004).

Signalling mechanisms

The microarray results indicated changes in the expression levels of genes involved in signal transduction that may also be involved in the leaf response to P deprivation. A gene encoding phospholipase C (PLC, EC 3·1·4·3) was significantly up-regulated in the leaf tissue of both genotypes. PLC catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] into diacylglycerol (DAG) and inositol triphosphate (IP$_3$), the latter diffusing through the cytosol releasing calcium from intracellular stores or being converted to inositol hexakisphosphate (IP$_6$), both of which may play roles as signalling molecules (Meijer and Munnik, 2003).

We also identified the up-regulation of an *ids4*-like gene in both genotypes, which has been identified in previous transcriptomic studies as being enhanced under P deficiency (Misson *et al.*, 2005; Guo *et al.*, 2008). The *ids4*-like gene up-regulated here shares most significant homology with *At2g45130* from *Arabidopsis* that contains an SPX domain, a region in PHO1 genes identified as being important for inorganic phosphate homeostasis (Wang *et al*, 2004). This gene, *AtSPX3*, has recently been proposed to play a role in a phosphate-signalling network (Duan *et al.*, 2008). It was shown that when *AtSPX3* was repressed by RNAi it resulted in augmentation of P-deficient symptoms and altered allocation of internal P, leading to the conclusion that *AtSPX3* plays an important role in plant adaptation to P deficiency (Duan *et al.*, 2008). The high induction of an *AtSPX3* homologue in the early stages of P deficiency in both genotypes of our study indicates it may have a similar role to play in adaptation to P deficiency in perennial ryegrass.

Aromatic secondary metabolites

A common physiological response in a wide variety of plants to long-term P limitation is the increase in anthocyanin content in leaf tissue (Hammond *et al.*, 2004) and a number of studies have demonstrated the up-regulation of genes involved in anthocyanin biosynthesis during P deficiency (Hammond *et al.*, 2003; Misson *et al.*, 2005). It is thought that anthocyanin helps protect the photosynthetic machinery during leaf senescence (Hoch *et al.*, 2001). The biosynthesis of anthocyanins involves the shikimate pathway to produce phenylalanine, which eventually leads to flavonoid biosynthesis (Fig. 2). The accumulation of anthocyanin is generally a feature of long-term P deficiency and the majority of the genes are

specifically induced during long-term deficiency (Misson *et al.*, 2005). In IRL-OP-02538-P leaves after 24 h of P deficiency, two transcripts involved in phenylalanine and flavonoid biosynthesis (anthranilate phosphoribosyltransferase, EC 2·4·2·18; and flavonol synthase/flavanone 3-hydroxylase, EC 1·14·11·9) were up-regulated, which may suggest the early onset of a secondary metabolite response in this genotype.

Summary

Although most metabolic profiling reports aimed at studying the responses of plants to P deficiency have not been performed at early stages of the response, it seems that a significant degree of metabolic control is evident in the early stages of such response. This was particularly evident in the leaves, suggesting that P sensing mechanisms readily signal to the leaf tissue eliciting a metabolic response. We also observed significant changes in the transcriptome, which pointed to the utilization of glycolytic bypasses, remodelling of lipid membranes and P-scavenging mechanisms under early P deficiency. Although at an early stage, these studies are providing insights at the biochemical and genetic level that will undoubtedly facilitate hypotheses testing via transgenic methodologies and ultimately identify a way forward to improve P-use efficiency in ryegrass by employing candidate genes and marker-assisted breeding approaches.

SUPPLEMENTARY DATA

Supplementary data are available online at w.aob.oxford-journals.org and consist of the following files. (1) Mean values for Pi removal from solution by 34 ecotypes and two cultivars. (2) Primer sequences used in real-time RT-PCR to verify array results. (3) Metabolomics quality control and PCA. (4) Genes significantly regulated (>two-fold, $P <$ 0·05) by P deficiency in leaf tissue. (5) Genes significantly regulated (>two-fold, $P < 0·05$) by P deficiency in root tissue.

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