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**Quantitative proteomics to study the response of wheat to contrasting
fertilisation regimes**

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Abstract

Negative environmental impacts from mineral fertilisers and pesticides used in conventional cropping have raised concern over the sustainability of arable crop production. Organic cropping uses alternatives that avoid many of these negative environmental effects; however crop yields can be significantly reduced possibly due to a lower proportion of plant available nutrients. To gain insights into the molecular effects of organic compared to conventional cropping systems on plant utilisation of nutrients, we used proteomics to analyze winter wheat (*Triticum aestivum*). Our aim was to investigate the effects of contrasting fertility management and crop protection regimes in organic and conventional cropping systems on the wheat flag leaf proteome and the association between the proteome and physiological traits. Wheat flag leaves were flash frozen, lyophilized, and milled prior to protein extraction (TCA/acetone) and analyzed using 2D gel electrophoresis (DIGE) and MALDI-TOF MS. The abundance of 111 protein spots varied significantly between fertilisation regimes. Flag leaf N and P composition were significant drivers of differences in protein spot abundance, including major proteins involved in nitrogen remobilisation, photosynthesis, metabolism and stress response. These results indicate molecular based mechanisms involved in the effect of contrasting cropping systems on nutrient utilisation and wheat grain yield. Using a functional genomics approach, we were able to identify proteins that are linked to causal genes, enabling the potential development of functional molecular markers for crop improvement in nutrient use efficiency.

Key words: nutrient use efficiency; fertilisation regimes; proteomics; functional molecular markers; winter wheat; Rubisco degradation; glutamine synthetase.

Introduction

There is increasing concern over the dependence of agricultural food production on mineral fertiliser (especially N, P and K) and pesticide usage, because these inputs are associated with significant negative environmental impacts and reduce the sustainability of crop production systems (Tilman et al. 2002). The reliance of food production on mineral nitrogen (N) fertilisers is of concern because their manufacture and/or use is associated with high levels of energy use and greenhouse gas emissions (e.g. CO₂, N₂O), in addition to eutrophication of fresh water and marine ecosystems (Tilman et al. 2002; Robertson and Vitousek 2009). In Europe, 11 Mt reactive N (out of 91 Mt globally) is introduced into the environment as mineral fertiliser annually (EFMA 2007). Also, of the mined minerals used as mineral fertiliser, phosphorus is likely to be the first that will become unavailable since currently known deposits could be depleted within 30-100 years (Fantel et al. 1985; Trehan and Sharma 2005; Cordell et al. 2009). Therefore, there is clear need for either a reduction in applied mineral fertilisers, or a complete replacement with alternative fertilisers. Composted organic fertilisers are an alternative currently used in organic agriculture, which can have the same yield potentials (when applied at similar N P K input levels) as mineral fertilisers for some crops / cropping systems (Herencia et al. 2007; Hepperly et al. 2009; Warman et al. 2009). The application of compost fertilisers can lead to further advantages such as increased inherent fertility of soils when used repeatedly over many years (Herencia et al. 2007; Hepperly et al. 2009; Warman et al. 2009), reduced environmental impacts (reduced N leaching and P run off), and increased soil organic matter content, structural stability, biological activity and invertebrate biodiversity (Eyre et al. 2009; Mäder et al. 2002). However, current environmental regulations limit the total annual application of livestock manure per farm to an average of 170 kg N/ha per calendar year (The council of the European communities 1991). For some crops (e.g. winter wheat) this level of organic

fertiliser input is insufficient to achieve the same yield levels currently obtained with mineral fertilisers (Mäder et al. 2002) due to differences in the required timing of compost application, and the availability of nitrogen within compost. In six years of field trials, yield of organically grown wheat crops (cultivar ‘Malacca’) were on average 40% lower than conventional crops and this was in all years primarily, due to insufficient N-supply from organic fertiliser (composted manure) used in the organic system (Cooper et al. 2011; QLIF 2009). In contrast, there was no indication of P and K limitation in organically grown crops. However, less efficient crop protection protocols used in organic systems (in particular for foliar Septoria control) also significantly contributed to the yield differential. Therefore, winter wheat fertilised with composted organic waste could be nitrogen deficient compared to winter wheat grown under mineral fertiliser.

For a reduction in available nitrogen levels to be a viable option, crop varieties that can maintain/increase current yields at reduced levels of N supply i.e. with improved Nutrient Use Efficiency (NUE) are needed. In wheat, NUE depends on two major processes: nutrient uptake efficiency by roots (the proportion of available nutrients taken up by the crop), and nutrient utilisation efficiency (yield per unit nutrient taken up). The processes that influence utilisation efficiency include both nutrient transport into plant tissues and subsequent remobilisation out of tissues. Nutrient remobilisation occurs upon leaf senescence to remobilize nutrients into young, photosynthesizing tissue (Mae 2004; Kant et al. 2011). A particularly important stage affecting crop yield is the efficiency of nutrient remobilisation from senescing flag leaves (the uppermost wheat leaf) into developing grain tissue (Dawson et al. 2008; Barraclough et al. 2010; Howarth et al. 2008). It is this stage of nutrient remobilisation that is the focus of this study. We investigate the effect of conventional and organic cropping regimes (that encompass methods of fertilisation and crop protection) on the

protein profile of wheat flag leaves in order to observe the effect of these contrasting cropping methods on processes involved in nutrient remobilisation.

The analysis of the protein profile of plant tissue is an optimal method for quantifying changes in protein abundance caused by cropping systems. Proteomics is the study of gene products, which enables the observation of the products of gene expression that have a physiological effect on the plant. By identifying these proteins we can then link the protein back to the gene. In this way, candidate genes for agronomic traits can be identified, leading to the development of functional molecular markers for accelerating and assisting crop breeding practices (Varshney et al. 2005). Transcriptomics (the study of gene expression) has previously been used to directly identify genes (in developing wheat grain tissue) that are involved in nitrogen metabolism and storage protein synthesis, which are differentially expressed in response to organic and conventional fertilisers (Lu et al. 2005). The main advantage of using a proteomics approach is that proteomics allows the observation of post-transcriptional changes to gene products that would not be identified in the transcriptome, such as protein degradation involved in important plant physiological processes like nitrogen remobilisation. Previous studies using this approach include Nawrocki *et al.* (Nawrocki et al. 2011), who used proteomics to compare the effect of organic and conventional cropping systems on cabbage and carrot roots. They found significant differences in the root protein profiles of cabbage and carrot due to contrasting cropping systems. However, this study was not able to discern which factor within cropping systems (e.g. different fertility management or different crop protection practices) had caused the differences in protein profiles. In a similar comparison of organic and conventional cropping systems, Lehesranta *et al.* (2007) report that only fertility management (not crop protection regime) had a significant effect on the protein profile of potato tubers.

The objectives of the study presented here were to compare the effect of contrasting components (fertility management and crop protection regime) of organic and conventional cropping systems on i) agronomic / physiological traits (wheat grain yield, nutrient and chlorophyll content of flag leaves and crop Nutrient Use Efficiency); ii) the wheat flag leaf proteome at three plant growth stages; iii) the association between the flag leaf proteome and agronomic / physiological traits. This is a first step to identifying functional molecular markers for subsequent marker assisted breeding of wheat with enhanced nutrient use efficiency.

Materials and Methods

Field experimental design – An experiment comparing contrasting components (fertilisation and crop protection regimes) of organic and conventional cropping systems was established in 2001 on a field with a uniform sandy loam at the Nafferton Experimental Farm (University of Newcastle). For this study winter wheat seeds of the variety Malacca were sown in late October 2008, on plots that had grown winter wheat during the 2008 season, using a commercial drill. The experiment is a split-plot design with crop protection (two contrasting treatments) as the main plot factor and fertility management (two contrasting treatments) as the subplot factor. This design allowed the experiment to be analyzed using a 2 × 2 factorial design with identification of the effects of both fertility management and crop protection treatments. The four treatments were: (1) fertilisation with composted cattle manure and organic crop protection (i.e. manual weeding only); (2) fertilisation with composted cattle manure and conventional crop protection (use of herbicides and fungicides according to British Farm Assured practice); (3) Mineral NPK fertilisation and organic crop protection; (4) Mineral NPK fertilisation and conventional crop protection. All treatments were replicated four times, generating 16 plots in total. Each plot measured 12 x 24m. Seed

used in conventional crop protection plots were supplied by Horizon Seeds (www.horizon.com) and was produced under conventional seed production conditions and treated with standard seed pesticide treatments. Seed used in organic crop protection subplots were also supplied by Horizon Seeds and produced to organic seed production standards (Soil Association, Bristol, UK) and were untreated. Further details for fertilisation and crop protection application, timings and rates are provided in Online Resource 1. Additional information on the elemental composition of the composted cattle manure is provided in Online Resource 2.

Chlorophyll content measurements – The content of chlorophyll in flag leaves was estimated using a Spad meter (Konica Minolta) following the standard protocol as described in the product manual. The average of 10 readings was recorded for 4 areas of each plot, at two dates; 15/Jun/2009 and 29/Jun/2009. Spad meter readings have previously been shown to be positively correlated to chlorophyll content, as quantified by HPLC (Lombard et al. 2010; Uddling et al. 2007).

Wheat leaf sampling – Wheat flag leaves were sampled from each of the 16 plots at three physiological growth stages: inflorescence emergence (growth stage 50, 5/Jun/2009), anthesis (growth stage 62, 19/Jun/2009) and at grain milk development (growth stage 71, 3/Jul/2009) generating 48 samples in total. The whole flag leaf lamina, excluding the leaf sheath was collected. Growth stages were measured on the Zadoks scale (Zadoks et al. 1974). No effect of treatments on the growth stage of wheat could be detected (e.g. flowering occurred at the same time). However, visual signs of senescence occurred approximately 1 week earlier in flag leaves of compost fertilised plants compared to mineral fertilised plants. Approximately 100 – 200 flag leaves from each plot were collected and immediately flash

frozen. Leaves from each plot were then lyophilized, milled (Retsch ultra centrifugal mill, 0.2mm mesh screen) and stored at -80°C until required.

Analysis of minerals and heavy metals in soil and leaves– Five cores of soil (0 to 30 cm) were randomly sampled within each plot and immediately mixed to form one composite sample per plot prior to sowing (1st October 2008). Soils were analysed for pH analysis (1:1 in water), NO₃-N and NH₄-N, total organic C, N and Mehlich-3 extractable macro and micronutrients as explained by Orr et al. (2011).

Wheat grain, flag leaves and straw were analyzed for total N by Dumas combustion using a LECO TruSpec Automated C/N Analyzer (LECO Corporation, USA) according to the Manufacturer's instructions. Other nutrients and metal elements (P, K, S, Ca, Mg, Na, Fe, Mn, Cu, Zn, Mo, Cd, Al, B, Ni) were determined by subjecting the grain, flag leaves and straw to acid digestion (H₂O₂, HNO₃) in a closed-vessel microwave reaction system (MarsExpress; CEM Corp., Matthews, NC, USA) and analyzing with an inductively coupled argon plasma optical emission spectrometer equipped with a CCD detector (Vista-Pro Axial; Varian Pty Ltd, Mulgrave, Australia) as previously described (Cooper et al. 2011).

Yield and Nutrient Use Efficiency Assessment – Wheat grain was harvested using a plot combine harvester (Claas Dominator 38; Claas U.K Ltd, Bury St Edmunds, UK). Grain samples were dried (hot air drying using an MT motor fed through a 3m x 1.5m x 0.70m wooden box with a meshed surface) and then immediately cleaned (Lainchbury HC1/7W grain cleaner, Blair Engineering, Blairgowrie, UK). Several standard parameters of NUE were calculated: grain harvest index (grain biomass / total aboveground biomass, N harvest

index (grain N / total N uptake), total N uptake (total amount of N contained in plant tissues at grain harvest), and N utilisation efficiency (grain yield / N uptake).

Protein profiling –

Protein extraction and 2D Difference Gel Electrophoresis (DIGE) – Proteins were extracted from 1g of freeze dried flag leaf meal using TCA/acetone precipitation (Isaacson et al. 2006). TCA/acetone precipitation is routinely used to extract proteins when requiring whole proteome analysis. This technique is known to extract essentially all non-membrane bound proteins, and will also extract many of the membrane bound proteins. The protein pellet was resolubilised in DIGE sample buffer containing 7M Urea, 2M thiourea, and 2% CHAPs. Non-protein contaminants were removed from the samples using the Clean-Up kit (GE Healthcare), and subsequently resolubilised in DIGE sample buffer. The pH was adjusted to 8.5 using pH10 DIGE sample buffer, and protein quantified using the Quant kit (GE Healthcare). A total of 50µg of each protein sample was labelled with fluorescent CyDyes (GE Healthcare). Of the 48 samples, 24 were labelled with Cy3 and 24 labelled with Cy5. In addition, an internal standard composed of 25ug of each of the 48 samples, was labelled with Cy2. Two samples (sample 1 with cy3 and sample 2 with cy5) were combined, with 50µg of internal standard, to be run on 2-D gels, giving a total of 24 gel samples. Samples were assigned to a dye (Cy3 or Cy5) and to a gel using a fully randomized design, as recommended. Single gel analyses were made from each biological sample. Samples were initially run on 18cm pH3-10 non linear strips (GE Healthcare) followed by 12.5% 2-D acrylamide gels.

Image analysis and DIGE data analysis – Gels were scanned immediately after electrophoresis using a DIGE scanner (GE Healthcare). Three images were generated from each gel (sample 1, sample 2, and internal standard) giving 72 images, which were analyzed using Progenesis SameSpots (Non Linear Dynamics). Protein spot volumes for each sample were normalized to the volume of the corresponding spot for the internal standard on each gel to correct for any gel – gel variation. Overall, 219 distinct spots were matched across the DIGE gels and used in statistical analyses.

Protein identification – Protein spots that were significantly ($p < 0.01$) influenced by cropping system across all four biological replicates were selected for identification. Spots were manually picked from preparative gels using gel picking tools (Harris Uni-CoreTM, with 2mm and 0.75mm diameter). All methods for preparative gels corresponded to the method for DIGE analytical gels (as described above), with three exceptions: (i) 500ug protein was used for each preparative gel; (ii) 6ul of Mark12 unstained standard ladder (Invitrogen) was included, to allow interpretation of protein spot molecular mass; (iii) preparative gels were post fixed (10% methanol and 7.5% acetic acid) overnight, stained with coomassie blue G-250 overnight, and then washed (7.5% acetic acid) to remove background stain. Digest of spots with trypsin and protein identification of spots by MALDI-TOF mass spectrometry was conducted by NEPAF (Newcastle upon Tyne) according to Ferry et al 2011; the m/z range for the peptides was between 700 and 4500 Daltons. Peptide Mass Fingerprints (PMF) were matched to wheat EST databases (in house database; Ferry et al 2011) and Swissprot using a modified MASCOT search. The search parameters used carbamidomethyl (C) as a fixed modification, oxidation (M) as a variable modification, and 1 missed cleavage.

Statistical analyses – The effects of crop protection and fertility management on wheat grain yield, flag leaf nutrient composition and protein expression were assessed using ANOVA derived from linear mixed-effects models (Pinheiro and Bates 2000). Crop protection and fertility management were fixed factors in the models, and trial blocks and crop protection were random factors (Crawley 2007). An additional three way ANOVA was also carried out to assess whether the abundance of proteins changed significantly over the three sampled growth stages. Analyses were carried out using the nlme library in the R statistical environment (R Development Core Team 2009). Residual normality was assessed using the qqnorm function in R (Crawley 2007), with no data showing serious violations from normality.

The relationships of protein spots to leaf nutrients were investigated with redundancy analyses (RDA), using the CANOCO package (Ter Braak and Šmilauer 1998). Automatic forward selection of the leaf nutrients within RDA was used and their significance calculated using Monte Carlo permutation tests.

Results and Discussion

Effect of fertilisation and crop protection regimes on wheat grain yield, nutrient composition of flag leaves, and Nitrogen Use Efficiency parameters – Grain yields were significantly reduced in compost fertilised plots compared with those that received mineral fertilisation ($F=300.5$, $p<0.0001$, Online Resource 4). Wheat grain yield was also significantly lower when organic crop protection was used compared to conventional crop protection ($F=27.2$, $p<0.05$) and there was a significant interaction between fertilisation and

crop protection effects ($F=12.9$, $p<0.05$). Interestingly, this interaction effect showed that pesticides had a greater yield enhancing effect in mineral compared to compost fertilised treatments. These results indicate that a greater proportion of the observed reduction in wheat yield in organic compared to conventional cropping systems was mainly due to differences in fertilisation regimes (rather than the omission of pesticides). This could be due to limitations in nutrient supply from the organic fertiliser used in this study (composted cattle manure), which. may also be true of other studies comparing organic and conventional systems (Mäder et al. 2002).

The nutrient status of wheat flag leaves was significantly affected by both fertilisation and crop protection regimes (Online Resource 5A-D). Fertilisation regime significantly altered the composition of all nutrients except Mo (N, P, K, Ca, Cu, Zn, S, Mg, Na, Mn, B, Cd, Fe, Al, $p<0.01$). Compared to the conventional fertilisation regime, flag leaves of wheat grown under compost fertilisation had reduced levels of N, K, Ca, Cu, Zn, Mn, Cd (at inflorescence, anthesis and grain development), S, Mg, B (at inflorescence and anthesis), and Fe (at inflorescence), and increased levels of P, Al (at inflorescence, anthesis and grain development), and Na (at inflorescence and anthesis). Crop protection significantly affected the concentration of K, S, Cu, Zn, Mo and Ca ($p<0.05$) in flag leaves. Compared to conventional crop protection, wheat flag leaves grown under organic crop protection had significantly decreased levels of Ca (at inflorescence), and significantly increased levels of K (at inflorescence, anthesis and grain development), S, Ca, Cu, Zn, and Mo (at anthesis and grain development) (Tables S3A-S3D).

The higher N composition in mineral fertilised compared to compost fertilised wheat flag leaves corresponded with the N composition within grain and straw tissue at final harvest; the N composition of both tissues was significantly higher in mineral fertilised compared to compost fertilised wheat (Online Resource 6). This led to a significant

difference in the total N uptake ($p < 0.001$), which was 2.5x higher in mineral fertilised wheat compared to compost fertilised wheat. However, despite the higher uptake of N in mineral fertilised wheat, a larger proportion of N in the flag leaves and straw of compost fertilised wheat was remobilized into its' wheat grain compared to the N taken up in mineral fertilised plants. This resulted in significantly higher N utilisation efficiency ($p < 0.001$) and Nitrogen Harvest Index (NHI, $p < 0.5$) in compost fertilised wheat (87.4%) compared to mineral fertilised leaves (83.5%). These results are consistent with published studies that show a pattern of greater N utilisation efficiency in wheat fertilised with low N input compared to high N input (Barracclough et al. 2010). High NHI in N deficient plants can occur due to the earlier onset of senescence (Crafts-Brandner et al. 1998), compared to delayed senescence in plants with higher N availability. Although delayed senescence allows prolonged photosynthesis (leading to higher yield), it also reduces the nitrogen utilisation efficiency due to a shorter period of remobilisation, which consequently results in lower nitrogen use efficiency (Masclaux-Daubresse et al. 2010; Le Gouis et al. 2000).

Effect of crop management on protein expression in flag leaves – A total of 219

spots were matched across gel images and analyzed by ANOVA for the effects of fertilisation and crop protection regimes on protein expression. Contrasting fertilisation regimes resulted in significant differential expression of 111 protein spots ($p < 0.01$). Five of these protein spots also changed in response to crop protection regimes, but with a lower level of significance ($p < 0.05$). The expression of some of these 111 proteins spots were also affected by an interaction between fertilisation and crop protection regimes: 15 protein spots at the $p < 0.05$ level, and a further 2 proteins at the $p < 0.01$ level. The 111 protein spots differentially

expressed in response to fertilisation treatment were selected for protein identification by Peptide Mass Fingerprinting (PMF). Of these, 58 proteins could be identified from 55 2D gel spots (Table 1).

Proteins up-regulated in wheat flag leaves grown under compost fertilisation –

Twenty six proteins were identified that were significantly ($p < 0.01$) up-regulated in wheat grown under compost compared to mineral fertilisation (Table 1a, Supplementary Table S5). Eleven of these proteins were identified as the large subunit of Rubisco (protein spots 7, 12, 15, 17, 37, 39, 59, 83, 109, 130, 134). Most notable was the position of these protein spots on the 2D gel (Online Resource 8), signifying that the molecular weights of these proteins were approximately 31kDa (protein spots 12, 37, 39), 22kDa (protein spots 7, 134), 21.5 – 14.4kDa (protein spots 59, 83, 130), and 14.4 – 6kDa (protein spots 15, 17, 109). Furthermore, multiple spots were observed at the same molecular weight but with different isoelectric points, which indicates that there are chemical differences in the protein spots caused by post-translational modification. Rubisco is known to contain 8 large chain subunits of around 55 kDa each, and 8 small chain subunits of around 14 kDa each. Therefore, the proteins spots identified as Rubisco large subunits could be degradation products, since they are all under 55 kDa. The functional role of Rubisco is in the Calvin cycle of photosynthesis, however it is also known to act as a major nitrogen storage compound. Changes in the regulation of Rubisco synthesis and degradation are initiated upon leaf senescence (after full leaf expansion) (Irving and Robinson 2006). Rubisco degradation products are transported out of chloroplast cells and may undergo further degradation before being remobilized in nitrogen sink tissues such as developing wheat grains (Mae et al. 1983; Mae 2004; Chiba et al. 2003). However, it has been previously postulated that the visual presence of Rubisco degradation products on electrophoretic gels indicates an accumulation of these products

332 within chloroplasts due to a bottleneck in the transport process (Irving and Robinson 2006).
333 By analyzing the proteome over three growth stages, we were able to observe three different
334 patterns of expression in Rubisco over time. Rubisco protein spots 7, 12, and 39 significantly
335 increased in abundance during the three observed growth stages (i.e. inflorescence through to
336 grain milk development), which would support the suggestion of accumulation of Rubisco
337 degradation products. Rubisco protein spots 15, 17, 37, 44, 59, and 109 peaked in abundance
338 at anthesis, and decreased at grain development. This could be due to an initial accumulation,
339 followed by either transport out of the flag leaf tissue to the developing grain or further
340 degradation into smaller products prior to transport. Spot 83 decreased in abundance over all
341 three observed growth stages. It is not known whether the degradation products originate
342 from the same Rubisco large subunit, or whether they are from separate subunits. It may be
343 that degradation products with the same expression profiles originated from the same Rubisco
344 subunits, and differences in expression profiles are caused by differences in the rate of
345 degradation across the eight Rubisco large subunits. This may be possible, if differences in
346 their structural stability make some subunits more susceptible to proteolysis than others. It is
347 known that Rubisco degradation by active oxygen species oxidatively modify specific
348 cysteine residues within the Rubisco complex (Garcia-Ferris and Moreno 1994). Although
349 not fully understood, this oxidation process is likely to alter the susceptibility of affected
350 cysteine residues and associated amino acids to cysteine proteolysis (Thoenen et al. 2007).
351 Although the mechanisms involved in Rubisco degradation are largely unknown it is thought
352 that it may take place within specific vacuoles with high proteolytic activity involving
353 multiple proteases (Martínez et al. 2008). Information on the peptides present in Rubisco
354 subunits in the present study indicates that they are degradation products of different subunits
355 (Online Resource 9).

Also up-regulated in compost fertilised (compared to mineral fertilised) flag leaves were a small subunit of Rubisco (protein spot 44), and a further 8 protein spots identified as Rubisco activase isoform 1. These proteins are all involved in the Calvin cycle of photosynthesis, as is glyceraldehyde-3-phosphate dehydrogenase A, which was also found to be up-regulated in these leaves. Two protein spots of serine hydroxymethyltransferase, known for its role in metabolic reactions within photorespiration and implicated in functioning to control cell damage (caused by reactive oxygen species) by abiotic stresses (Moreno et al. 2005), were shown to be up-regulated at anthesis. Lipoxygenase 2, a protein which is associated with senescing tissues and plays a role in cell wall degradation (Siedow 1991), was also up-regulated at anthesis.

Proteins up-regulated in wheat flag leaves grown under mineral fertilisation –

Thirty one proteins were significantly up-regulated in flag leaves of wheat grown under mineral fertilisation when compared to compost fertilised flag leaves (Table 1b). Three of these proteins spots were identified as Rubisco large subunit (protein spot 2 of approximately 55kDa, and protein spots 75 and 131 of between 31 – 21.5kDa). A further four protein spots were identified as Rubisco small subunit (protein spots 194 of 36.5kDa, 213 of 31 – 21.5kDa, 174 and 175 of 14kDa). These molecular masses are consistent with those previously reported for the 55kDa Rubisco large subunit and the 14kDa small subunits, which indicates that these are intact functioning subunits (Schneider et al. 1992). Therefore their up-regulation in mineral fertilised leaves suggest that these leaves have greater photosynthetic activity compared to compost fertilised plants. This would agree with the measurements of chlorophyll content, which showed that flag leaves of conventionally fertilised plants had a significantly greater chlorophyll content ($p < 0.0001$, Online Resource 10) and therefore photosynthetic capacity. These results give further evidence that the higher nitrogen levels in

the mineral fertilisation regime resulted in delayed senescence and prolonged photosynthesis leading to increased grain yield, and a lower proportion of N remobilized to the grain. A reduction in N remobilisation efficiency could be a contributing factor to the reduction in nutrient use efficiency under high nitrogen fertilisation. The up-regulation of two Rubisco large subunits with 21.5 – 31kDa mass indicates that Rubisco degradation was also occurring in mineral fertilised flag leaves at the three observed growth stages. The up-regulation of different Rubisco degradation products in the contrasting fertilisation regimes could simply be due to differences in the onset, and therefore progression of senescence, or due to a more complex difference in the Rubisco degradation process, such as the expression of different proteolytic enzymes caused by different nutrient levels (Thoenen et al. 2007; Martínez et al. 2008).

Most of the identified Rubisco protein spots (except for spot 194) significantly changed in expression across growth stage ($p < 0.05$), or an interaction between growth stage and fertilisation regime could be detected. The abundance of Rubisco large subunits (protein spots 2, 97, and 131) and one Rubisco small subunit (protein spot 194) increased over time, a further two Rubisco small subunits (protein spots 174, 175) decreased (Fig. 1) and Rubisco small subunit spot number 213 peaked at anthesis. Changes in these expression profiles could be due to a combination of continual low level biosynthesis of Rubisco that is known to occur during senescence (Irving and Robinson 2006; Mae et al. 1983; Suzuki et al. 2001) and differential degradation of Rubisco subunits as observed in compost fertilised leaves. The up-regulation of catalase in mineral fertilised leaves may be significant in terms of its possible role in regulating the activity of active oxygen species, which contribute to the degradation of Rubisco (Garcia-Ferris and Moreno 1994). Alternatively, catalase could have been up-regulated in response to the up-regulation of NADH dehydrogenase subunit I (has a role in oxidative phosphorylation – a metabolic pathway) in these mineral fertilised leaves.

Glutamine synthetase (isoform GS2c), which has a role in nitrogen metabolism and remobilisation, was up-regulated in flag leaves of mineral fertilised wheat (compared to compost fertilised wheat) at anthesis. Protein degradation is known to release ammonia as well as amino acids (Kant et al. 2011), which can be assimilated by cytosolic glutamine synthetase in senescing leaves for nitrogen remobilisation to the grain (Bernard et al. 2008) and levels of flag leaf ammonia content and glutamine synthetase are positively correlated (Fontaine et al. 2009). The up-regulation of glutamine synthetase in mineral fertilised plants compared to compost fertilised plants was positively associated with leaf nitrogen concentrations. This indicates that glutamine synthetase was more abundant in mineral fertilised plants compared to compost fertilised plants due to higher N uptake in mineral fertilised plants, possibly due to greater plant availability of N (supplied as mineral N).

Other proteins that were found to be up-regulated in mineral fertilised plants included Rubisco activase isoform 1, which is associated with photosynthetic activity via its' interaction with Rubisco (Portis 2003). The occurrence of several spots of the same proteins may be due to the hexaploid nature of the wheat genome, with copies of the protein contributed from each of the three genomes (Donnelly et al. 2005). Several other proteins that were up-regulated in mineral fertilised leaves are also known for their role in photosynthesis such as oxygen-evolving enhancer protein 1 and 2, putative oxygen-evolving complex precursor, cytochrome b6-f complex iron-sulfur subunit and dehydroascorbate reductase. The latter protein is involved in the maintenance of photosynthesis due to its role in the regeneration of ascorbic acid (Chen and Gallie 2008).

Associations between nutrient composition and protein expression in flag leaves –

We conducted multivariate analyses to investigate the association between nutrient composition in flag leaves (as an indication of nutrient supply/availability), and protein abundance at the corresponding growth stages (Fig. 2-4). Axis 1 in the analysis accounted for 72% of variation in the nutrient and proteomics data at all three growth stages, with a further 10-17% accounted for by axis 2 (Table 2). Phosphorus was a main driver for Rubisco regulation in all three growth stages (Fig. 2a-c). Phosphorus concentration in flag leaves associated positively with the abundance of Rubisco subunits that were up-regulated in compost fertilised leaves, and negatively with Rubisco subunits that were up-regulated in mineral fertilised leaves. This indicates that the higher relative phosphorus supply to compost fertilised plants was associated with up-regulation of some Rubisco subunits. Previous studies indicate that phosphorus has a role in regulating the uptake of nitrogen, and the level of nitrogen stored as Rubisco (Warren and Adams 2002; Osaki et al. 1993). Nitrogen was a strong driver of the expression of the Rubisco large and small subunits that were up-regulated in leaves grown under mineral fertilisation (Fig. 2b,c).

In Fig. 3a-c we present the association between nutrient composition and identified proteins other than those involved in nitrogen metabolism, for each of the three growth stages. Two clusters formed on each plot, representing positive associations between nutrients and proteins up-regulated in either mineral fertilised flag leaves, or up-regulated in compost fertilised leaves. The abundance of protein spots that were not identified (Fig. 4a-c) also formed separate clusters depending on whether they were up-regulated in the compost or mineral fertilised plants. Together, the data presented in figures 3 and 4 shows that there is a strong relationship between nutrient availability and protein expression in the wheat flag leaf at each of the three observed growth stages.

Conclusion

The overall aim of this research was to investigate the differential effect of conventional and organic cropping systems on the wheat flag leaf proteome and the association between differences in the flag leaf proteome and agronomic / physiological traits (wheat grain yield, nutrient and chlorophyll content in flag leaves and Nutrient Use Efficiency parameters). We found that the most significant changes to the proteome were due to the contrasting fertilisation regimes rather than the contrasting crop protection regimes. This is similar to previous studies on the effects of organic and conventional cropping systems on the potato tuber proteome (Lehesranta et al. 2007). Differences in the proteome may have been due to either the different levels of individual nutrients available to wheat, or the different types of fertilisation input. The organic fertilisation regime (composted cattle manure) led to lower N uptake compared to the convention fertilisation regime (mineral NPK). The association between nutrient composition and the abundance of proteins (e.g. those involved in photosynthesis and metabolism) indicated an earlier onset of senescence in cattle manure fertilised leaves compared to conventional fertilised leaves. Although an earlier onset of senescence shortens the period of photosynthesis, it allows a longer period of N remobilisation from flag leaves to developing grains, resulting in higher Nitrogen utilisation efficiency as observed in this study and others (Kant et al. 2011; Mae 2004). Increasing nitrogen utilisation efficiency is one approach to improve crop nitrogen use efficiency. This work has improved our understanding of how contrasting fertilisation regimes affect nitrogen utilisation efficiency through their impacts on nitrogen remobilisation from the leaves to the grain. In this study, plant response to contrasting fertilisation regimes was studied in a single wheat genotype in a single year to initially demonstrate a cause and effect response of contrasting crop management regimes. The data presented here demonstrates our ability to identify a molecular response to fertilization that is associated with genes involved in NUE.

However, this study does not enable extrapolation to other wheat varieties and neither does it enable generalisations in the response of wheat to contrasting fertilisation regimes to be made at this stage. Further steps will involve testing multiple wheat varieties to look for genetic variability in nitrogen utilisation efficiencies, and to investigate how genotype interacts with nitrogen source (organic or mineral) and rate to affect key NUE parameters including N utilisation efficiency. Using a proteomics approach, the genomic pathways controlling agronomic traits such as nutrient use efficiency can be linked to the respective encoding genes to enable the development of functional molecular markers for accelerating and assisting future crop breeding programmes (Varshney et al. 2005). However, the use of such tools for increasing the efficiency and precision of crop improvement are still in their infancy and caution should be used when interpreting data sets to identify potential candidate genes.

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Tables

Table 1: Proteins up-regulated in flag leaves of wheat in response to (A) compost fertilisation, (B) mineral fertilisation, at three growth stages: 1, inflorescence; 2, anthesis; 3, grain milk development. Additional data for identification scores and sequence coverage is provided in Online Resource 7.

Table 1A

Spot ID	Similar protein name	Function / metabolism	Fold increase of proteins ¹		
			1	2	3
7	RuBisCo large subunit	Calvin cycle	1.13	2.41*	3.72**
12	RuBisCo large subunit	Calvin cycle	0.62	1.45	3.21***
15	RuBisCo large subunit	Calvin cycle	1.72**	4.46**	3.60**
17	RuBisCo large subunit	Calvin cycle	1.45	4.34**	3.54**
27	Lipoxygenase 2	Senescence	1.86*	1.92**	1.58*
37	RuBisCo large subunit	Calvin cycle	1.04	2.38*	3.09***
39	RuBisCo large subunit	Calvin cycle	0.70	1.52	3.27**
40	RuBisCo activase isoform 1	Rubisco activation	1.56	1.78**	1.77*
44	RuBisCo small subunit ^{2, 3}	Calvin cycle	2.24**	2.97**	1.67**

59	RuBisCo large subunit ³	Calvin cycle	1.80**	2.79**	1.99*
69	Rubisco activase A	Rubisco activation	1.42	2.30**	1.88**
73	Unknown		2.00**	1.82***	2.19**
83	RuBisCo large subunit	Calvin cycle	2.64**	2.21**	1.68***
84	RuBisCo activase isoform 1	Rubisco activation	2.06*	2.60**	1.59***
86	Serine hydroxymethyltransferase	Photorespiration; metabolic reactions	1.87*	2.07***	1.80*
101	RuBisCo activase A	Rubisco activation	1.95*	2.17**	1.39*
109	RuBisCo large subunit	Calvin cycle	1.31	1.95**	1.42*
125	Glyceraldehyde-3-phosphate dehydrogenase A ³	Calvin cycle	1.15,	1.92**	1.41
129	RuBisCo activase isoform 1	Rubisco activation	1.67*	1.85**	1.18
130	RuBisCo large subunit ^{2, 3}	Calvin cycle	1.42**	1.98*	1.37
134	RuBisCo large subunit	Calvin cycle	1.84*	1.73**	1.87*
138	RuBisCo activase isoform 1	Rubisco activation	1.50	1.54*	1.30**
144	RuBisCo activase	Rubisco activation	1.82*	1.69**	1.19*
147	RuBisCo activase isoform 1 ²	Rubisco activation	1.11	1.72***	1.35
163	Serine hydroxymethyltransferase	Photorespiration; metabolic reactions	1.23	1.54**	1.14
166	RuBisCo activase isoform 1 ³	Rubisco activation	1.44*	1.35**	0.94

Table 1B

Spot ID	Similar protein name	Function / metabolism	Fold increase of proteins ¹		
			1	2	3
2	RuBisCo large subunit	Calvin cycle	2.87	3.66*	1.78
14	Mitochondrial aldehyde dehydrogenase	Acetaldehyde oxidation	1.82	2.73***	1.87*
19	Oxygen-evolving enhancer protein 1	Photosystem II	2.45***	4.45***	2.73***
28	Beta-1,3-glucanase mRNA	PR protein / Senescence	1.14	1.54*	3.04***
34	Catalase-1	Stress response	1.62	3.05***	1.52*
54	Oxygen-evolving enhancer protein 1	Photosystem II	2.85***	3.33***	1.51*
62	Cytochrome b6-f complex iron-sulfur subunit	Photosynthesis	1.01	1.49*	1.67**
68	Catalase-1	Stress response	1.63	2.73***	1.52**
71	Oxygen-evolving enhancer protein 1	Photosystem II	1.88**	3.03***	2.10***
76	Putative oxygen-evolving complex precursor ³	Photosystem II	1.76**	2.76**	1.67**
82	O-methyltransferase ZRP4	Cellular metabolism	1.72	2.16**	1.12
90	Oxygen-evolving enhancer protein 2	Photosystem II	1.20	1.38**	1.35*
97	RuBisCo large subunit	Calvin cycle	1.76*	1.86**	1.01
118	Cu/Zn superoxide dismutase	Oxidative stress protective system	0.79	1.11	1.53***

131	RuBisCo large subunit	Calvin cycle	1.72	1.99***	1.03
143	Putative oxygen-evolving complex precursor	Photosystem II	1.37	2.00**	1.49**
149	RuBisCo activase isoform 1	Rubisco activation	1.32*	1.60***	1.38***
159	RuBisCo activase isoform 1 ³	Rubisco activation	1.19*	1.74*	1.40**
164	RuBisCo activase isoform 1	Rubisco activation	1.56*	1.51*	1.39**
169	Peptidylprolyl isomerase (cyclophilin-like protein)	Protein folding	1.27*	1.33*	1.36**
174	RuBisCo small subunit	Rubisco activation	0.94	1.30*	1.32***
175	RuBisCo small subunit	Rubisco activation	0.99	1.18	1.25***
189	RuBisCo activase isoform 1	Rubisco activation	1.36	1.69***	1.41**
190	RuBisCo activase isoform 1	Rubisco activation	1.27	1.75***	1.47**
193	RuBisCo activase isoform 1	Rubisco activation	1.24	1.66***	1.42**
194	RuBisCo small subunit	Calvin cycle	1.17	1.64*	1.55**
213	Mixture:		1.04	1.54**	1.21*
	RuBisCo small subunit	Calvin cycle			
	Dehydroascorbate reductase	Maintenance of photosynthetic function			
215	Mixture:		1.39**	1.49***	1.20*
	Oxygen-evolving enhancer protein 2	Photosystem II			

	NADH dehydrogenase subunit I	Oxidative phosphorylation			
216	Mixture:		1.16	1.49**	1.21
	Plastid glutamine synthetase isoform GS2c	N metabolism			
	RuBisCo activase isoform 1	Rubisco activation			

Notes:

¹ Asterix refer to the level of significance as tested in ANOVA (*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.000$).

²Proteins whose volume was significantly ($p<0.05$) higher in flag leaves of wheat not treated with conventional crop protection. Significant fold increases at inflorescence: protein spot 44, 1.63; protein spot 130, 1.39. Significant fold increases at anthesis: protein spot 147, 1.29.

³Proteins whose volume was influenced by an interaction effect (fertilisation x crop protection). See Online Resource 12 for mean data illustrating interaction effect.

633 **Online Resource legends**

634 Online Resource 1: Field trial agronomic management.

635 Online Resource 2: Nutrient analysis of the composted cattle manure, with estimated amount
636 applied.

637 Online Resource 3: Soil quality and nutrient status of field plots prior to fertilisation.

638 Online Resource 4: Values for ANOVA for grain yield across contrasting fertilisation and
639 crop management regimes. Letters above the columns refer to Tukey test for significant
640 differences between means ($p < 0.05$).

641 Online Resource 5A-D: Means and ANOVA data for nutrient analysis of wheat flag leaves
642 sampled at three growth stages: 1, inflorescence; 2, anthesis; 3, grain milk development.
643 Values for ANOVA data show F-values followed by asterix denoting p-levels: $*=p < 0.05$,
644 $**=p < 0.01$, $***=p < .001$; ns = non significant.

645 Online Resource 6: Means and ANOVA of Nutrient Use Efficiency (NUE) parameters
646 calculated for wheat grown under contrasting fertilisation and crop protection management:
647 Grain Harvest Index (GHI), Nitrogen Harvest Index (NHI), Nitrogen Uptake and Nitrogen
648 Utilisation Efficiency (NUE). Values for ANOVA data show F-values followed by asterix
649 denoting p-levels: $*=p < 0.05$, $**=p < 0.01$, $***=p < .001$; ns = non significant.

650 Online Resource 7: Data on protein identification search with wheat EST databases and
651 Swissprot.

652 Online Resource 8: Image of the DIGE reference gel (pH3-10 non-linear). The red arrows
653 point to Rubisco and glutamine synthetase protein spots that were up-regulated in flag leaves

of compost and mineral (bold) fertilised wheat. The numbers on the right indicate the molecular mass.

Online Resource 9: Sequence coverage information for spots identified as Rubisco large and small subunits. Matched peptides are shown in bold red.

Online Resource 10: Means and ANOVA data for wheat flag leaf chlorophyll content, measured at two dates with a Spad meter.

Online Resource 11: Multivariate analysis of Nutrient and Proteomics data for flag leaves sampled at three growth stages (1, inflorescence; 2, anthesis; 3, grain milk development.

Online Resource 12: Means and ANOVA data for volume of protein spots that were affected by the interaction effect. Values for ANOVA data show F-values followed by asterix denoting p-levels: $\ast = p < 0.05$, $\ast\ast = p < 0.01$, $\ast\ast\ast = p < .001$; ns = non significant. These values are for data at inflorescence, except for protein spot 166 that is for anthesis.

Figure legends

Figure 1: Change in abundance of Rubisco in (A) compost fertilised flag leaves and (B) mineral fertilised flag leaves at three growth stages. For most protein spots, growth stage was significant ($p < 0.05$) as a main effect and / or as an interaction effect (growth stage x fertilisation). In figure 1A, the volume of spots 130 and 134 were not significantly affected by either growth stage or the interaction effect. In figure 1B, the volume of spot 194 was not significantly affected by growth stage.

Figure 2: Multivariate analysis of nutrient data and regulation of Rubisco subunits at (A) inflorescence, (B) anthesis and (C) grain milk development. Proteins circled with a solid line

676 were up-regulated in leaves of compost fertilised wheat. Proteins circled with a dashed line
677 were up-regulated in leaves of mineral fertilised wheat.

678 Figure 3: Multivariate analysis of nutrient data and regulation of all other identified proteins
679 at (A) inflorescence, (B) anthesis and (C) grain milk development. Proteins circled with a
680 solid line were up-regulated in leaves of compost fertilised wheat. Proteins circled with a
681 dashed line were up-regulated in leaves of mineral fertilised wheat.

682 Figure 4: Multivariate analysis of nutrient data and regulation of all non identified proteins at
683 (A) inflorescence, (B) anthesis and (C) grain milk development. Proteins circled with a solid
684 line were up-regulated in leaves of compost fertilised wheat. Proteins circled with a dashed
685 line were up-regulated in leaves of mineral fertilised wheat.

686

Figure 1

Figure 2

Figure 3

Figure 4

Quantitative proteomics to study the response of wheat to contrasting fertilisation regimes

Journal: Molecular Breeding

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Online Resource 1: Field trial agronomic management

Date	Plots	Details
2 nd Oct 2008	Organic fertilisation	Application of composted cattle manure (at a rate of 170kg N ha ⁻¹). See Table S2 for elemental composition.
9 th Oct 2008	All	Wheat seeds (Malacca) drilled at 400 seeds m ⁻²
21 st Nov 2008	Conventional fertilisation	Fertilised with phosphorus (64 kg P ₂ O ₅ ha ⁻¹) and Potassium (96 kg K ₂ O ha ⁻¹) as 0:20:30
24 th Feb 2009	Conventional crop protection	Application of herbicides (IPU, 2.5 l ha ⁻¹ and Pendimethalin, 2 l ha ⁻¹) in 200 l water
13 th Mar 2009	Conventional fertilisation	Fertilised with “Nitram” Nitrogen (80kg N ha ⁻¹)
16 th , 25 th , 31 st Mar and 17 th Apr 2009	Organic crop protection	Weeding with Einbock tined weeder
20 th Apr 2009	Conventional crop protection	Application of herbicide (Fluroxypyr, 0.75l ha ⁻¹) and Optica (1.5l ha ⁻¹) in 200l water
	Conventional fertilisation	Fertilised with “Nitram” Nitrogen (130kg N ha ⁻¹)
6 th May 2009	Conventional crop protection	Application of fungicide (Bravo, 1l ha ⁻¹ ; Proline, 0.4l

		ha ⁻¹ ; Justice, 0.125l ha ⁻¹) and growth regulator
		(Agriguard chlormequat, 2l ha ⁻¹) in 200l water
1 st Jun 2009	Conventional crop protection	Application of fungicide (Bravo, 1l ha ⁻¹ ; Opus, 0.6l ha ⁻¹ ; Comet, 0.3l ha ⁻¹) in 200l water
25 th Aug 2009	All	Grain harvested with combine harvester

Online Resource 2: Nutrient analysis of the composted cattle manure, with estimated amount applied

Element	Composition in composted cattle manure	Estimated amount applied in 5.2 t dry compost / ha (kg ha ⁻¹)
N	3.24%	170
P	0.906%	48
K	2.78%	146
C	38.3%	2010
Ca	2.06%	108
Mg	0.8%	42
S	0.686%	36
Na	0.507%	27
Fe	1270 mg/kg	6.7
Al	733 mg/kg	3.8
Mn	360 mg/kg	1.9
Zn	218 mg/kg	1.1
Cu	66.1 mg/kg	0.35
B	27.2 mg/kg	0.14
Ni	3.30 mg/kg	0.017

Mo	3.27 mg/kg	0.017
Pb	2.52 mg/kg	0.013
Cd	0.434 mg/kg	0.0023

Online Resource 3: Soil quality and nutrient status of field plots prior to fertilisation

Factor	pH	N (mg/g)	C (%)	C:N ratio	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	S (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Fe (mg/kg)
Organic CP											
Compost Fertilized	6.86	2.46	2.79	11.41	43.56	53.19	2138.97	13.31	213.2	19.79	352.38
	±0.07	±0.09	±0.06	±0.67	±4.06	±6.49	±96.08	±0.76	±10.62	±1.23	±30.18
Mineral Fertilized	6.51	2.51	2.85	11.39	47.45	49.55	2010.66	13.75	220.15	23.21	374.17
	±0.12	±0.09	±0.04	±0.59	±5.21	±4.53	±70.73	±0.53	±11.65	±7.86	±47.08
Conventional CP											
Compost Fertilized	6.85	2.53	2.84	11.27	39.29	46.11	2115.21	12.89	207.23	18.19	354.1
	±0.04	±0.16	±0.1	±0.58	±6.41	±5.07	±86.30	±0.49	±4.78	±1.10	±34.57
Mineral Fertilized	6.34	2.5	2.85	11.42	48.23	46.97	1991.84	13.27	206.87	15.003	357.32
	±0.06	±0.08	±0.06	±0.53	±6.66	±3.58	±41.03	±0.14	±4.12	±1.69	±37.66

Online Resource 4

Online resource 5A-D: Means and ANOVA data for nutrient analysis of wheat flag leaves sampled at three growth stages: 1, inflorescence; 2, anthesis; 3, grain milk development. Values for ANOVA data show F-values followed by asterix denoting p-levels: *=p<0.05, **=p<0.01, ***=p<.001; ns = non significant.

A	N (%)			P (%)			K (%)			S (%)		
Factor	1	2	3	1	2	3	1	2	3	1	2	3
Means (± SE)												
Conventional CP												
Mineral fertilised	4.27	4.07	3.21	0.283	0.282	0.239	1.27	1.23	1.02	0.344	0.358	0.332
	±0.045	±0.054	±0.075	±0.007	±0.013	±0.010	±0.078	±0.065	±0.035	±0.007	±0.012	±0.012
Compost fertilised	2.97	2.80	2.17	0.296	0.397	0.386	1.07	0.93	0.68	0.285	0.323	0.320
	±0.048	±0.047	±0.056	±0.012	±0.021	±0.024	±0.059	±0.045	±0.033	±0.006	±0.006	±0.010
Organic CP												

Mineral fertilised	4.38	4.01	3.26	0.296	0.312	0.246	1.39	1.36	1.12	0.357	0.405	0.364
	±0.116	±0.282	±0.054	±0.003	±0.006	±0.003	±0.040	±0.021	±0.065	±0.012	±0.006	±0.014
Compost fertilised	3.26	3.24	2.33	0.324	0.449	0.450	1.29	1.18	0.80	0.313	0.392	0.394
	±0.138	±0.397	±0.024	±0.003	±0.022	±0.035	±0.032	±0.027	±0.041	±0.005	±0.009	±0.013
ANOVA												
Main effects												
Fertilisation (FE)	158.5,	17.2,	467.0,	36.7,	171.4,	102.5,	33.3,	208.2,	409.2,	69.6,	17.7,	ns
	***	**	***	**	***	***	**	***	***	***	**	
Crop protection (CP)	ns	ns	ns	ns	ns	ns	20.9, *	24.1, *	14.8, *	ns	31.3, *	15.4, *
2-way interactions												
FE x CP	ns	ns	ns	ns	ns	ns	ns	14.6, **	ns	ns	ns	ns

B	Ca (%)			Na (mg kg⁻¹)			Mg (%)			Fe (mg kg⁻¹)		
Factor	1	2	3	1	2	3	1	2	3	1	2	3
Means (± SE)												
Conventional CP												
Mineral fertilised	0.925	1.116	1.25	33.8	42.2	29.3	0.265	0.350	0.401	108.5	82.1	79.9
	±0.065	±0.058	±0.117	±7.13	±6.58	±1.91	±0.015	±0.013	±0.020	±11.82	±4.00	±6.43
Compost fertilised	0.619	0.791	0.91	52.0	56.3	35.2	0.217	0.326	0.377	82.3	87.2	80.5
	±0.072	±0.062	±0.075	±8.31	±7.51	±6.72	±0.013	±0.013	±0.018	±2.10	±22.21	±3.35
Organic CP												
Mineral fertilised	0.872	1.110	1.19	27.1	47.0	38.1	0.260	0.366	0.419	100.1	80.1	77.6
	±0.061	±0.067	±0.083	±4.20	±3.67	±11.47	±0.016	±0.017	±0.016	±1.77	±2.26	±2.22

Compost fertilised	0.539	0.738	0.85	30.3	63.3	41.1	0.209	0.340	0.421	75.1	71.1	81.0
	±0.061	±0.053	±0.082	±3.38	±1.78	±10.53	±0.012	±0.010	±0.015	±3.05	±4.77	±2.82
ANOVA												
Main effects												
Fertilisation (FE)	294.6,	98.4,	93.2,	55.1,	28.7,	ns	61.3,	7.1, *	ns	19.4,	ns	ns
	***	***	***	***	***		***			**		
Crop protection (CP)	12.8, *	ns	ns	20.9, *	24.1, *	14.8, *	ns	ns	ns	ns	ns	ns
2-way interactions												
FE x CP	ns	ns	ns	26.7**	ns	ns	ns	ns	ns	ns	ns	ns

C	Cu (mg kg ⁻¹)			Zn (mg kg ⁻¹)			Mn (mg kg ⁻¹)			Mo (mg kg ⁻¹)		
Factor	1	2	3	1	2	3	1	2	3	1	2	3
Means (± SE)												
Conventional CP												
Mineral fertilised	7.27	6.86	5.25	18.5	15.7	12.2	34.4	39.4	45.6	0.783	0.82	0.92
	±0.119	±0.137	±0.157	±0.663	±0.458	±0.167	±3.14	±5.37	±1.56	±0.226	±0.08	±0.182
Compost fertilised	5.87	5.55	4.21	13.9	11.1	8.1	20.1	18.4	15.9	0.665	0.85	0.75
	±0.129	±0.140	±0.088	±0.528	±0.301	±0.099	±0.93	±1.18	±1.48	±0.108	±0.11	±0.103
Organic CP												
Mineral fertilised	7.49	7.96	6.44	19.5	18.6	13.6	48.4	54.4	52.9	0.940	1.11	1.18
	±0.279	±0.250	±0.242	±0.292	±0.519	±0.201	±4.11	±5.44	±8.54	±0.137	±0.18	±0.176

Compost fertilised	6.21	6.19	5.47	13.2	11.6	9.4	21.8	20.8	18.1	0.970	1.34	1.25
	±0.249	±0.082	±0.130	±0.482	±0.111	±0.125	±0.50	±1.53	±1.95	±0.066	±0.16	±0.143
ANOVA												
Main effects												
Fertilisation (FE)	128.6,	303.9,	109.6,	114.4,	232.3,	993.8,	74.1,	47.8,	51.1,	ns	ns	ns
	***	***	***	***	***	***	**	***	***			
Crop protection (CP)	ns	23.5, *	113.9,	ns	20.0, *	90.1, **	ns	ns	ns	ns	16.2, *	22.2, *

2-way interactions												
FE x CP	ns	6.5, *	ns	ns	9.1, *	ns	6.6, *	ns	ns	ns	ns	ns

D	B (mg kg ⁻¹)			Al (mg kg ⁻¹)			Cd (µg kg ⁻¹)			Ni (µg kg ⁻¹)		
Factor	1	2	3	1	2	3	1	2	3	1	2	3
Means (± SE)												
Conventional CP												
Mineral fertilised	11.0	6.92	5.05	34.1	19.9	23.5	33.6	52.9	85.6	63.8	23.1	74.7
	±0.52	±0.18	±0.251	±5.59	±1.15	±2.37	±10.40	±5.12	±8.09	±13.4	±12.21	±7.72
Compost fertilised	8.6	4.96	4.44	36.3	18.7	32.8	14.0	26.7	11.1	91.5	28.1	52.7
	±1.08	±0.37	±0.362	±3.60	±0.72	±4.36	±5.81	±4.89	±5.40	±30.9	±9.57	±9.28
Organic CP												
Mineral fertilised	10.9	6.65	5.14	26.4	15.3	22.9	41.4	52.4	80.1	39.9	32.0	46.3
	±0.65	±0.29	±0.298	±1.82	±0.85	±1.44	±4.12	±3.71	±15.58	±4.9	±8.22	±15.03

Compost fertilised	8.5	5.64	5.21	31.8	18.6	27.6	11.8	23.2	24.6	31.8	13.4	51.1
	±0.84	±0.44	±0.449	±1.12	±1.81	±1.25	±3.33	±3.67	±7.81	±14.8	±4.36	±8.64
ANOVA												
Main effects												
Fertilisation (Fe)	29.8,	30.2,	ns	ns	ns	35.6,	14.3,	54.7,	76.3,	ns	ns	ns
	***	***				**	**	***	**			
Crop protection (CP)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
2-way interactions												
Fe x CP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Online Resource 6: Means and ANOVA of Nutrient Use Efficiency (NUE) parameters calculated for wheat grown under contrasting fertilisation and crop protection management: Grain Harvest Index (GHI), Nitrogen Harvest Index (NHI), Nitrogen Uptake and Nitrogen Utilization Efficiency (NUtE). Values for ANOVA data show F-values followed by asterix denoting p-levels: *=p<0.05, **=p<0.01, ***=p<.001; ns = non significant.

Factor	Grain dry wt (t/ha)	Straw dry wt (t/ha)	GHI	Grain N (%)	Straw N (%)	NHI	N Uptake (Kg/ha)	NUtE (kg grain/kg N uptake)
Means (± SE)								
Compost fertilised	4.23 ± 0.29	2.70 ± 0.24	52.75 ± 1.82	1.45 ± 0.02	0.35 ± 0.01	0.87 ± 0.01	70.86 ± 4.51	59.63 ± 1.36
Mineral fertilised	7.75 ± 0.43	5.01 ± 0.32	54.65 ± 1.73	2.00 ± 0.06	0.58 ± 0.03	0.84 ± 0.01	182.36 ± 4.95	42.42 ± 1.88
Organic CP	5.29 ± 0.55	4.03 ± 0.46	49.67 ± 1.12	1.81 ± 0.12	0.49 ± 0.05	0.83 ± 0.01	120.92 ± 20.11	47.76 ± 3.48
Conventional CP	6.69 ± 0.84	3.68 ± 0.56	57.74 ± 0.79	1.65 ± 0.10	0.44 ± 0.04	0.88 ± 0.01	132.30 ± 22.78	54.29 ± 3.37
ANOVA								
Main effects								
Fertilisation (Fe)	300.5, ***	62.2, ***	ns	164.0, ***	57.4, ***	6.2, *	629.3, ***	155.1, ***
Crop protection (CP)	27.2, *	ns	38.7, **	12.7, *	ns	ns	ns	17.4, *

2-way interactions								
Fe x CP	12.9, *	ns	ns	ns	ns	ns	ns	ns

Online Resource 7: Data on protein identification search with wheat EST databases and Swissprot

Spot				Number of matching peptides	Number of searched peaks	Sequence coverage (%)	Score	Expect	Database searched
7	gi 32966580 AAP92166.1	Rubisco large chain	<i>Triticum aestivum</i>	14	107	49	150	2.60E- 010	EST
12	gi 32966580 AAP92166.1	RuBisCo large chain	<i>Triticum aestivum</i>	28	107	40	161	2.10E- 011	EST
14	gi 15128580 BAB62757.1	Mitochondrial aldehyde dehydrogenase	<i>Hordeum vulgare subsp. Vulgare</i>	15	93	39	117	5.30E- 007	EST
15	gi 210972720 CAR48129.1	Rubisco large chain	<i>Triticum aestivum Triticum</i>	10	65	71	99	3.40E- 005 5.30E-	EST
17	gi 210972720 CAR48129.1	Rubisco large chain	<i>aestivum</i>	13	71	71	127	008	EST

19	gi 147945622 ABQ52657.1	Oxygen-evolving enhancer protein 1	<i>Leymus chinensis</i>	18	124	63	154	1.10E-010	EST
27	gi 2429087 AAB70865.1	Lipoxygenase 2	<i>Hordeum vulgare subsp. Vulgare</i>	8	83	52	75	0.0087	EST
28	gi 68250406 AA Y88778.1	Beta-1,3-glucanase mRNA	<i>Triticum aestivum</i>	10	62	55	84	0.001	EST
34	gi 2493543 Q43206.1	Catalase-1	<i>Triticum aestivum</i>	12	80	37	75	0.0076	EST
37	gi 14017580 NP_114267.1	RuBisCo large chain	<i>Triticum aestivum</i>	16	55	36	118	4.20E-007	Swissprot*
39	gi 14017580 NP_114267.1	RuBisCo large chain	<i>Triticum aestivum</i> <i>Hordeum vulgare subsp. vulgare</i>	23	107	43	143	1.40E-010 2.10E-007	Swissprot*
40	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>vulgare subsp. vulgare</i>	19	71	45	121	007	EST

44	gi 11990897 BAB19812.1	RuBisCo small subunit	<i>Triticum</i>	12	69	80	176	6.60E-	EST
			<i>aestivum</i>					013	
54	gi 195619530 ACG31595.1	Oxygen-evolving enhancer protein 1	<i>Zea mays</i>	21	133	82	183	1.30E-	EST
								013	
59	gi 32966580 AAP92166.1	RuBisCo, large chain	<i>Triticum</i>	11	124	46	96	6.80E-	EST
			<i>aestivum</i>					005	
62	gi 68566191 Q7X9A6.1	Cytochrome b6-f complex iron-sulfur subunit	<i>Triticum</i>	10	104	87	130	2.60E-	EST
			<i>aestivum</i>					008	
68	gi 2493543 Q43206.1	Catalase-1	<i>Triticum</i>	16	74	32	118	4.20E-	EST
			<i>aestivum</i>					007	
71	gi 195619530 ACG31595.1	Oxygen-evolving enhancer protein 1	<i>Zea mays</i>	15	128	70	165	8.40E-	EST
								012	
			<i>Hordeum</i>					2.60E-	
69	gi 167096 AAA63163.1	Rubisco activase A	<i>vulgare subsp.</i>	22	89	49	150	010	EST
			<i>Vulgare</i>						
73	gi 194708314 ACF88241.1	Unknown	<i>Zea mays</i>	9	57	39	70	0.024	EST

79	gi 134290407 ABO70330.1	Putative oxygen-evolving complex precursor	<i>Triticum aestivum</i>	11	92	67	146	6.60E-010	EST
82	gi 162460240 NP_001105689.1	O-methyltransferase ZRP4	<i>Zea mays</i>	8	62	48	60	0.026	Swissprot*
83	gi 14017580 NP_114267.1	RuBisCo large chain (fragment)	<i>Triticum aestivum</i>	6	65	49	84	0.00094	EST
84	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum vulgare subsp. Vulgare</i>	23	115	71	195	8.40E-015	EST
86	gi 115589736 ABJ15727.1	Serine hydroxymethyltransferase	<i>Triticum monococcum</i>	15	75	72	154	1.10E-010	EST
90	gi 131394 Q00434.1	Oxygen-evolving enhancer protein 2	<i>Triticum aestivum</i>	15	81	68	182	1.70E-013	EST
97	gi 14017580 NP_114267.1	RuBisCo large chain	<i>Triticum aestivum</i>	7	39	56	92	0.00019	EST
101	gi 167096 AAA63163.1	RuBisCo activase A	<i>Hordeum vulgare subsp.</i>	16	127	69	128	4.20E-008	EST

			<i>Vulgare</i>							
109	gi 32966580 AAP92166.1	RuBisco, large chain	<i>Triticum</i>	9	78	45	95	8.90E-	EST	
			<i>aestivum</i>					005		
118	gi 1568639 AAB67990.1	Cu/Zn superoxide dismutase	<i>Triticum</i>	8	81	60	80	0.0029	EST	
			<i>aestivum</i>							
125	gi 162461856 NP_001105414.1	Glyceraldehyde-3-phosphate dehydrogenase A	<i>Zea mays</i>	11	58	33	79	0.0034	EST	
129	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	22	70	52	177	5.30E-	EST	
			<i>vulgare subsp.</i>					013		
			<i>Vulgare</i>							
130	gi 32966580 AAP92166.1	RuBisCo, large chain	<i>Triticum</i>	10	81	45	102	1.70E-	EST	
			<i>aestivum</i>					005		
			<i>Hordeum</i>					1.30E-		
131	gi 118430396 YP_874661.1	RuBisCo large chain	<i>vulgare subsp.</i>	9	55	48	103	005	EST	
			<i>Vulgare</i>							
134	gi 131974 P28420.1	RuBisCo large subunit,	<i>Harpagophytum</i>	11	114	21	63	0.014	Swissprot*	

		fragment	<i>grandidieri</i>						
138	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	19	61	47	148	4.20E-	EST
			<i>vulgare subsp.</i>					010	
			<i>Vulgare</i>						
143	gi 134290407 ABO70330.1	Putative oxygen-evolving complex precursor	<i>Triticum</i>	10	93	64	146	6.60E-	EST
			<i>aestivum</i>					010	
144	gi 37783283 AAP72270.1	RuBisCo activase	<i>Triticum</i>	8	73	68	79	0.0036	EST
			<i>aestivum</i>						
147	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	21	92	62	208	4.20E-	EST
			<i>vulgare subsp.</i>					016	
			<i>Vulgare</i>						
			<i>Hordeum</i>					8.40E-	
149	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>vulgare subsp.</i>	23	129	48	135	009	EST
			<i>Vulgare</i>						
159	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	16	79	44	115	8.40E-	EST
			<i>vulgare subsp.</i>					007	

			<i>Vulgare</i>						
163	gi 115589736 ABJ15727.1	Serine	<i>Triticum</i>	12	80	62	109	3.30E-	EST
		hydroxymethyltransferase	<i>monococcum</i>					006	
164	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	22	75	55	156	6.60E-	EST
			<i>vulgare subsp.</i>					011	
			<i>Vulgare</i>						
166	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	14	79	67	148	4.20E-	EST
			<i>vulgare subsp.</i>					010	
			<i>Vulgare</i>						
169	gi 133741925 ABO37960.1	Peptidylprolyl isomerase	<i>Triticum</i>	13	107	46	74	0.012	EST
		(cyclophilin-like protein)	<i>aestivum</i>						
174	gi 11990901 BAB19814.1	RuBisCo small subunit	<i>Triticum</i>	10	119	86	80	0.0027	EST
			<i>aestivum</i>						
175	gi 11990897 BAB19812.1	RuBisCo small subunit	<i>Triticum</i>	14	93	79	133	1.30E-	EST
			<i>aestivum</i>					008	
189	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	32	104	56	173	1.30E-	EST

			<i>vulgare subsp.</i>					012	
			<i>Vulgare</i>						
190	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	26	122	58	135	8.40E-	EST
			<i>vulgare subsp.</i>					009	
			<i>Vulgare</i>						
193	gi 167096 AAA63163.1	RuBisCo activase isoform 1	<i>Hordeum</i>	39	146	61	224	1.10E-	EST
			<i>vulgare subsp.</i>					017	
			<i>Vulgare</i>						
194	gi 11990901 BAB19814.1	RuBisCo small subunit	<i>Triticum</i>	10	67	65	77	0.0055	EST
			<i>aestivum</i>						
213		Mixture:					70		
	gi 11990897 BAB19812.1	RuBisCo small subunit	<i>Triticum</i>	11	78	81	108	4.20E-	EST
			<i>aestivum</i>					006	
	gi 259017810 ACV89491.1	Dehydroascorbate reductase	<i>Triticum</i>	11	78	75	99	3.20E-	EST
			<i>aestivum</i>					005	
215		Mixture:					81		

216	gi 131394 Q00434.1	Oxygen-evolving enhancer protein 2	<i>Triticum aestivum</i>	8	59	37	65	0.0085	EST
	gi 14017627 NP_114312.1	NADH dehydrogenase subunit I	<i>Triticum aestivum</i>	6	59	36	61	0.025	EST
		Mixture:					184		
	gi 71362640 AAZ30062.1	Plastid glutamine synthetase isoform GS2c	<i>Triticum aestivum</i>	19	148	36	90	0.00024	EST
		RuBisCo activase isoform 1	<i>Hordeum vulgare subsp. Vulgare</i>	32	148	66	144	1.1e-09	EST

Online Resource 9: Sequence coverage of peptides used for protein match to Rubisco large and small subunits. Matched peptides are shown in bold red.

Protein	Sequence coverage
spot ID	
2	1 MSPQTETKAG VGFQAGVKDY KLTYTPEYE TKDTDILAAF RVSPQPGVPP 51 EEAGAAVAEE SSTGTWTTVW TDGLTSLDRY KGRCYHIEPV AGEDSQWICY 101 VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI PPTYSKTFQG 151 PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRACY ECLRGGLDFT 201 KDDENVNSQP FMRWRDRFVF CAEAIYKSQA ETGEIKGHYL NATAGTCEEM 251 IKRAVFAREL GVPIVMHDYL TGGFTANTTL AHYCRDNGLL LHIHRAMHAV 301 IDRQKNHGMH FRVLAKALRM SGGDHIHSGT VVGKLEGERE MTLGFVDLLR 351 DDFIEKDRAR GIFFTQDWVS MPGVIPVASG GIHVWHMPAL TEIFGDDSVL 401 QFGGGTLGHP WGNAPGAAAN RVALEACVQA RNEGRDLARE GNEIIRAACK 451 WSPELAAACE VWKAIKFEFE PVDITDKKV
7	1 EREMTLGFVD LLRDDFIEKD RARGIFFTQD WVSMPGVIPV ASGGIHVWHM 51 PALTEIFGDD SVLQFGGGTL GHPWGNAPGA AANRVALEAC VQARNEGRDL 101 AREGNEIIRA ACKWSPELAA ACEVWKAIF EFEPVDITDK
12	1 MSPQTETKAG VGFKAGVKDY KLTYTPEYE TKDTDILAAF RVSPQPGVPP 51 EEAGAAVAEE SSTGTWTTVW TDGLTSLDRY KGRCYHIEPV AGEDSQWICY 101 VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI PPTCSKTFQG 151 PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRACY ECLRGGLDFT 201 KDDENVNSQP FMRWRDRFVF CAEAIYKSQA ETGEIKGHYL NATAGTCEEM 251 IKRAVFAREL GVPIVMHDYL TGGFTANTTL AHYCRDNGLL LHIHRAMHAV 301 IDRQKNHGMH FRVLAKALRM SGGDHIHSGT VVGKLEGERE MTLGFVDLLR 351 DDFIEKDRAR GIFFTQDWVS MPGVIPVASG GIHVWHMPAL TEIFGDDSVL 401 QFGGGTLGHP WGNAPGAAAN RVALEACVQA RNEGRDLARE GNEIIRAACK 451 WSPELAAACE VWKAIKFEFE PVDITDK
15	1 ACYECLRGGL DFTKDDENVN SQPFMRWRDR FVFCAEAIYK SQAETGEIKG 51 HYLNATAGTC EEMIKRAVFA RELGVPIVMH DYLTGGFTAN TTLAHYCRDN 101 GLLLHIHRAM HAVI
17	1 ACYECLRGGL DFTKDDENVN SQPFMRWRDR FVFCAEAIYK SQAETGEIKG 51 HYLNATAGTC EEMIKRAVFA RELGVPIVMH DYLTGGFTAN TTLAHYCRDN 101 GLLLHIHRAM HAVI
37	1 MSPQTETKAG VGFKAGVKDY KLTYTPEYE TKDTDILAAF RVSPQPGVPP 51 EEAGAAVAEE SSTGTWTTVW TDGLTSLDRY KGRCYHIEPV AGEDSQWICY 101 VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI PPTCSKTFQG 151 PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRACY ECLRGGLDFT 201 KDDENVNSQP FMRWRDRFVF CAEAIYKSQA ETGEIKGHYL NATAGTCEEM 251 IKRAVFAREL GVPIVMHDYL TGGFTANTTL AHYCRDNGLL LHIHRAMHAV 301 IDRQKNHGMH FRVLAKALRM SGGDHIHSGT VVGKLEGERE MTLGFVDLLR 351 DDFIEKDRAR GIFFTQDWVS MPGVIPVASG GIHVWHMPAL TEIFGDDSVL 401 QFGGGTLGHP WGNAPGAAAN RVALEACVQA RNEGRDLARE GNEIIRAACK

451 WSPELAAACE VWK**AIKFEFE PVD**TIDK

39 1 MSPQTETKAG VGFKAGVKDY KLTYYTPEYE TKD**T**DILAAF RVSPQPGVPP
51 **EEAGAAVA**AE SSTGTWTT**VW** TDGLTSLDRY KGRCYHIEPV AGEDSQWICY
101 VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI PPTYSK**T**FGG
151 PPHGIQ**V**ERD KLNKYGRPLL GCTIKPKLGL SAKNYGR**AC**Y ECLRGGLD**F**T
201 **K**DDENVNSQ**P** FMRWRDR**F**VF CAEAIYKSQA ETGEIKGHYL NATAGTCEEM
251 **I**KRAVFAREL GVPIVMHDYL TGGFTANTTL AHYCRDNGLL LHIHRAMHAV
301 IDR**Q**KNHGMH FRVLAKALRM SGGDHIHSGT VVGK**L**EGERE MTLGFVDLLR
351 **D**DFIEKDRAR GIFFTQDWVS MPGVIPVASG GIHVWHMPAL TEIFGDDSVL
401 QFGGGTLGHP WGNAPGAAAN R**V**ALEAC**V**QA RNEGRDLARE **G**NEI**R**AAACK
451 **WSPELAAACE VWKAIKFEFE PVD**TIDK

44 1 **K**FETLSYLP**P** LSTEALLK**Q**V DY**L**IRSKWVP CLEFSKVGFI FREHNASPGY
51 **Y**DGRYW**T**MWK **L**PMFGCTDAT QVINEVEEVK KEY**P**DAYVRI IGFDNMRQ**V**Q
101 **C**VSFIAFK**P**P GCEESG**K**A

59 1 **E**REMTLGFVD LLRDDFIEK**D** RARGIFFTQD WVSMPGVIPV ASGGIHVWHM
51 PALTEIFGDD SVLQFGGGTL GHPWGNAPGA AANR**V**ALEAC **V**QARNEGRDL
101 AR**E**GNEI**R**A ACK**WSPELAA ACEVWKA**IKF EFEPVD**T**IDK

83 1 MSPQTETKAG VGFKAGVKDY KLTYYTPEYE **T**KD**T**DILAAF RVSPQPGVPP
51 **EEAGAAVA**AE SSTGTWTT**VW** TDGLTSLDRY KGRCYHIEPV AGEDSQWICY
101 VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI PPTYSK**T**FGG
151 **P**PHGIQ**V**ERD

97 1 **M**SPQTETKAG VGFKAGVKDY KLTYYTPEYE **T**KD**T**DILAAF RVSPQPGVPP
51 **EEAGAAVA**AE SSTGTWTT**VW** TDGLTSLDRY KGRCYHIEPV AGEDSQWICY
101 VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI PPTYSK**T**FGG
151 **P**PHGIQ**V**ERD KLNKYGR**P**

109 1 **E**REMTLGFVD LLRDDFIEK**D** RARGIFFTQD WVSMPGVIPV ASGGIHVWHM
51 PALTEIFGDD SVLQFGGGTL GHPWGNAPGA AANR**V**ALEAC **V**QARNEGRDL
101 AR**E**GNEI**R**A ACK**WSPELAA ACEVWKA**IKF EFEPVD**T**IDK

130 1 **E**REMTLGFVD LLRDDFIEK**D** RARGIFFTQD WVSMPGVIPV ASGGIHVWHM
51 PALTEIFGDD SVLQFGGGTL GHPWGNAPGA AANR**V**ALEAC **V**QARNEGRDL
101 AR**E**GNEI**R**A ACK**WSPELAA ACEVWKA**IKF EFEPVD**T**IDK

131 1 RHEAQTETKA GVGFQAGVKD YK**L**TYYTPEY E**T**KD**T**DILAA FRVSPQPGV**P**
51 **P**EEAGAAVAA ESSTGTWTT**V** WTDGLTSLDR YKGRCYHIEP VAGEDSQWIC
101 YVAYPLDLFE EGSVTNMFTS IVGNVFGFKA LRALRLEDLR IPPTYSK**T**FGG
151 **G**PPHGIQ**V**ER DKLNKYGRPL LGCTIKPKLG LSAKNYGRAC YECLRGGLD**F**
201 TK**D**DENVNSQ **P**FMRWRDRFV FCAEAIYKSQA

174 1 **P**PLSTEALLK QVDYLIRSKW VPCLEFSKVG F**V**REHNSSP GYYDGRYW**T**M
51 **W**KLPMFGCTD ATQVLNEVEE VKKEY**P**DAYV RVIGFDNLRQ VQCVSFI**A**R
101 **P**PGCEESG**K**A

175 1 **G**MQVWPIEGI KKFETLSYLP PLSTEALLKQ VDYLIRSKWV PCLEFSKVGFI
51 IFREHNASPG YYDGRYW**T**MW KLPMFGCTDA TQVINEVEEV KKEY**P**DAYV**R**
101 **I**IGFDNMRQV QCVSFI**A**FKP PGCEESG**K**A

194	1	MAPAVMASSA TSVAPFQGLK	STAGLPVSRR SSSAGLSSVS NGGRIRCMQV
	51	WPIEGIKKFE TLSYLPPLST EALLKQVDYL	IRSKWVPCLE FSKVGFVFRE
	101	HNSSPGYYDG RYWTMWKLPM FGCTDATQVL	NEVEEVKKEY PDAYVRVIGF
	151	DNLRQVQCVS FIAFRPPGCE ESGKA	
213	1	MAPAVMASSA TSVAPFQGLK	STAGLPVSRR SSSAGLSSVS NGGRIRCMQV
	51	WPIEGIKKFE TLSYLPPLST EALLKQVDYL	IRSKWVPCLE FSKVGFVFRE
	101	HNSSPGYYDG RYWTMWKLPM FGCTDATQVL	NEVEEVKKEY PDAYVRVIGF
	151	DNLRQVQCVS FIAFRPPGCE ESGKA	

Online Resource 10: Means and ANOVA data for wheat flag leaf chlorophyll content, measured at two dates with a Spad meter.

Spad reading		
Factor	16/06/2009	29/06/2009
Means ($\pm$ SE)		
Conventional CP		
Mineral fertilised	45.6 $\pm$ 0.8	48.1 $\pm$ 0.8
Compost fertilised	33.7 $\pm$ 0.6	32.7 $\pm$ 1.4
Organic CP		
Mineral fertilised	45.5 $\pm$ 0.4	46.8 $\pm$ 1.0
Compost fertilised	33.5 $\pm$ 1.0	33.8 $\pm$ 1.0
ANOVA		
Main effects		
Fertilisation (Fe)	p<0.0001	p<0.0001
Crop protection (CP)	ns	ns
2-way interactions		
Fe x CP	ns	ns

Online resource 11: Multivariate analysis of Nutrient and Proteomics data for flag leaves sampled at three growth stages (1, inflorescence; 2, anthesis; 3, grain milk development.

		1	2	3
Eigen value	Axis 1	71.8	72.3	72.6
(%)	Axis 2	17.1	11.5	10.5
Drivers	N	2.82, 0.022	2.65, 0.044	24.51, 0.002
(F, P)	P	1.24, 0.362	26.14, 0.002	1.06, 0.416
	K	0.00, 1.000	0.81, 0.518	0.76, 0.636
	S	1.69, 0.152	0.82, 0.558	1.11, 0.410
	Ca	27.56, 0.002	1.03, 0.422	2.41, 0.050
	Mg	0.80, 0.546	1.30, 0.310	1.88, 0.180
	Na	1.59, 0.190	0.69, 0.662	1.40, 0.252
	Fe	1.62, 0.174	3.28, 0.032	1.37, 0.224
	Mn	3.13, 0.228	0.00, 1.000	0.00, 1.00
	Cu	1.50, 0.196	0.79, 0.500	1.36, 0.198
	Zn	0.85, 0.518	0.80, 0.560	1.58, 0.142
	Mo	2.41, 0.062	1.79, 0.130	0.51, 0.614
	Cd	1.45, 0.272	2.34, 0.010	1.44, 0.182
	Al	1.90, 0.124	0.83, 0.550	2.04, 0.062
	B	2.85, 0.052	1.34, 0.242	2.93, 0.008

Online Resource 12: Means and ANOVA data for volume of protein spots that were affected by the interaction effect. Values for ANOVA data show F-values followed by asterix denoting p-levels: *=p<0.05, **=p<0.01, ***=p<.001; ns = non significant. These values are for data at inflorescence, except for protein spot 166 that is for anthesis.

Protein Spot							
Factor	44	59	76	125	130	159	166
Means ($\pm$ SE)							
Conventional CP							
Mineral fertilised	0.76	0.72	1.21	0.95	0.76	0.92	0.82
	± 0.104	± 0.101	± 0.176	± 0.116	± 0.112	± 0.063	± 0.121
Compost fertilised	1.09	0.88	0.94	0.68	0.87	0.91	1.33
	± 0.220	± 0.233	± 0.194	± 0.120	± 0.169	± 0.157	± 0.172
Organic CP							
Mineral fertilised	0.74	0.71	1.37	0.96	0.85	1.18	0.94
	± 0.054	± 0.054	± 0.139	± 0.107	± 0.020	± 0.112	± 0.008
Compost fertilised	2.28	1.69	0.53	1.52	1.42	0.85	1.05
	± 0.285	± 0.248	± 0.116	± 0.306	± 0.130	± 0.134	± 0.057
ANOVA							
Main effects							

Fertilisation (Fe)	31.0, **	16.4, **	23.2, **	ns	15.7, **	8.5, *	16.4, **
Crop protection (CP)	12.2, *	ns	ns	ns	14.0, *	ns	ns
2-way interactions							
Fe x CP	13.1, *	8.4, *	6.0, *	6.6, *	7.15, *	7.4, *	6.9, *