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RESEARCH PAPER

Co-ordinated expression of amino acid metabolism in response to N and S deficiency during wheat grain filling

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Abstract

Increasing demands for productivity together with environmental concerns about fertilizer use dictate that the future sustainability of agricultural systems will depend on improving fertilizer use efficiency. Characterization of the biological processes responsible for efficient fertilizer use will provide tools for crop improvement under reduced inputs. Transcriptomic and metabolomic approaches were used to study the impact of nitrogen (N) and sulphur (S) deficiency on N and S remobilization from senescing canopy tissues during grain filling in winter wheat (*Triticum aestivum*). Canopy tissue N was remobilized effectively to the grain after anthesis. S was less readily remobilized. Nuclear magnetic resonance (NMR) metabolite profiling revealed significant effects of suboptimal N or S supply in leaves but not in developing grain. Analysis of amino acid pools in the grain and leaves revealed a strategy whereby amino acid biosynthesis switches to the production of glutamine during grain filling. Glutamine accumulated in the first 7 d of grain development, prior to conversion to other amino acids and protein in the subsequent 21 d. Transcriptome analysis indicated that a down-regulation of the terminal steps in many amino acid biosynthetic pathways occurs to control pools of amino acids during leaf senescence. Grain N and S contents increased in parallel after anthesis and were not significantly affected by S deficiency, despite a suboptimal N:S ratio at final harvest. N deficiency resulted in much slower accumulation of grain N and S and lower final concentrations, indicating that vegetative tissue N has a greater control of the timing and extent of nutrient remobilization than S.

Key words: Affymetrix, grain filling, metabolomics, nitrogen, sulphur, transcriptomics, wheat.

Introduction

Global population growth is increasingly putting pressure on agricultural production, leading to demands for higher yields from arable land. Higher yields of both food and energy crops usually demand increased fertilizer inputs; however, current application levels often have negative environmental impacts. Not only is the production of fertilizers hugely energy consuming, but only 30–50% of Nitrogen (N) fertilizer applied is taken up by crops, the remainder being lost by denitrification or leaching into terrestrial ecosystems, causing problems of eutrophication and contamination of drinking water (Vitousek *et al*., 1997; Cassman *et al*., 2003). The importance of improving crop fertilizer use efficiency and increasing grain yield and quality is therefore clear. The challenge of maintaining sustainability in agricultural systems is certain to be one of the leading social scientific problems of the 21st century (Tilman, 1999; Tilman *et al*., 2002).

N fertilizer application is directly linked to wheat grain yield and quality (protein content) (Good *et al*., 2004; Barneix, 2007). Sulphur (S) nutrition is more specifically

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associated with levels of glutenin in the endosperm and the ratio of glutenin to other grain storage proteins, which is responsible for dough elasticity and loaf quality (Zhao *et al*., 1997, 1999*b*,*c*; Shewry and Halford, 2002). During growth of the wheat crop, N and S are accumulated in the vegetative tissues and are then redistributed to the developing seed during the concurrent processes of vegetative tissue senescence and grain development (Dalling, 1985).

Amino acids are the major form in which N is remobilized from the leaf to the grain during grain filling. The leaves of wheat plants grown under high N accumulate free amino acids from recently reduced nitrate which are subsequently loaded into the phloem (Caputo and Barneix, 1997; Lalonde *et al*., 2003, 2004). During senescence, amino acids for remobilization are provided by the proteolysis of leaf proteins such as Rubisco (which contributes up to 50% of the total leaf protein and 30% of total leaf N), which are degraded by developmentally regulated cysteine endopeptidases and peptide hydrolases (Chandlee, 2001; Buchanan-Wollaston *et al*., 2003; Feller *et al*., 2008). Free amino acids are major components of both the phloem and xylem sap in wheat: during vegetative growth, phloem amino acid concentrations have been measured at 260 mM, eight times the concentration of nitrate ions. Aspartate (Asp) and glutamate (Glu) were the predominant components, comprising ~50% of the total amino acids (Hayashi and Chino, 1986). However, during leaf senescence, the Asp and Glu pools decrease, glutamine (Gln) becoming the predominant free amino acid in both leaf and phloem extracts (Simpson and Dalling, 1981). This shift in amino acid balance during grain filling is a programmed strategy for N remobilization during reproductive development and has potential for exploitation for the improvement of N use efficiency.

S is taken up by the roots as sulphate (reviewed in Hawkesford *et al*., 2003) and transported in its inorganic form to developing leaf tissues, with expanding leaves being particularly strong sinks (Anderson, 2005). In the leaves, S is either stored as sulphate or reduced and incorporated into an organic form by the reductive sulphate assimilation pathway. This series of reactions takes place in the plastids and produces the amino acid cysteine (Cys) which is used to synthesize a wide range of S-containing organic molecules such as methionine (Met) and glutathione (GSH) (Schmidt and Jager, 1992; Hell, 1997; Hawkesford and Wray, 2000; Leustek *et al*., 2000; Kopriva and Rennenberg, 2004). Canopy proteins such as Rubisco may act as a store of these S-containing amino acids (Gilbert *et al*., 1997). During wheat grain development, S is transported to the grain in the phloem (Wang *et al*., 1994). Approximately 75% of S found in the endosperm cavity during grain development is in the form of sulphate: the remaining 25% comprises organic soluble compounds such as Met and GSH, which are particularly important as S transport compounds in S-deficient plants (Fitzgerald *et al*., 2001). The Met derivative *S*-methylmethionine (SMM) is also an important form of transported S in plants: SMM is produced from Met by adenosylmethionine:methionine *S*-methyltransferase and may be present in the phloem at concentrations exceeding that of GSH (Bourgis *et al*., 1999). It has been hypothesized that GSH and SMM represent the transportable organic forms of Cys and Met, respectively, in a sink demand-driven system (Anderson and Fitzgerald, 2003).

Whilst the physical processes of N and S remobilization have been studied in detail, the genetic control of these processes and their contribution to agronomic productivity are less well understood. Studies at the metabolic and genetic level using genomic-era analytical techniques will aid in providing novel insights into the regulation of the many contributing traits involved in N and S remobilization during wheat grain filling and may suggest targets for the enhancement of these processes in arable crops. In this report a metabolomic and transcriptomic assessment of the leaf and grain following anthesis in field-grown winter wheat plants with varying N and S fertilizer applications is presented. Material was harvested from the Broadbalk winter wheat experiment at Rothamsted Research (Harpenden, UK), which is the longest continually running scientific experiment in the world and has been providing agronomic data on wheat crop nutrition for 164 years (Poulton, 1995). With the advent of modern analytical technologies the experiment is a valuable resource for genetic and metabolomic studies (Bearchell *et al*., 2005; Lu *et al*., 2005).

Materials and methods

Plant material

All tissues were harvested from Rothamsted's Broadbalk winter wheat experiment (variety Hereward; RAGT Seeds Ltd, Cambridge, UK) in 2005 from plots 6 (N1), 9 (N2), and 14 (–S). N was applied at 48 kg N ha^{-1} as NH_4NO_3 for N1 and 192 kg N ha^{-1} for N2. –S was a sulphur-deficient plot identical to N2 but with K_2SO_4 fertilizer replaced by KCl at a rate of 90 kg K ha^{-1}. N was applied in a single dressing in mid-April. The fertilizer rate N2 (192 kg N ha^{-1}) is the typical rate used by UK winter wheat farmers. None of the plots was limiting for P, K, or Mg. Harvesting was carried out at anthesis and at subsequent 7 d intervals for 49 d. Anthesis dates of N1, N2, and –S plants varied by <24 h. For N partitioning analysis, 0.5 m^2 cuts of wheat plants were taken and the main stem separated into leaf [leaf 1 (flag), leaf 2, leaf 3, remainder of leaves], stem, and ear fractions. For transcriptomic and metabolomic analysis, leaves 2 and 3 from 10 plants were harvested from three replicate zones within the above plots, pooled, and stored in liquid N. Whole grain was extracted manually into liquid N from the central section of ears from the same plants.

Physiological analyses

Total N measurements were determined using a CNS-2000 combustion analyser (LECO, St Joseph, MI, USA) and tissue S by induction-coupled plasma-atomic emission spectrometry (ICP-AES)

(Applied Research Laboratories, Accuris, Switzerland) according to the manufacturer's instructions. SPAD-502 meter (Konica Minolta, Ramsey, NJ) readings were taken from 10 replicate main stem leaves, midway along the leaf blade and half way between the central vein and the leaf edge. Least significant difference errors were calculated by two-way analysis of variance (ANOVA) using GenStat v10 (VSN International, Hertfordshire, UK).

Nuclear magnetic resonance (NMR) profiling

NMR sample preparation was carried out according to the procedures described in Ward *et al*. (2003) and Baker *et al*. (2006). NMR extractions into 80:20 D_2O:CD_3OD containing 0.05% d_4 TSP (1 ml) were performed for three technical replicates, of 15 mg, for each biological sample.

^{1}H-NMR spectra were acquired under automation at 300 °K on an Avance Spectrometer (Bruker Biospin, Coventry, UK) operating at 600.0528 MHz and equipped with a 5 mm selective inverse probe. Spectra were collected using a water suppression pulse sequence with a relaxation delay of 5 s. Each spectrum was acquired using 128 scans of 64 000 data points with a spectral width of 7309.99 Hz. Spectra were automatically Fourier transformed using an exponential window with a line broadening value of 0.5 Hz. Phasing and baseline correction were carried out within the instrument software. ^{1}H chemical shifts were referenced to d_4-TSP at δ0.00.

^{1}H-NMR spectra were automatically reduced, using Amix (Analysis of MIXtures software, Bruker Biospin), to ASCII files containing integrated regions or 'buckets' of equal width (0.01 ppm). Spectral intensities were scaled to the d_4-TSP region (δ0.05 to –0.05). The ASCII file was imported into Excel for the addition of sampling/treatment details. The regions for unsuppressed water (δ4.865–4.775), d_4-MeOH (δ3.335–3.285), and d_4-TSP (δ0.05 to –0.05) were removed prior to importing the data set into SIMCA-P 11.0 (Umetrics, Umea, Sweden) for multivariate analysis.

Quantitative amino acid analysis

Amino acids were quantified using an EZfaast gas chromatography–mass spectrometry (GC–MS) amino acid analysis kit (Phenomenex, Cheshire, UK) with amino acid standards from Sigma (Dorset, UK). Freeze-dried analytical samples (15±0.03 mg) were suspended in 0.9 ml of 80:20 H_2O:MeOH and 0.1 ml of 0.75 mM norvaline solution [in 20% (v/v) aqueous methanol]. Samples were extracted for 10 min at 50 °C. After centrifugation (10 min at 16 000 *g*), the supernatant (0.75 ml) was transferred to a clean vial and evaporated to dryness under vacuum.

Samples were reconstituted in 20% (v/v) aqueous methanol (200 µl) and amino acids isolated and derivatized according to the manufacturer's instructions (EZfaast manual). The organic phase was diluted 1:5 with 80:20 iso-octane:chloroform, and a 2 µl aliquot (splitless injection) was analysed by GC–MS using a Hewlett Packard 5970 MSD coupled to a 5890 gas chromatograph fitted with a Zebron Amino acid ZB-AAA column (10 m×0.25 mm i.d.; Phenomenex, Cheshire, UK), Agilent 7683 automatic liquid sampler, and split/splitless injector. Mass spectra were acquired at 70 eV over 45–450 *m/z* from 3.60 to 13.00 min with an acquisition rate of 1.98 Hz. The GC injector and transfer line were both held at 280 °C. Helium (50 kPa, constant pressure) was used as the carrier gas. The oven temperature was kept at 75 °C for 2 min and then ramped to 320 °C at 25 °C min^{-1}, with a further hold at this temperature for 1.2 min. Data were quantified using MassLynx 4.0 (Waters, Manchester, UK). Quantification of the amino acid peaks was done using extracted ion chromatograms as described by Baker *et al*. (2006).

Transcriptomic analysis

RNA was extracted from plant material described above using the method of Verwoerd *et al*. (1989), purified using RNeasy Plus columns (Qiagen, Valencia, CA, USA), and hybridized in three biological replicates to wheat gene chips as described in the GeneChip® Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). Data were pre-processed using the GC-RMA algorithm (Wu *et al*., 2004) and analysed using GeneSpring 7.2 (Agilent Technologies, Santa Clara, CA, USA). Data were normalized to the median expression across all chips, with raw values <0.1 converted to 0.1.

Functional annotations of probe sets representing the various amino acid biosynthetic pathways were determined from the Affymetrix standard annotation (July 2006 wheat release) and from the closest *Arabidopsis* homologue identified by blastx searching against GenBank protein sequence databases. Remaining pathway steps with no assigned Affymetrix probe set were identified, where alternative plant gene sequences were available, by blast searching these against wheat Affymetrix probe set targets to find highly homologous (>80%) matches. A full list of probe sets with corresponding function and GenBank accessions is available as supplementary data at JXB *online* (Table S3).

For expression analysis, probe sets with absolute 'raw' data values <25 in all treatments and time points were classed as 'not expressed' and ignored. Two-way ANOVA tests were then performed, and probe sets showing no significant change in expression [P >0.05 using Benjamini and Hochberg (1995) multiple testing correction] between fertilizer treatment or days post-anthesis (dpa) were also ignored. Remaining probe sets with the same annotated function were grouped by expression profile using quality threshold (QT) clustering (Pearson correlation >0.8). Cluster groups of the probe sets and representative expression data are detailed in Table S3 at *JXB* online.

Results

Establishment of N and S partitioning and remobilization during grain filling

Following anthesis, cell expansion and nuclear division establishes the cellular structure of the wheat grain that will eventually reach maturity. Subsequently the grain endosperm accumulates starch, oil, and protein, reaching its maximum fresh weight by ~21 dpa. Between 21 and 30 dpa, the pericarp fuses with the maternal epidermis, the endosperm fills with starch and protein, and the embryo fully develops by ~30 dpa (Wilson *et al*., 2004). In order to assess the rate of nutrient remobilization from vegetative tissues to grain during this period and to study the effect of fertilizer application on senescence processes, total N and S contents of individual plant parts and leaf chlorophyll measurements [using a SPAD (soil-plant analyses development) meter] were determined. Leaves 1 (flag) to 3 and whole main stems were harvested weekly from anthesis to 49 dpa from control (N2; 192 kg N ha^{-1}), N-deficient (N1; 48 kg N ha^{-1}), and S-deficient (–S; same N application as N2 but with no S) field plots (Fig. 1).

SPAD measurements followed a similar pattern in all treatments, where a developmental series of chlorophyll

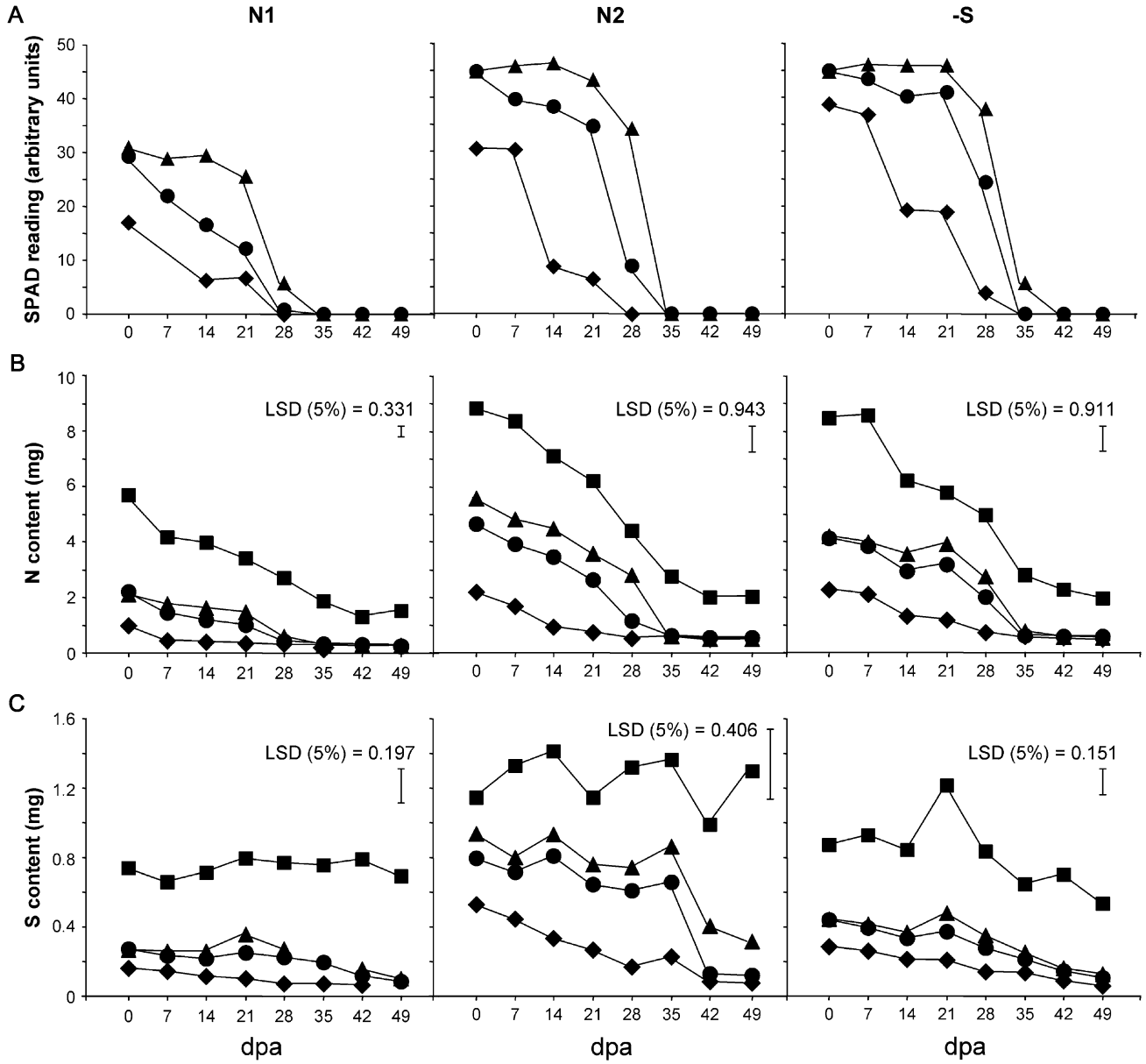

Fig. 1. Post anthesis measurements of (A) chlorophyll (SPAD), (B) nitrogen, and (C) sulphur content of winter wheat (*T. aestivum* var Hereward) tissues during grain filling. Stems (filled squares) and leaves 1 (filled triangles), 2 (filled circles), and 3 (filled diamonds) (numbered from the flag leaf down) were harvested from control (N2), N-deficient (N1), and S-deficient (–S) plots. Contents were measured per plant part. Least significant difference (LSD) error bars were calculated from three biological replicates using a two-way ANOVA to test for significance at the 5% (P <0.05) level. SPAD readings were mean measurements from the leaves of 10 replicate main stems.

degradation was observed, first in leaf 3 followed sequentially by leaf 2 and finally leaf 1 (Fig. 1A). Leaves of N2 and –S plants had higher maximum SPAD readings than those of N1 plants throughout the post-anthesis period. Leaf 1 of all plants showed no degradation until 21 dpa, before reducing to 0 by 35–42 dpa.

Paralleling the SPAD measurements, increased leaf N was observed from the lower to the upper leaves in all plants at anthesis (Fig. 1B). Vegetative tissue N was exported from leaves 1–3 and stem during senescence in parallel rather than following the sequential pattern seen for the decrease in the SPAD measurements. N content in leaf 3 of N1 plants reached a minimum level by 7 dpa compared with 28 dpa in N2 and –S plants. Leaves 1 and 2 contained significantly more N than leaf 3 and depleted to the minimum by 28 dpa in N1 and 35 dpa in N2 and –S. In all treatments the stems had higher absolute N contents than the leaves on a per organ basis, although the concentrations were much lower. Stem N was decreasing even in the late stages of grain filling (up to 42 dpa). No significant differences in tissue N were observed between N2 and –S plants other than in –S leaf 1 at anthesis. This

may reflect a slightly reduced maximum N accumulation potential of S-deprived plants.

The pattern of S remobilization in vegetative plant parts differed from that observed for N (Fig. 1C). S remobilization occurred initially at a much slower rate than observed with N for all fertilizer treatments. No significant reduction in total S was measured in any plant part between anthesis and 49 dpa from the N1 treatment. Similarly, N2 plants remobilized tissue S much less rapidly than N, and contained significantly higher S levels in all plant parts than N1 or –S plants up to 35 dpa. Consequently, whilst –S plants accumulated levels of tissue N comparable with the N2 controls (Fig. 1B), the level of S accumulation was significantly lower, comparable with N1 plants (two-way ANOVA, data not shown).

The import of N and S into the grain between 14 and 49 dpa was determined (Fig. 2). From 14 dpa, grain N and S accumulated in parallel, reflecting the increase in grain biomass. N1 plants showed no significant difference in grain N until 28 dpa (Fig. 2A) or grain S until 35 dpa (Fig. 2B), but subsequently reached a plateau in the acquisition of both elements. This was due to decreased grain expansion and fewer grains/ear in the N-deficient treatment, leading to reduced overall yield. Concentrations (percentage N and S) remained relatively constant throughout the 49 d period, albeit with significantly lower N concentrations in N1 grain (Fig. 2C). N2 and –S plants showed no significant difference throughout the post-anthesis time course in either grain N or S, although final harvest grain N:S ratios were increased to 16.2 in the –S treatment compared with 14.3 for N2 (data not shown).

Nutritional status affects the metabolite profile of leaf but not of grain during early grain filling

To examine the metabolic processes of nutrient remobilization and grain filling with varying fertilizer inputs, metabolite profiling using ^{1}H-NMR spectroscopy was performed on grain and pooled leaf 2 and 3 samples between anthesis and 28 dpa, when the grain is most actively importing remobilized N and S. Metabolite availability in the leaf during the grain-filling period will have implications for the ability of the developing grain to reach optimal size and weight, and consequently for crop yield.

^{1}H-NMR profiles from polar solvent extracts of leaves between anthesis and 28 dpa, and whole grain between 7 and 28 dpa (no grain present at anthesis), were compared using principal component analysis (PCA) to separate samples according to the major divergent metabolite pools. Field-grown samples from the three fertilizer treatments were compared. PCA revealed contrasting effects of

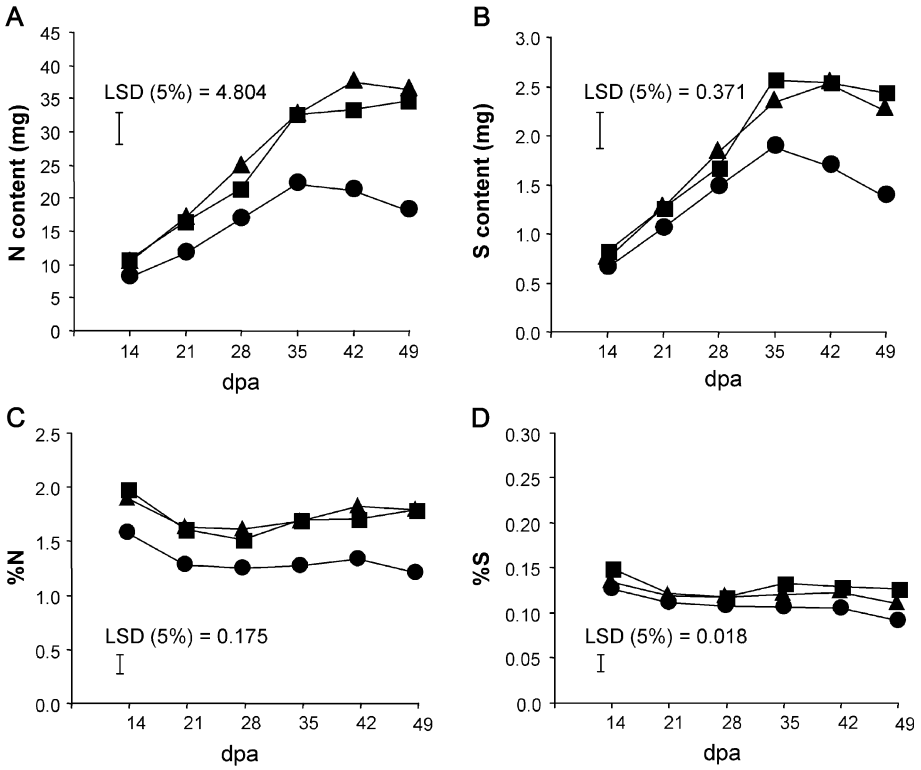

Fig. 2. Nitrogen and sulphur accumulation in wheat grain post-anthesis. (A) Total N and (B) total S accumulation expressed on a mg per ear basis. (C) Percentage N and (D) percentage S concentration in the developing grain. Tissues were harvested from control (N2, filled squares), N-deficient (N1, filled circles), and S-deficient (–S, filled triangles) plots. Least significant difference (LSD) error bars were calculated from three biological replicates using two-way ANOVA to test for significance at the 5% (P <0.05) level.

fertilizer application on the NMR profiles of leaf compared with grain tissues (Fig. 3). Separation of NMR profiles from leaf samples was observed on the basis of fertilizer treatment by principal components 2 and 3 (Fig. 3A). This indicated a fertilizer-dependent variation in leaf metabolite composition throughout grain filling. No separation was observed on the basis of dpa (Fig. 3C). N1 samples separated from N2 and –S samples in different areas of the PCA scores plot. N2 and –S samples resided in the same region of the plot but also showed some separation. A small overlap between N1 and N2 samples was observed where the N2 28 dpa leaf samples were positioned between the N1 7 and 14 dpa groups (Fig. 3A, C), indicating metabolic similarities between these tissues.

All grain NMR profiles grouped closely by dpa (Fig. 3D) and not by treatment (Fig. 3B), indicating that unlike in the leaf tissue, the metabolic composition of wheat grain was mainly developmentally controlled, and that the nutritional status of the vegetative tissues had a comparatively minor effect.

The principal metabolite changes which characterized the separations in the leaf tissue due to nutritional treatment consisted predominantly of carbohydrate signals,

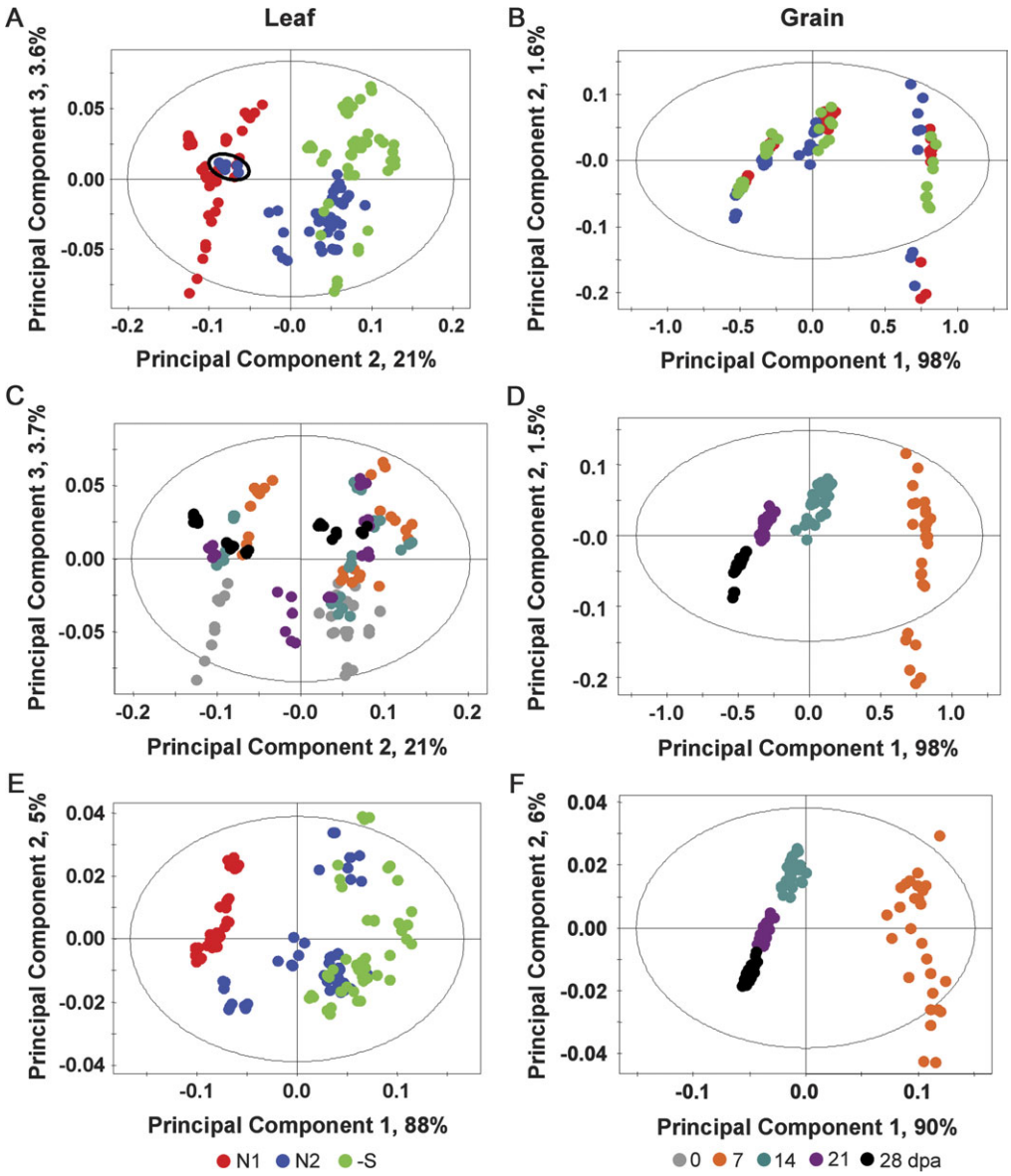

Fig. 3. Principal component analysis (PCA) of ^{1}H-NMR profiles of wheat leaf (A, C, E) and whole grain (B, D, F) tissue between anthesis and 28 dpa. Data points represent positions of three biological and three technical replicates for each sample separated two-dimensionally by principal changes in the NMR spectra. (A, B) PCA plots of complete NMR data sets coloured by fertilizer treatment. (C, D) PCA plots of complete NMR data sets coloured by dpa. (E, F) PCA plots of NMR data sets after removal of the carbohydrate region, coloured by fertilizer treatment for leaf, and dpa for grain samples. The position of the 28 dpa N2 leaf samples overlapping with N1 is circled in A.

glycine betaine, choline, and amino acids (Table S1 at *JXB* online). In the N1 treatment, clear reductions were evident in glycine betaine, choline, and γ-aminobutyric acid relative to the other two treatments. These reductions were accompanied by an elevation in carbohydrate signals, currently unassigned but believed to relate to linked fructose metabolites. The principal metabolite changes in N2 leaf material across the 28 d period were a decrease in the concentration of sucrose and glycine betaine from anthesis to 28 dpa.

The principal metabolites separating grain tissues developmentally (by dpa) were decreasing levels of glucose, maltose, sucrose, and glutamine (Table S2 at *JXB* online). PCA of the NMR spectra after removal of the dominant carbohydrate region allowed examination of the smaller but important peaks of the aliphatic and aromatic profile regions as discussed in Baker *et al.* (2006). As shown in Fig. 3E and F, results from this model were similar to the full spectra models (Fig. 3A, D). Separation of N2 and N1 samples was observed in leaf tissues, but again separation of grain samples was predominantly dependent on developmental stage after anthesis rather than fertilizer treatment. Removal of the dominant carbohydrate peaks did not change the behaviour of the principal metabolites identified above, but did show changes in the aromatic region of the spectra (tyrosine and tryptophan) which had previously not been identified.

Leaf and grain amino acid survey post-anthesis

Amino acids are the major form of N transported from leaf to grain in wheat (Caputo and Barneix, 1997; Lalonde *et al.*, 2003, 2004). NMR profiling suggested differences in amino acid levels across the data set which were validated using amino acid analysis by GC–MS. Concentrations of individual free amino acids were determined in leaf and grain samples between anthesis and 28 dpa. Total amino acid content of leaves (2 and 3 pooled) and grain (Fig. 4) and individual amino acids as a percentage of the total pool (Fig. 5) are presented. Dry weight concentrations of free amino acids in the leaf decreased ~2-fold to 3-fold between anthesis and 28 dpa in all treatments (Fig. 4A). In agreement with previous reports (Caputo and Barneix, 1997), significantly lower concentrations of total free amino acids were measured in the leaves of the N-deficient plants. No significant differences were observed between N2 and –S leaves until 28 dpa. Concentrations between 250 (N1) and 350 (–S) mmol kg^{-1} of free amino acids were measured in the grain at 7 dpa. This was followed by a rapid decrease in total free amino acid concentrations between 7 and 14 dpa. No significant differences were observed between grain amino acid concentrations after 21 dpa in any treatment (Fig. 4B).

Figure 5 shows the free amino acid pools for leaves and grain at N1, N2, and –S in the post-anthesis time course. The use of pool percentage rather than total concentration allows changes in the predominance and importance of individual amino acids to be shown, which would otherwise be masked by the large changes of overall concentration during the time course, as shown in Fig. 4. In the leaf, Glu was the major pool component in all plants, with Asp, alanine (Ala), lysine (Lys), serine (Ser), and leucine (Leu) each generally occurring at >10% abundance. Glu levels remained high in all treatments. Asp, Ala, Lys, and Ser pools reduced in size throughout the 28 d time course. These decreases were balanced predominantly by an increase in Gln (to between 9.2% and 15.0% of the total) and also in trytophan (Trp; to between 1.0% and 3.8% of the total) by 28 dpa, particularly in N1 leaves.

Gln made up 50–60% of the free amino acids in grain tissue at 7 dpa, probably reflecting its role as a major transported amino acid. Gln levels fell rapidly, and Glu, Asp, Ala, Ser, glycine (Gly), and Trp became the most

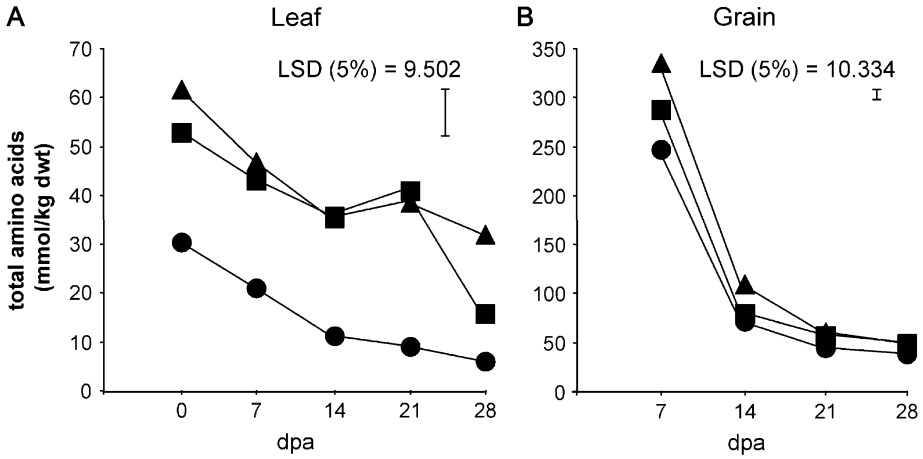

Fig. 4. Total free amino acid concentrations in (A) leaf and (B) grain of field-grown wheat post-anthesis from control (N2, filled squares), N-deficient (N1, filled circles), and S-deficient (–S, filled triangles) plots. Least significant difference (LSD) error bars were calculated using a two-way ANOVA to test for significance at the 5% (P <0.05) level.

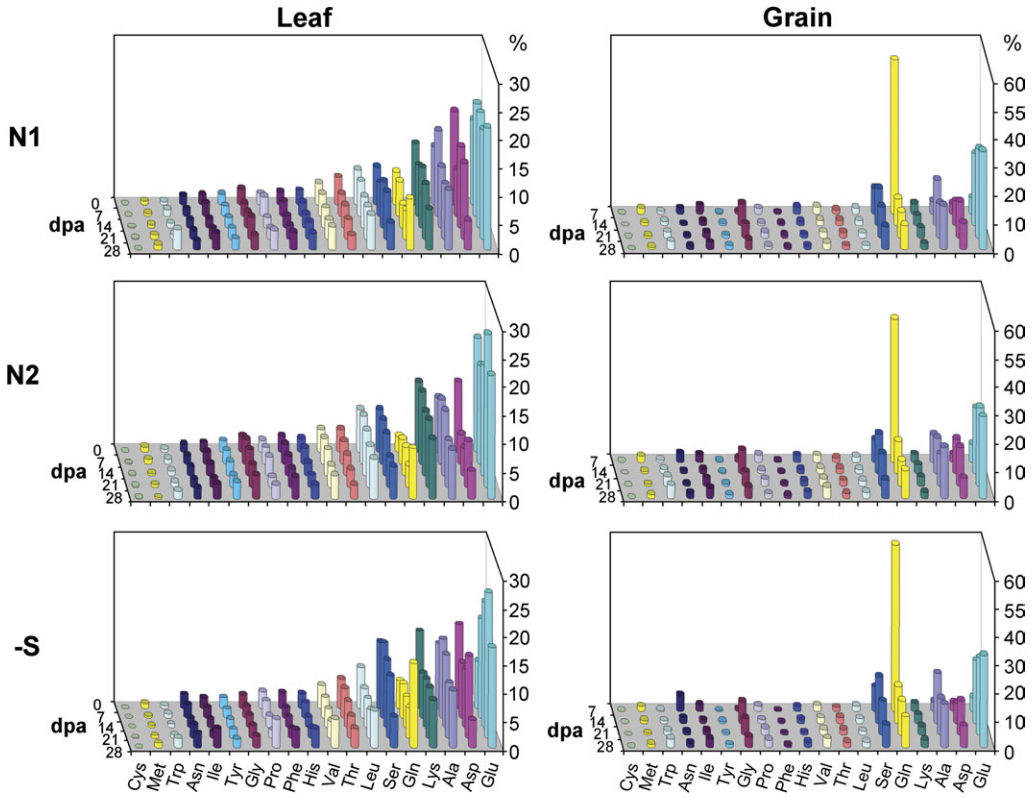

Fig. 5. Amino acid compositions of wheat leaf and grain tissues post-anthesis from control (N2), N-deficient (N1), and S-deficient (–S) plots. The contribution of each individual amino acid to the total free amino acid pool is represented to highlight changes in pool composition post-anthesis. Standard abbreviations are used. Arg was below the detection limits of GC–MS in all samples.

abundant in terms of percentage composition by 14 dpa. It is likely that these amino acids were synthesized *in situ* in the second week of development, utilizing Gln delivered to the grain. In the grain, little variation in free amino acids was observed between treatments. This correlated with the NMR data which showed the metabolic profile of grain to be more closely linked to developmental stage than fertilizer treatment. The only major observable difference between the grain amino acid profiles was the increased pool of asparagine (Asn) in –S plants at 7 dpa which suggested Asn to be more predominant as a form of transported N in S-deficient plants.

Genetic regulation of amino acid metabolism post-anthesis

Transcriptome analysis of leaf amino acid metabolism was performed using the Affymetrix wheat GeneChip and GeneSpring microarray data analysis software (Agilent Technologies). Leaf tissues sampled at anthesis, and 7, 14, and 21 dpa were analysed for the same three biological replicate samples used for metabolite profiling (Figs 3–5). Selected expression data of amino acid metabolism pathways are displayed as an expression heatmap in Fig. 6. A complete set of expression data is included in Table S3 at *JXB* online. The pathways of amino acid metabolism are displayed in the major pathway groups as described in Singh (1999). Each arrow in Fig. 6 represents a single enzymatic step and is numbered by the corresponding gene annotation. In many cases, multiple probe sets were found to encode any given enzyme, and are listed in Table S3 at *JXB* online. Probe sets annotated as having the same function but differing in expression profile are likely to represent different isoforms, homeologues, and orthologues of any given gene under varying transcriptional control.

In tissues from N1-grown plants, many genes have very low expression or have profiles considerably reduced in expression after anthesis compared with N2 and –S. This was particularly evident in the pathway of arginine (Arg) biosynthesis from Glu via ornithine [probe set reference numbers: 1, 2, 4, 5, 6] (Fig. 6). In addition, there was significantly lower expression in the final steps of synthesis of many amino acids in N1 leaves. For example, arginosuccinate lyase [6] (synthesizing Arg), chloroplastic Gln synthetase GS2 isoform [14], threonine (Thr) synthase [26], Ala aminotransferases [36], *O*-acetylserine (thiol) lyase (synthesizing Cys) [46], prephenate dehydratase [62] [synthesizing phenylalanine (Phe)], and Met synthase [49] had very low expression after anthesis compared with N2 and –S leaves.

Adenosylmethionine:methionine *S*-methyltransferase [50] which produces SMM from Met was also reduced in expression. In contrast, Trp synthase [60] had elevated

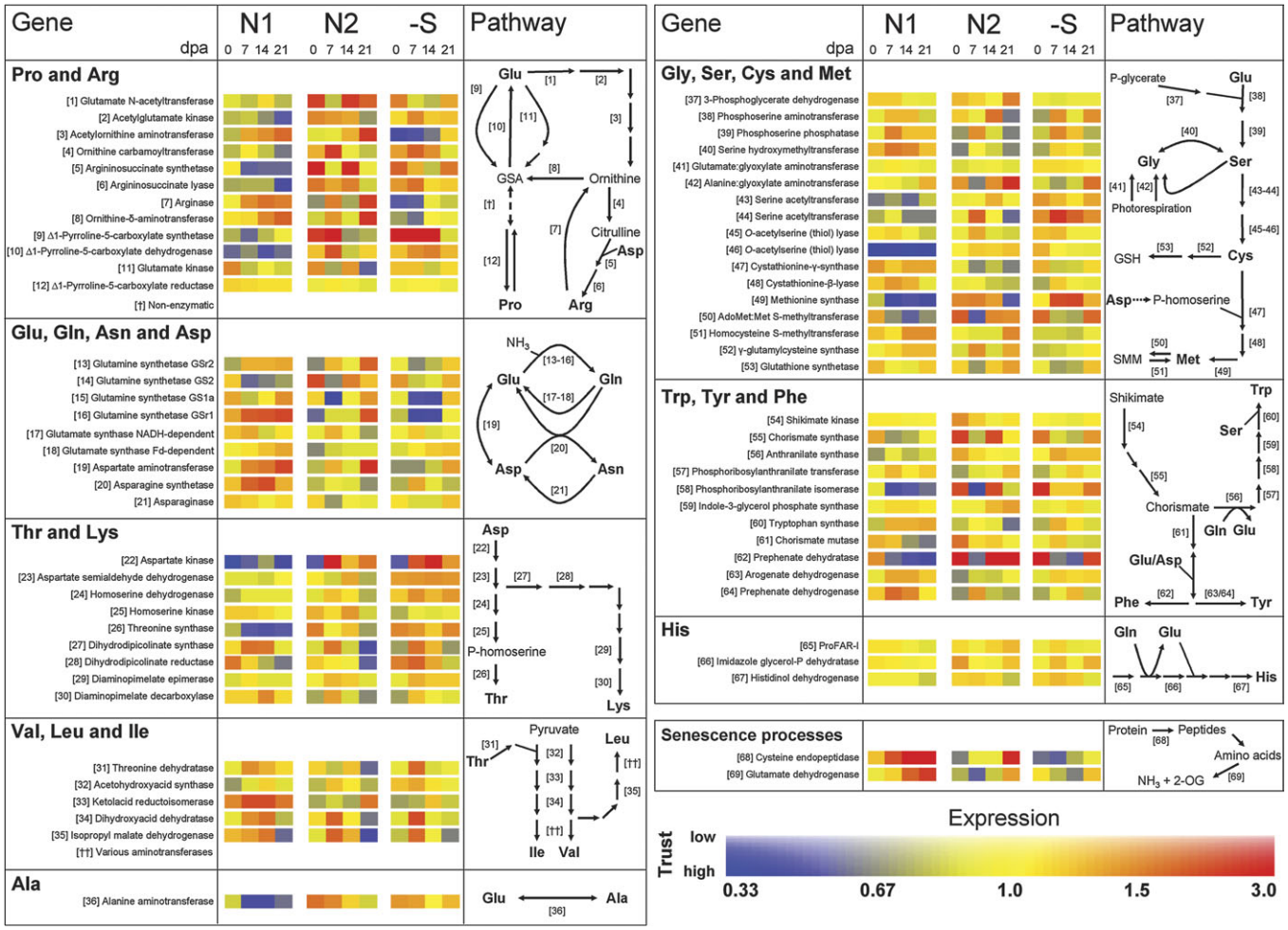

Fig. 6. Heatmap representation of amino acid biosynthetic pathway gene expression in the 21 d following anthesis in control (N2), N-deficient (N1), and S-deficient (–S) wheat plants. Each expression value was calculated from Affymetrix data of three biological replicates using GeneSpring microarray expression software. The normalized expression level of each individual gene is shown according to the horizontal axis of the continuous scale colour bar key. Colour bar limits were set to show 3-fold up- or down-regulation from the median expression value of each gene (blue=low expression, red=high expression). Trustworthiness of the expression value is represented in the vertical axis of the colour bar key (dark or unsaturated colours=low trust, bright or saturated colours=high trust). A complete data set of expression profiles is available in Supplementary Table S3 at *JXB* online. GSA=γ-glutamyl semialdehyde; P-homoserine=phosphohomoserine; P-glycerate=phosphoglycerate; GSH=glutathione; SMM=*S*-methylmethionine; 2-OG=2-oxoglycerate; ProFAR-I=phosphoribosyl formimino-5-amino-1-phosphoribosyl-4-imidazole carboxamide isomerase.

expression in N1 leaves. Various other genes were also more highly expressed in N1 plants or had expression increasing between anthesis and 21 dpa compared with N2 and –S, such as Asp aminotransferases [19], Asn synthetase [20], ketolacid reductoisomerase [33], phosphoserine phosphatase [39], arogenate dehydrogenase [63]/prephenate dehydrogenase [64], and cytosolic Gln synthetases GSr2 [13], GS1a [15], and GSr1 [16].

Genes encoding enzymes involved in the biosynthesis of the Met precursor homocysteine from Cys [47, 48] were also more highly expressed in N1 compared with N2/–S plants.

Between anthesis and 21 dpa, N2 leaves showed an increase in expression of Glu *N*-acetyltransferase [1] which diverts Glu towards ornithine production, whereas expression of Δ1-pyrroline-5-carboxylate synthetase [9] and Glu kinase [11] was reduced. These enzymes are both involved in the conversion of Glu to γ-glutamyl semialdehyde (GSA), as an intermediate in proline (Pro) biosynthesis, and are potential competitors for the Glu pool. N2 leaves also exhibited lower expression of ferredoxin-dependent Glu synthase [18] than N1 and –S plants, in which an increase in expression was observed between anthesis and 21 dpa.

Leaves from the –S treatment, whilst most similar to those from N2 plants, showed differences in expression of several genes involved in amino acid metabolism. Acetylornithine aminotransferases [3], an intermediate step in the synthesis of ornithine from Glu, increased in expression between 0 and 21 dpa in all treatments, but had 2-fold to 3-fold lower expression in –S. The same regulation by –S treatment was observed for both arginase [7] and

ornithine-δ-aminotransferase [8] of the Arg degradation pathway. –S leaves showed higher expression of Asp semialdehyde dehydrogenase [23] and homoserine dehydrogenase [24], both of which are involved in the production of Thr and Lys from Asp. Similarly the S-deficient leaves had higher expression of serine acetyltransferase [43, 44] which produces *O*-acetylserine, the critical precursor for Cys biosynthesis (Hell *et al*., 2002; Kopriva and Koprivova, 2003; Hawkesford *et al*., 2006).

Figure 6 also shows the expression of Cys endopeptidase [68] and Glu dehydrogenase [69] (GDH), which are associated with senescence in plant tissues. GDH activity increased in wheat leaves post-anthesis (Peeters and Vanlaere, 1992, 1994) and Cys endopeptidases are involved in the catabolism of proteins during senescence in *Arabidopsis*. The *Arabidopsis SAG12* gene is a Cys endopeptidase specifically involved in senescence-related proteolytic activity (Lohman *et al*., 1994; Otegui *et al*., 2005). The expression of both (Fig. 6) corresponded to the level of senescence in leaf tissues (Fig. 1B), increasing in expression after anthesis in all samples and having higher expression levels in N1 plants.

Discussion

In order to ensure sustainable world crop production, increased grain yields are required from arable lands without concomitant increases in fertilizer inputs. As with the first 'green revolution' of the 1960s to 1970s (Kendall and Pimentel, 1994; Peng *et al*., 1999), this will be achieved by a combination of improved farming practice and genetic improvement of modern wheat cultivars. A detailed understanding of wheat grain production and fertilizer use efficiency will be essential to meet these aims.

In this study, physiological measurements have been combined with transcriptomic and metabolomic analysis to provide novel insights into the remobilization of nutrients to the developing wheat grain. The aim was to determine the relationship of senescence and nutrient remobilization with grain filling and the effect of reduced fertilizer application on the contribution of these key processes to grain yield and quality.

Specific responses in gene expression related to development and to the N-deficient and S-deficient crops have been identified, indicating the importance of shifts in expression of amino acid metabolism and other genes during leaf senescence. The developmental timings of these processes are potential targets for improving the efficiency of nutrient remobilization during wheat grain production.

The independence of grain development from fertilizer application and nutritional status of the plant canopy has been demonstrated. Differing strategies for the remobilization of N and S to the developing grain have also been highlighted. These findings confirm vegetative remobilization processes as a target for the genetic improvement of N and S use efficiency and consequently grain yield and quality in wheat.

Following anthesis, nutrients (particularly N) are remobilized from senescing vegetative tissues to the developing grain. Figure 1B shows the N remobilization processes from the leaves and stem tissues. As expected, all vegetative tissues of control N2 plants contained significantly higher total N contents than N-deficient (N1) plants. These differences in stored reserves are a major determinant of the grain yield potential of the crop. A comparison of leaf N content (Fig. 1B) and SPAD measurements (Fig. 1A) revealed that senescence processes commence before decreases in leaf chlorophyll were detected. N content of N2 leaf 1 tissue was reduced by >20% by 21 dpa before any drop in SPAD reading was recorded. This demonstrated that senescence was initiated prior to any reduction in photosynthetic capacity caused by chlorophyll degradation.

S-deficient plants were shown to accumulate and remobilize N comparably with N2 plants, although lower leaf 1 N content at anthesis may have reflected a slightly lower capacity for N accumulation caused by the S deficiency. Optimal plant growth requires an N:S ratio of ~15:1 for protein production (Dijkshoorn and van Wijk, 1967; Zhao *et al*., 1999*a*), and an increase in this ratio, due to the S deficiency, was the most likely cause of lower N accumulation in leaf 1 of the –S plants.

Importantly, N-deficient N1 plants not only contained less N to remobilize, but additionally were shown to have completed N remobilization 7–14 d earlier than observed for the N2 and –S plants (Fig. 1B). In order to maximize the export of vegetative N reserves to the developing grain, optimal alignment of transportable nutrient availability and grain filling is important and would be a trait with potential for breeding crops, producing improved yields on low fertilizer inputs.

Whereas S-deficient plants accumulated levels of N comparable with those of control N2, N-deficient plants accumulate a significantly lower tissue S concentration (Fig. 1C). This reflected the reduced protein production of N-deficient plants and highlights the control N availability has on S uptake and accumulation (Koprivova *et al*., 2000). S remobilization from vegetative tissues also followed a very different pattern from that observed for N. Remobilization was later, only showing significant reductions in leaves 1 and 2 of N2 plants at 42 dpa, well after the completion of N remobilization in the vegetative tissues in this study. N1 plants showed no significant remobilization of S reserves after anthesis. Despite these differences, both N and S accumulated in the grain at the same rate in N2 and –S plants up to 49 dpa (Fig. 2A, B), although final grain N:S ratios of –S plants were >16, the

recommended maximum for bread-making grain quality in the UK (Zhao *et al*., 1999*a*). Only in the N1 grain was ear N and S accumulation significantly reduced, reaching maximum levels by 35 dpa (Fig. 2A, B). This reduced N/S content is likely to be due to fewer grain numbers per ear and the completion of vegetative senescence and subsequent reduction in the availability of N for export during grain development.

S accumulation in canopy tissues of –S plants was significantly lower than in the controls (N2) and was only significantly remobilized from leaves 1 and 2 after 35 dpa. However, this did not coincide with increases in grain S for –S and N2 plants during the first 28 dpa (Fig. 2A). This indicated sources additional to the canopy analysed for the S imported into grain. Likely sources are from increased uptake of sulphate from the soil, or from sulphate storage pools in the root. Consequently, an application of fertilizer S at anthesis may prove a successful strategy to boost grain S content and bread-making quality (Tea *et al*., 2003, 2007).

For a more detailed insight into the contribution of leaf senescence and grain development to wheat productivity, NMR metabolite profiling was performed on leaf and grain samples between anthesis and 28 dpa. Analysis of the principal changing metabolite pools revealed distinct differences between leaf and grain tissues of N1, N2, and –S plants. Whilst fertilizer application had a significant effect on the leaf metabolome profile, very little variation was observed in grain (Fig. 3). In the PCA, N1, N2, and –S leaf tissues grouped separately by fertilizer treatment. Overlap of the N2 and N1 samples (circled in Fig. 3A) indicated that at 28 dpa, N2 leaves closely resembled those of N1 plants between 7 and 14 dpa, in terms of metabolism. As with the physiological N data in Fig. 1, this demonstrated that senescence in N1 plants occurred earlier than in the higher N treatment (N2).

In contrast, the grain metabolome was largely independent of the nutritional status of the crop and more closely determined by its developmental stage (Fig. 3D). This indicated that whilst leaf metabolite composition was significantly affected by fertilizer treatment, metabolite composition of the grain was developmentally regulated following anthesis. This has implications for the improvement of grain filling under reduced fertilizer inputs. The capacity for optimal grain yield will depend on the timing of senescence/remobilization processes and availability of nutrient recycling relative to grain development. Therefore, optimizing the genetic timing of remobilization to coincide with temporally regulated grain development may provide a target for maximizing grain yield under reduced fertilizer inputs. Exploitation of available genetic diversity for senescence timing, from both wild relative species and commercially available crop varieties, may prove useful in breeding varieties with improved yields and protein contents for reduced fertilizer input systems. Similar approaches have recently been investigated using functional 'stay-green' mutants which senesce late into the development of grain and have the potential to produce higher yields due to their protracted photosynthetic capacity and higher leaf N contents. However, such mutants can also be associated with high grain carbohydrate:protein ratios and low N remobilization if senescence is delayed beyond grain filling (Borrell and Hammer, 2000; Borrell *et al*., 2001; Spano *et al*., 2003; Uauy *et al*., 2006*a*,*b*).

One of the principal metabolite pools changing after anthesis as indicated by the NMR analysis, particularly in the grain tissue, was the amino acid Gln. Gln is a major transported form of N in wheat during reproductive growth (Simpson and Dalling, 1981). Figure 5 represents a comprehensive analysis of free amino acid pools in the leaves and grain of wheat post-anthesis. In leaves, Glu remained the predominant amino acid pool throughout the 28 d following anthesis; however, by 28 dpa, the Gln pool increased substantially under all conditions.

In the grain, high levels of free amino acids accumulated in the first 7 d after anthesis (Fig. 5), and >50% of the total pool was in the form of Gln, indicative of a role as the major transported amino acid in wheat. –S plants also showed elevated levels of Asn, implicating this amino acid as a potentially important transported form of N in S-deficient plants. The grain of wheat plants grown at low S has previously been reported to accumulate free Asn, and all plant tissues limited in protein synthesis when grown in adequate N supply accumulate Asn (Shewry *et al*., 2001). Under severe S limitation, up to 30 times more Asn can accumulate in the grain, levels which may produce the carcinogen acrylamide when the flour is used in baking (Muttucumaru *et al*., 2006; Lea *et al*., 2007).

By 14 dpa all grain samples showed ~3-fold reductions in free amino acids (Fig. 4) and the amino acid pools had redistributed from predominantly Gln to other forms, most notably Glu, Ser, Ala, Asp, and Gly. Together with data showing linear increases in total grain N in the 28 dpa period (Fig. 2B), it can be concluded that the transported free amino acids (mainly Gln) accumulated in the grain up to 7 dpa are used to synthesize other amino acids and protein by 14 dpa.

Transcriptome analysis was used to provide information on the genetic control of the amino acid composition of leaves after anthesis (Fig. 6). Gene expression patterns of N2 and –S leaves showed similar trends, with a few key exceptions in the pathways of Cys biosynthesis, transportable organic S (SMM, GSH) biosynthesis, ornithine production, and Arg breakdown. Higher expression of serine acetyltransferase [43, 44] and *O*-acetylserine (thiol) lyase [45] may be expected to maximize Cys production under low S availability. Significantly increased Met synthase [49] expression in S-deprived leaves may

indicate a requirement for Met, and its transported derivative SMM, after anthesis. Increased Met synthase expression was not observed in S-starved *Arabidopsis thaliana* seedlings (Nikiforova *et al*., 2003); however the metabolic requirement for organic S-containing compounds is likely to be different for a developing dicotyledonous seedling compared with a cereal leaf subject to sink demands during grain development.

The major differences in N1 gene expression between anthesis and 21 dpa were a down-regulation of many of the terminal steps of the various amino acid pathways (Phe, Met, Cys, Ala, Thr, Arg, and chloroplastic Gln). This strategy limited production of amino acids which were less important for remobilization during senescence. Elevated expression of Trp synthase [60] in N1 plants, increasing up to 21 dpa, was an exception to this and is reflected by the increased contribution of Trp to the free amino acid pool at 21 dpa in N1 plants (Fig. 5). The regulation of amino acid pools via transcription in this way contrasts with the general response of amino acid metabolism under abiotic stress conditions. Most amino acid pools in stressed *A. thaliana* were shown to be regulated by the transcription of catabolic enzymes, with biosynthetic enzymes remaining largely unresponsive in an assessment of publicly available array data (Less and Galili, 2008). Pro was the exception to this model, with its production as an osmolyte being induced under stress by transcription of Δ1-pyrroline-5-carboxylate synthetase [9]. In the present study, the reduction in expression of Pro biosynthetic genes and Pro concentrations after anthesis indicates a reduced requirement for Pro as an osmolyte in senescing tissue. This highlights the specialized response of amino acid biosynthesis during senescence, which is distinct from that under abiotic stress.

An increase in expression of Glu synthases (particularly the ferredoxin-dependent form [18]) and the cytosolic Gln synthetase isoforms GSr2[13], GS1a [15], and GSr1[16], may be seen as a strategy to divert amino acid synthesis into Gln, the major transportable form of N. The contrasting expression patterns of the GS2 and GS1 forms, which decrease and increase, respectively, after anthesis, show a differential regulation of the GS gene family, as previously described in the leaves of maize and wheat plants during grain development (Kichey *et al*., 2005; Martin *et al*., 2006). The differential regulation of the family acts to balance the functions of primary nitrate assimilation into Gln and, during senescence, the reassimilation of ammonium released during proteolysis. GS expression is controlled antagonistically by N and C metabolites in plants in order to balance the Gln and amino acid requirements of the cell. Sucrose induces GS expression, whereas amino acids such as Asp, Asn, Glu, and Gln suppress the induction. Additionally, the carbon backbone precursor to Glu, 2-oxoglutarate (2-OG), induces the expression of GS1 but not GS2 isoforms (Oliveira and Coruzzi, 1999; Gutierrez *et al*., 2008). It can be speculated that the balance of sucrose, free amino acids, and 2-OG is regulating the expression of the GS family both temporally and between fertilizer treatments in the present study. Rapid decreases in leaf amino acids were observed in N1, N2, and –S leaves (Fig. 4A), coinciding with increases in cytosolic GS expression. Higher expression of GSr2 [13], Gs1a [15], and GSr1 [16] in N1 compared with N2 leaves may be caused by the equilibrium between photosynthetic capacity (and consequently sucrose availability) and amino acid content.

N1 plants also exhibited reduced expression of genes involved in the production of ornithine and citrulline from Glu [1, 2, and 4]. Ornithine is the point of entry for the biosynthesis of polyamines such as putrescine, spermidine, and spermine (Verma and Zhang, 1999), which are used to store excess organic nitrogen in plant tissues. Down-regulation of ornithine biosynthesis after anthesis in N1 plants may serve to ensure the N-limited Glu pool is channelled towards Gln for transport and grain production, rather than to redundant storage compounds.

Transcriptomic studies in *A. thaliana* have identified a range of enzymes with senescence-specific roles in protein degradation, including Asp, Cys proteases and endopeptidases, and also Ser carboxypeptidases (Buchanan-Wollaston *et al*., 2003, 2005). Expression of these genes is fundamental to both the onset and progression of senescence processes. The binding properties of specific Cys protease promoters with extracts of young and old leaves have implicated these genes in the initial signalling of senescence via either a repressor or activator system (Noh and Amasino, 1999). The expression pattern of a Cys endopeptidase [68] is included in Fig. 6. Expression complies with the findings of previous transcriptomic studies, increasing as senescence progresses. A Glu dehydrogenase (GDH) [69] is shown also to be a marker of senescence. GDH has been implicated as a late senescence marker in tobacco, which is repressed by sucrose and therefore increases in expression as photosynthetic capacity declines (Masclaux *et al*., 2000; Masclaux-Daubresse *et al*., 2005). In the present study, GDH expression is induced between anthesis and 21 dpa, but lags behind the Cys endopeptidase response, in line with previous reports. Expression of both genes indicates the advanced onset and development of senescence in N1 plants compared with N2 and –S.

N and S nutrition are two of the key determinants of grain yield and bread-making quality in wheat, and this study, of grain filling in N and S-deficient plants, has provided some important insights into genetic, metabolic, and physiological processes under reduced fertilizer inputs. N-deficient plants were shown to accumulate less total N, S, and free amino acids than control plants by anthesis and started senescing earlier than both control and S-deficient plants. This demonstrated that the N, but

not S, status of the vegetative crop canopy regulated nutrient remobilization and senescence in wheat. In contrast, metabolomic analysis showed that grain development occurred independently of the nutritional status of vegetative plant parts.

During leaf senescence, amino acid biosynthesis was regulated in favour of Gln production at the expense of a number of other amino acids, and Gln was the major transported form of amino acid imported during early grain development. These data suggest that nutrient remobilization, controlled genetically by senescence, and channelling of amino acid biosynthesis, presents a strategic target for the optimization of wheat grain production at low fertilizer inputs by synchronizing the timing of these processes with temporally controlled grain development.

Data deposition

Microarray data are deposited in ArrayExpress under the accession E-MEXP-1415. NMR and amino acid data are available from the BBSRC National Centre for Plant and Microbial Metabolomics (MeT-RO) at http://www.metabolomics.bbsrc.ac.uk

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Principal metabolite differences determining leaf PCA separations by fertilizer treatment in Fig. 3.

Table S2. Principal metabolite differences determining grain PCA separations by dpa in Fig. 3.

Table S3. Affymetrix probe sets representing amino acid metabolism genes with functional annotation, GenBank accession numbers, expression profile clustering, and expression heatmap data.

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