Running title: Protein turnover in barley leaves

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Proteins with high turnover rate in barley leaves estimated by proteome analysis combined with *in planta* isotope labelling

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**One Sentence Summary**: Proteins turnover at different rates in plant tissues, and these have been quantified using stable isotope labelling of nitrogen and peptide mass spectrometry of leaf tissue from hydroponically grown barley.
Footnotes:

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Abstract

Protein turnover is a key component in cellular homeostasis, however there is little quantitative information on degradation kinetics for individual plant proteins. We have used $^{15}$N labelling of barley (Hordeum vulgare) plants and GC-MS analysis of free amino acids and LC-MS analysis of proteins to track enrichment of $^{15}$N into the amino acid pools in barley leaves, and then into tryptic peptides derived from newly synthesised proteins. Using information on the rate of growth of barley leaves combined with the rate of degradation of $^{14}$N labelled proteins, we calculate the turnover rates of 508 different proteins in barley and show they vary by $>100$ fold. There was approximately a nine hour lag from label application until $^{15}$N incorporation could be reliably quantified in extracted peptides. Using this information and assuming constant translation rates for proteins during the time course, we were able to quantify degradation rates for several proteins that exhibit half-lives on the order of hours. Our workflow, involving a stringent series of MS spectra filtering steps, demonstrates that $^{15}$N labelling can be used for large-scale LC-MS studies of protein turnover in plants. We identify a series of abundant proteins in photosynthesis, photorespiration, and specific subunits of chlorophyll biosynthesis that turnover significantly more rapidly than the average protein involved in these processes. We also highlight a series of proteins that turnover as rapidly as the well-known D1 subunit of PSII. While these proteins need further verification for rapid degradation in vivo, they cluster in chlorophyll and thiamine biosynthesis.
Introduction

New proteins need to be synthesised while others must be degraded so that plants can respond to the daily requirements of cellular maintenance while also allowing progression through different developmental stages. Ubiquitinated proteins are targeted to the proteasome which is responsible for a large part of the protein degradation process in both plants and animals (Vierstra, 2009). Its action is supplemented by vacuolar protein degradation during autophagy (Araujo et al., 2011) as well as compartment-specific ATP-dependent metalloproteases (Janska et al., 2013). The plant cell proteome differs most markedly from other eukaryotic cells by the abundance of chloroplasts which can account for up to 3/4 of soluble protein in green plant tissues (Huber et al., 1976). Besides capturing energy from sunlight into fixed carbon, chloroplasts are also responsible for vitamin and co-factor generation as well as other anabolic processes needed to synthesise amino acids, lipids and pigments (Rolland et al., 2012). Determining the turnover characteristics of chloroplast proteins relative to those in other parts of the plant cell will be important for understanding how metabolic functions are coordinated across plant organelles.

Over the lifecycle of plants, the turnover of the chloroplast proteome is critical on several occasions. First, plastid protein degradation and the cytosolic ubiquitination system are known to be a critical factor in de-etiolation, the process of converting etioplasts to chloroplasts (Ling et al., 2012). Second, under carbon starvation induced by leaf shading or darkness, catabolism of plastid proteins via autophagy is important in selectively degrading some proteins and tissues in order to sustain others (Araujo et al., 2011). Third, amino acids derived from degradation of the photosynthetic machinery are the primary source of nitrogen during the seed-filling process (Masclaux et al., 2000), therefore plastid degeneration during natural senescence is central to the yield potential of crops. The protein content of chloroplasts is also modified on a shorter time scale in order to respond to environmental perturbations, to account for changing metabolic processes in diurnal cycles, and to renew machinery required for the steady state operations of the organelle. As a result, turnover of plastid proteins contributes to the significant energetic cost of protein synthesis required for cell maintenance (Penning De Vries, 1975). The most studied protein that exhibits fast turnover in plants is the D1 subunit protein of PS II in chloroplasts. The half-life of D1 is inversely correlated with light intensity and environmental stress conditions are also
associated with faster degradation of D1 (Aro et al., 1993; Marutani et al., 2012; Mittal et al., 2012). Plants generate their circadian rhythms by regulating gene expression and protein abundance over the diurnal cycle (McWatters and Devlin, 2011), and recently these pathways have been shown to be interconnected with plastid proteins involved in thiamine metabolism (Bocobza et al., 2013). A deeper understanding of protein turnover dynamics within the plastid will enhance our understanding of plastid maintenance (van Wijk and Baginsky, 2011; Nevo et al., 2012), and also allow researchers to make more informed decisions regarding the energetic costs of genetic engineering strategies in the plastid compartment (Maliga and Bock, 2011).

The simultaneous measurement of protein degradation rate ($K_d$) and synthesis rate ($K_s$) for a range of proteins in parallel is a new proteomic tool available to biologists, resulting from improved mass spectrometry (MS) equipment, computational power, and algorithm development. Large datasets have been generated in yeast, mammalian cell culture as well as in intact animals (Price et al., 2010; Cambridge et al., 2011; Schwanhausser et al., 2011). Proteomic analyses of protein turnover are just beginning to gather momentum in plants. Several studies have assessed the utility of various metabolic labels ($^2$H, $^{13}$C, and $^{15}$N) for this purpose (Yang et al., 2010; Chen et al., 2011; Li et al., 2012a). More focused reports have refined our understanding of the dynamics in the assembly of mitochondrial electron transport complexes using these tools (Li et al., 2012b; Li et al., 2013). Most recently, we performed a shotgun study of mitochondrial proteins from Arabidopsis cell culture, measured $K_d$ values of 224 proteins and assessed the turnover of several protein complexes, by adaptation of this approach to assess larger scale LC-MS/MS datasets (Nelson et al., 2013).

The important next step for utility of this approach for plant biologists is to conduct and analyse whole plant isotope labelling experiments in order to define the turnover of proteins in planta. Application of these techniques to a model species such as Arabidopsis will build upon the large body of information already available for this species (Lamesch et al., 2012). However, because there are significant differences between Arabidopsis and agriculturally relevant crop species, it is important to conduct studies in crops that can generate targets for geneticists and breeders.

Here we assess the utility of $^{15}$N labelling to better understand protein turnover during the steady-state operation of plant leaves, with an emphasis on chloroplast proteins. We have analysed leaves from barley (Hordeum vulgare), which have been a long standing model for photosynthesis research, chloroplast biology, and genetic dissection of photosynthesis and
photorespiration (Christopher and Mullet, 1994; Leegood et al., 1996; Dal Bosco et al., 2003; Rollins et al., 2013). Barley is also an important cereal crop for a variety of food and feed products (Schulte et al., 2009), and recent genome sequencing has scaffolded most of its expressed genes to a genetic map, opening new opportunities for in depth proteome analysis of this species and linkage to quantitative trait loci of agronomic importance (Mayer et al., 2012). We have examined $^{15}$N-labelling at the amino acid and protein levels and discuss the advantages and limitations of this method. We report $K_d$ values for more than 500 protein groups, with most of these being localized to the chloroplast, and compare turnover rates for proteins across different organelles and functional categories. We observed a series of abundant protein subunits in photosynthesis, photorespiration and in co-factor synthesis pathways that are degraded much more rapidly than the average barley or plastid protein involved in these processes. We also highlight a series of proteins that turnover rapidly which have not previously been described in terms of their degradation kinetics, and show that they cluster into certain metabolic pathways, specifically branches of chlorophyll and thiamine biosynthesis.

Results

Labelling of amino acid pools in barley leaves

In order to determine the viability of $^{15}$N protein labelling in planta, we first examined the rate and consistency of label incorporation into amino acid pools in the leaves of hydroponically-grown barley plants. After 25 days of plant growth and at 2.5 hours into the light period we swapped hydroponic media containing KNO$_3$ with natural abundance levels of $^{14}$N (99.6%) and $^{15}$N (0.4%) for media containing K$^{15}$NO$_3$ (98% $^{15}$N) and began collecting whole plants for GC-MS amino acid analysis in leaves. As can be seen in Figure 1A, $^{15}$N labelling increased in an approximately linear fashion through the light period and then began to level off later in the photoperiod. Glu, which is one of the entry points for inorganic nitrogen, was rapidly labelled. Other abundant amino acids (Ala, Asp, Ser, Thr) also incorporated the $^{15}$N label quickly. Other lower abundance amino acids such as Val and Ile were labelled more slowly. These results are in general agreement with prior data from metabolic labelling of barley plants (Thiele et al., 2008) and revealed 15% to 30% of many of the major amino acid pools were $^{15}$N labelled in leaves after ~10 hours of labelling. Alternatively, when the $^{15}$N label was applied 2.5 hrs after dark (Figure 1B), very little label
was incorporated into any of the amino acids. However, when the lights came on at 570 minutes the label was rapidly incorporated into barley leaf amino acids. This observation is consistent with the reduced activity of nitrate reductase in the dark and with other observations in barley (Aslam and Huffaker, 1982; Gojon et al., 1986; Abdel-Latif et al., 2004).

**Labelling of proteins and calculation of protein Kₐ in barley leaves**

In order to track protein turnover using stable isotope labelling and peptide MS, the first step involves swapping unlabelled or natural abundance (NA) amino acid pools for labelled or heavy (H) amino acids (Cambridge et al., 2011; Zhang et al., 2011; Trotschel et al., 2012; Li et al., 2012a). Following this label swap, proteins can be divided into two groups: NA (unlabelled) and H (labelled). The NA population contains proteins that were present prior to the label swap, and only experience degradation. In contrast, the H population contains newly synthesised proteins and is subject to both synthesis and degradation. For our calculations, we define relative isotope abundance (RIA) as the proportion of the total population that carries the NA label (RIA = NA/(H+NA)). As proteins are degraded over time, new copies of a protein are synthesised. This corresponds to a decrease in the proportional size of the NA population, and a reciprocal increase in the proportional size of the H population. Protein degradation rate (Kₐ) is calculated by tracking the decrease in the proportional size of the NA population over time using established procedures,(Pratt et al., 2002; Cambridge et al., 2011; Zhang et al., 2011; Li et al., 2012a), with details provided in Supplementary Methods. Plant tissues also grow over time which contributes to H but not to NA, we account for this dilution effect by incorporating measurements of plant growth rates into our calculations, outlined in detail in Supplementary Methods.

Ideally, the label swap would be instantaneous, but in reality when using whole plants there is a delay in delivery of the label associated with root uptake of $^{15}$NO$_3^-$, transport through the plant, and incorporation into amino acids by the nitrogen assimilation machinery. Thus, it is important to take this lag into account when choosing time points for sampling, to ensure that the tissue of interest is adequately labelled, allowing reliable measurements of Kₐ. To make high quality measurements, it is important to wait until the amino acid precursor pools reach a certain isotope enrichment threshold in order to maximize the chance that newly synthesized proteins will possess an isotopic label, or alternatively stated minimizes
the amount of newly synthesized protein that possesses no label. The fraction of the labelled population that will have zero labelled nitrogens is equal to $^{14}$N-enrichment$_{\text{Amino Acids}}$ #Nitrogens. For example consider a typical tryptic peptide of 15 residues made of amino acids with an average $^{15}$N-enrichment of 20%. In this case, <1% of peptides derived from a newly synthesised protein would have no labelled residues (Zhang et al., 2011). Therefore, based on our amino acid labelling data (Figure 1), we selected 12 hours as our first time point to assess protein turnover.

For our proteomic analysis of protein turnover rate in barley leaves, we also analysed leaf tissue sampled at 24, 48, 72, 96, 144, and 192 hours from the initiation of labelling. Images of barley plants from 25 days to 33 days old are provided in Supplementary Figure 1. An example of our workflow is provided in Figure 2. Proteins were separated using SDS-PAGE, gels cut into sequential fractions, proteolyzed with trypsin, and peptides analysed by LC-MS/MS. Isotopic labelling of proteins and peptides of an intermediate state of labelling has been reported to result in a loss of protein identifications (Whitelegge et al., 2004; Price et al., 2010). To counter this we applied a modified approach whereby proteotypic peptides generated for barley leaf samples were cross-extracted across all sample data files, so as not to suffer the negative consequences of reduced peptide and protein identifications from tandem mass spectrometry experiments with partially labelled samples (Price et al., 2010; Trotschel et al., 2012). Tandem mass spectrometry data were searched against a list of barley proteins (Mayer et al., 2012) using the Mascot search algorithm (Perkins et al., 1999). The search results from all samples were then filtered and combined using the Trans Proteomic pipeline (TPP) (Keller et al., 2005) (Figure 2A), in order to generate a list of proteotypic peptides (TPP peptide probability $\geq 0.8$), which were then used to determine high quality protein identifications with a protein probability $\geq 0.95$. Then for each peptide and across all samples, extracted ion chromatograms (EICs) were generated for all relevant isotopes. Next, natural abundance (NA) and heavy (H) peptide populations were assigned using a previously described non-negative least squares algorithm, whereby the H population is not defined by one value that represents an average of all the labelled peptide, but rather models the labelled peptides as several sub-populations based on the number of $^{15}$N atoms present in the peptide (Price et al., 2010). Because of the complex nature and wide dynamic range in abundance of proteins present in a crude lysate, we found that a significant portion of our peptide measurements were of low quality, due either to poor signal-to-noise or chemical noise, which in many cases corresponded to similarly eluting peptides that were close in mass.
order to remove these poor measurements from the dataset, we established a series of filters that removed low fidelity measurements as shown in Figure 2B and explained in detail in the Supplementary Methods.

As an example, the ATLAQLGYEKLDIIGR peptide from the ferredoxin linked glutamine oxoglutarate aminotransferase (MLOC_70866) is shown in Figure 3A. Examples of RIA are shown from samples at 24, 48, 72 and 96 hr, with the modelled fits to the isotopic abundances immediately beneath. The negative of the natural log of RIA measurements for all measurements for a protein were used to determine a degradation rate for each protein (Kd) (Figure 3B) as detailed in Supplementary Methods. The appearance of peptide populations with a larger degree of 15N enrichment through time in the proteins would be consistent with enrichment in the translational amino acid pool as plants grow and are exposed to the isotopic label and is consistent with studies in plants and mammals (Price et al., 2010; Li et al., 2012a). To more clearly demonstrate this, the average 15N-enrichment levels for the labelled peptide fraction for all identified peptides at each sampling time were then calculated (Figure 3C). At 12 h, the earliest time point, we had a median enrichment of 18% and the enrichment then rose steadily to 75% in the plants sampled after 8 d of labelling. The 18% enrichment in the labelled peptide fraction at 12 hours is consistent with the amino acid labelling profiles in Figure 1A.

A key assumption in the way we have conducted our calculation of protein turnover is that steady-state (constant Kd and Ks) is a reasonable approximation for the duration of the experiment. We assessed the validity of this assumption in two ways. First, we looked at growth rates over the course of the experiment (Supplementary Figure 2) and found that the values fit well to an exponential growth curve with an R^2 = 0.94 determined via non-linear regression. The growth constant (k=0.14, or 14% per day) is incorporated into our calculations of protein Kd to account for the dilution effect due to growth throughout the experiment. Second, we determined whether specific protein abundance levels varied during the experiment by measuring the abundance of proteins for which three or more peptides were observed in each time point. Our method for measuring protein abundance involved integrating the area under the EIC curve for the three most intense peptides derived from a given protein, using established protocols (Silva et al., 2006; Ahn et al., 2013) with a detailed explanation provided in the Supplementary Methods. From our dataset, we had 39 proteins that were observed by three or more peptides at all time points (Supplementary Table 3) with the abundances graphed in Supplementary Figure 3 and data provided in
Supplementary Table 4. Additionally, for each protein we conducted a Spearman rank correlation test for the relative abundance versus time and after correcting for multiple testing, none of the proteins showed a significant trend in protein abundance relative to time. This suggests that the cellular proteome was quite consistent across the duration of the experiment. Therefore, both analyses support our assertion that plants were undergoing steady-state exponential growth, validating our choice of mathematical approaches to calculate protein K_d.

The fastest degrading barley proteins and calculations of half-lives in the light

A list of the most rapidly degraded proteins is provided in Table 1. The proteins came from several different functional categories but all proteins with known localization were from the plastid. The fastest turning over protein with a K_d of 1.65 d^{-1} was THI1, an enzyme involved in thiamine metabolism. The second and third most rapidly degraded proteins are involved in tetrapyrrole metabolism, these were Mg-chelatase (GUN5) and an iron-binding protein CRD1. The D1 subunit of PSII was the fourth fastest in the dataset. Because D1, THI1, and GUN5 appeared to be turning over so quickly and were difficult to measure beyond 24 hours, we could not conduct a regression for these proteins. In order to account for the possibility that the abundance of these proteins varied over time, we also calculated K_d in an alternative fashion that considers potential changes in protein abundance and is described thoroughly in Supplementary Methods (Li et al., 2012a). As can be seen in Table 1, the proteins with the fastest K_d values using the first method of calculation also exhibited the highest K_d values via the alternative calculation, supporting the argument that these proteins are indeed degraded quickly. Proteins from several other functional categories were also observed in the top 10 including an RNA binding protein, a kinase, and a disulphide isomerase. Because synthesis and turnover of several proteins on this list are known to be higher in the light (Tang et al., 2003; Pal et al., 2013), we were concerned we were underestimating half-lives of this fastest set by considering data across all time points in our sampling regime, which encompassed long periods of darkness. To counteract this diurnal effect for the rapidly turning over subset, we also provide an estimate of half-life for proteins observations in the first 12 hour time point, taking into account the lag in labelling time calculated from the average X-intercept in the regression (Figure 3B). The median X-intercept from the regression values for all proteins was 8.6 hrs. As can be seen in Table 1,
based on these calculations a number of the most rapidly labelled proteins possess predicted half-lives of around 2 hours.

**Distribution of $K_d$ values for barley proteins across functional classes and subcellular location**

Considering peptides distinct for a protein group and with a probability of 0.8 or greater, we were able to measure $K_d$ for 508 proteins when requiring three or more quantitation events. The full list of protein $K_d$ values filtered with these thresholds is provided in Supplementary Table 2 with the list of measured peptides provided in Supplementary Table 3. A histogram of $K_d$ values for all proteins is shown in Figure 4A. The median value for all proteins is 0.08 d$^{-1}$, with most proteins falling between 0 and 0.2 d$^{-1}$. Next, we wanted to determine what role biological function or subcellular localization might play in protein turnover rate. Because the Arabidopsis genome and its gene complement is well annotated, we used the BLAST algorithm (Altschul et al., 1990) to select the closest Arabidopsis homolog for each barley protein so that we could take advantage of the extensive resources that have been developed for Arabidopsis research. We obtained biological functional categorization from the MapCave website (Thimm et al., 2004) ([http://mapman.gabipd.org/web/guest/mapcave](http://mapman.gabipd.org/web/guest/mapcave)), with the data presented in Figure 4B. Next, we defined subcellular localizations for this set of proteins using the SUBAcon naïve Bayesian classifier (Tanz et al., 2013) and this information is provided in Figure 4C.

Based on functional annotation grouping, we observed that cell wall associated proteins had the slowest turnover rate, followed by proteins involved in respiratory oxidative phosphorylation and protein synthesis. The fastest turnover rates were found in pigment and cofactor biosynthesis enzymes, followed somewhat distantly by secondary metabolism and photorespiratory components (Supplementary Table 2). When organellar $K_d$ values were compared by ANOVA, there were no significant differences between groups.

**Protein turnover for subunits of protein complexes**

The coordinated assembly and operation of protein complexes makes it likely that most subunits of a complex will exhibit similar turnover characteristics. Exceptions to this
rule might indicate, i) that individual subunits are being more rapidly damaged than other proteins in the complex and replaced, ii) that these subunits are peripheral to the complex and assembled separately, or iii) that these subunits are inducible and only present under certain conditions. Nine protein complexes with more than six subunits were represented in our dataset. These included the plastid and mitochondrial ATP synthases, the cytosolic and plastidic ribosomal large and small subunit complexes, the photosynthetic electron transport chain PSI and PSII complexes, and a complex of Calvin cycle-related enzymes. These complexes showed average $K_d$ values of 0.04 to 0.11 d$^{-1}$, however, in some cases there were outlier subunits that had $K_d$ value more than two standard deviations higher than the complex mean and were significantly different when assessed by an outlier test (Table 2).

**Cytosolic and plastidic ribosomes** – We measured turnover rates for 75 ribosomal protein groups in barley across the cytosolic and plastid ribosomes. Ribosome protein turnover was fairly slow with median half-lives of approximately 11 days, which was one of the slower functional categories observed (Figure 4A). This trend is consistent with other work from mice, where ribosomal proteins were turning over at slower rates compared to many other complexes (Price et al., 2010). In a study of mammalian cell culture, ribosomal subunits possessed different turnover rates (Cambridge et al., 2011) and the authors suggested that certain ribosomal proteins are generated in excess with rapid degradation of proteins that are not stabilized by insertion into complexes. This view is supported by the observation that when proteasomal activity was chemically blocked in animal cells ribosome subunits preferentially accumulate in the nucleolus (Lam et al., 2007). In the cytosolic 60S complex we observed $K_d$ values for 24 proteins, and from the 40S subunit we observed values for 17 proteins. There were no proteins from either group that were noticeably different from their respective complexes. On the whole, barley chloroplast ribosome 50S and 30S subunits were turning over at a similar rate to their cytosolic counterparts. The two exceptions to this were RPL33, belonging to the 50S subunit, which was turning over 3.5 SD faster than the mean and was significantly different via Dixon Q-test ($p << 0.001$). Additionally, from the 30S complex S18 was 2.2 SD from the mean for this complex but was not significantly different by Dixon Q-test ($p = 0.10$). Both of these subunits are plastid encoded. Overall 6 of the 35 chloroplast ribosomal subunits identified in this study were plastid-encoded and the other four plastid encoded subunits had turnover rates within 40% of the mean of the nuclear-encoded subunits.
**Photosystems** - In PSII, one protein was turning over 4.7 standard deviations faster than the mean for the complex (p << 0.001 via Dixon Q-test). This subunit was PSBA, also known as D1, and is the best known and most studied rapid turnover protein in plants. The oxidative damage, removal and replacement of D1 in PSII has been repeatedly measured (Aro et al., 1993; Christopher and Mullet, 1994; Tyystjärvi et al., 1994). Based on our calculations using only the 12-hour time point data, it has a half-life in the light of as little as 2.4 hours (Table 1). Much slower than D1 but still double the average Kd of PSII and significantly different (p<<0.001 via Dixon Q-test), was PSBC, alternatively named CP43. This observation is similar to prior observations made in algae (Yao et al., 2012) and land plants (Christopher and Mullet, 1994) and is consistent with the close proximity of D1 and CP43 in the PSII structure (Kato and Sakamoto, 2009). In PSI there was one faster turnover subunit, PSIP, that has a Kd of 0.09 compared to the complex mean of 0.05, but its turnover rate was not significantly different by Dixon Q-test. Interestingly, the homolog of this protein in Arabidopsis has recently been shown to have a role entirely independent of PSI as a curvature thylakoid protein (CURT1A; Armbruster et al., 2013), providing a reason why it would not have the same turnover characteristics as other PSI subunits.

**ATP synthases** – The structure of both the plastid and mitochondrial ATP synthases have been extensively studied (Hamasur and Glaser, 1992; Yoshida et al., 2001; Sunamura et al., 2012). We have shown previously that the subunits from both complexes turnover at comparable rates in Arabidopsis cell culture (Nelson et al., 2013). In barley leaves no subunit’s turnover rate was more than 2 standard deviations from the mean for each complex or significantly different by Dixon Q-test, suggesting the complexes turnover largely as intact units in planta.

**Calvin Cycle** – Reports over several decades have noted different combinations of Calvin cycle protein complexes, including early reports of association between Rubisco with other Calvin cycle enzymes and ferredoxin reductase (Suss et al., 1993). Additionally, there are detailed analyses of the role of CP12 in complexing with and modifying the activity of Calvin cycle enzymes, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and PRK (phosphoribulokinase) (Wedel et al., 1997; Carmo-Silva et al., 2011; Gontero and Maberly, 2012), as well as a recent report of mega-dalton complexes of GAPDH with small and large subunits of Rubisco, and 400 kDa complexes of Rubisco, GAPDH and PRK (Behrens et al., 2013). Combining this larger group of enzymes, we noted a similar turnover rate of 0.11 d^{-1} with the notable exception of CP12 (Kd =0.37 d^{-1}), which was 2.3 standard deviations faster
than the mean for the Calvin cycle group and significantly different via Dixon Q-test (p = 0.002).

**Fast turnover of enzymes in select metabolic pathways**

Analysis of functional groups (Figure 4) and individual protein turnover rates (Supplementary Table 1) revealed certain metabolic pathways that were over-represented amongst fast turnover proteins. Multiple enzymes of thiamine and tetapyrrole biosynthesis were found to possess high turnover rates, along with peroxisomal enzymes of the photorespiratory cycle. We examined these pathways more closely to determine if turnover rates might be informative in understanding the regulation or function of these pathways (Figure 5).

**Thiamine synthesis** - THIC and THI1 turn over very quickly and are involved in thiamine biosynthesis, which is ultimately used to make thiamine pyrophosphate (TPP), an important enzymatic co-factor (Figure 5A). The protein with the largest \( K_d \) value in the entire dataset was THI1, which converts glycine and NAD\(^+\) to adenosine diphosphate 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid, a precursor to thiazole. Mutants for this protein require thiamine supplementation for growth (Papini-Terzi et al., 2003). Using the modified method of calculating \( K_d \) in the light, the half-life of this protein would be 1.9 hours during illumination (Table 1). To our knowledge, there are no reports of turnover for this protein in plants. Additionally, we observed a \( K_d \) of 0.69 d\(^{-1}\) for THIC, which converts S-adenosyl methionine and 5-aminoimidazole ribonucleotide to hydroxymethylpyrimidine phosphate and is the rate limiting step in thiamine biosynthesis (Bocobza et al., 2013). These plastid enzymes were the only two enzymes in this pathway for which we obtained data, but these results clearly indicate that enzymes in both branches leading to thiamine synthesis are turned over several times a day.

**Tetapyrrole biosynthesis** – We measured degradation rates for several enzymes involved in tetapyrrole biosynthesis, which bifurcates into a branch for chlorophyll biosynthesis and a branch for heme biosynthesis (Figure 5B). In the upper portion of the pathway prior to the split, we have made estimates of turnover for glutamyl tRNA synthetase, glutamate semialdehyde aminotransferase, porphobilinogen deaminase, uroporphyrinogen decarboxylase, and coprogeon oxidase, with \( K_d \) values of 0.11, 0.03, 0.17, -0.04 and 0.16 d\(^{-1}\), respectively.
Although there were no measurements of $K_d$ for proteins with clearly defined enzymatic function in the heme branch, we measured unusually fast turnover rates for several enzymes involved in the $\text{Mg}^{2+}$ branch of tetrapyrrole biosynthesis, which is the branch associated with chlorophyll biosynthesis. Most prominent is the rapid turnover of Mg-chelatase H subunit (GUN5), which was the second fastest degraded protein in the dataset with a predicted half-life of 2 hours during illumination. GUN5 is one of three subunits in the Mg-chelatase complex that converts protoporphyrin IX to Mg-protoporphyrin IX in the first step in the chlorophyll branch of tetrapyrrole biosynthesis, and is a key player in chloroplast retrograde signalling (Kindgren et al., 2012). Another subunit of this complex, CHLI, had a more typical degradation rate of 0.11 d$^{-1}$. In the next step of the pathway, Mg-protoporphyrin IX methyl transferase (CHLM) catalyses the methylation of Mg-protoporphyrin IX to Magnesium-protoporphyrin IX monomethyl ester (MgMPPE). CHLM was degraded at a faster rate than the median protein in our dataset, but had a more typical rate in comparison to other enzymes in this branch of tetrapyrrole biosynthesis ($K_d = 0.19 \text{ d}^{-1}$). The MgMPPE is then converted to protoclerophyllide via MGMPPE cyclase, which contains at least one soluble and two membranous subunits (Rzeznicka et al., 2005). CRD1 is one of the membranous subunits of this complex and was the third most rapidly degraded protein in our dataset with an estimated half-life of 2.4 hours during illumination. In the last step of chlorophyll biosynthesis, NADPH:Pchlide oxidoreductase (POR) is responsible for the catalysis of protochlorophyllide to chlorophyllide and is composed of two subunits in dicots (PORA & PORB), but only one subunit in monocots. We observed POR as turning over rapidly with a $K_d$ of 0.27 d$^{-1}$, which is three times the median decay rate for all observed barley leaf proteins. Finally, there was one heme-binding protein (SOUL) classified as involved with tetryapyrrole metabolism but with no known specific function and had a $K_d$ of 0.15 d$^{-1}$.

**Photorespiration** – The most rapidly degraded protein in this pathway was an isoform of the peroxisomal glycolate oxidase ($K_d$ 0.27 d$^{-1}$) (Figure 5C), with two other isoforms having somewhat lower $K_d$ values (0.24 d$^{-1}$ & 0.12 d$^{-1}$). This is closely followed by three other peroxisomal located enzymes involved in the pathway: the serine/glyoxylate aminotransferase (SGAT, $K_d$ 0.26 d$^{-4}$), the peroxisomal malate dehydrogenase ($K_d$ 0.26 d$^{-1}$) and the hydroxypyruvate reductase ($K_d$ 0.16 d$^{-1}$). This is in contrast to the mitochondrial photorespiratory components (average $K_d$ of 0.12 d$^{-1}$) and the plastid localized enzymes, glycerate kinase and phosphoglycolate phosphatase, which respectively initiate and complete the cycle ($K_d$ average 0.04 d$^{-1}$).
Discussion

Protein turnover is integral in maintaining cellular function, particularly in response to environmental stress (Araujo et al., 2011). In addition, the cost of protein synthesis/degradation is also known to be a significant energetic cost for plants (Penning De Vries, 1975; Hachiya et al., 2007). Measurement of protein turnover kinetics as a proteomic technique is just beginning in planta, with prior large scale work conducted in plant cell culture (Li et al., 2012a; Nelson et al., 2013). Assuming that Kᵦ and Kᵦ are constant across the duration of our experiment, we have used mass spectrometry to acquire steady-state measurements of degradation rates for a selection of more than 500 of the most abundant leaf proteins in the major crop species barley under control conditions and show more than 100-fold differences between their half-lives. This highlights the highly differential cost of maintaining the major components of the proteome at steady-state levels. For example, we have documented the investment required to maintain the abundance of rapidly degraded proteins such as GUN5 and THI1. While it is interesting to consider these fast turning over proteins on the list, the total cost of synthesis is also a function of the protein’s abundance. While the Kᵦ values for the large and small subunits of Rubisco were close to the median value for all proteins, the abundance of these proteins in the cell will dominate the associated costs of protein maintenance. However, there are various improvements that can be made and challenges to be addressed in future protein turnover studies in intact plants to maximise the value and accuracy of calculations, especially for proteins possessing faster turnover rates. These include measurement of lag in labelling, determining diurnal patterns of amino acid and protein synthesis, alternative label options for proteins turning over at different rates, biological interpretation of aberrant turnover rates in pathways and protein complexes, and non-steady state analysis of changes in turnover characteristics.

Defining lag and diurnal effects in labelling to extend dynamic range

When applying isotopic labels to intact plants for quantifying protein turnover, one of the primary concerns is the lag from label application to incorporation into amino acids and proteins. In a prior study of $^2$H$_2$O labelling in Arabidopsis seedlings, proteins incorporated the isotopic label quickly, but high levels of deuterium were problematic due to the resulting
physiological changes (Yang et al., 2010). In an assessment of a pulse-chase $^{13}$CO$_2$ labelling approach in leaves from Arabidopsis during which plants were grown in $^{13}$CO$_2$ and the gas label exchanged for $^{12}$CO$_2$, a measurable but diffuse isotopic envelope was observed after 24 hours of label swap. To our knowledge, there are no proteomic studies in intact plants that have assessed the utility of $^{15}$N for protein turnover studies. Based on results from amino acid data and X-intercept data from regression results of protein measurements, we see approximately a nine hour lag between label application and robust protein labelling of 25 day old barley plants. This value is comparable to or less than the delays reported for labelling of tissues from mice (Price et al., 2010; Zhang et al., 2011).

The lag in label incorporation into proteins is probably due to several reasons. First, the $^{15}$NO$_3$ must be taken up in the root, transported to the leaves via transpiration, reduced to ammonia and assimilated into amino acids. Second, the labelling of translational amino acid pools with $^{15}$N is potentially further retarded by competing with NH$_3$ generated by photosynthesis in leaves for reassimilation into amino acids. Earlier work reported that reassimilation of photosynthetic NH$_3$ was significantly larger than new primary NH$_3$ acquisition (Keys et al., 1978). Interestingly, photosynthesis is required for efficient reduction of NO$_3$ to NH$_3$ (Rachmilevitch et al., 2004; Bloom et al., 2010). In barley, while there is extensive exchange of reduced N between tissues (Gojon et al., 1986; Abdel-Latif et al., 2004) studies suggest that leaves account for the vast majority of nitrate reduction under normal conditions with most of this reduction occurring in the light (Aslam and Huffaker, 1982; Gojon et al., 1986; Abdel-Latif et al., 2004).

It is also interesting to note that for many of the amino acids observed in our study there was a drop in the relative isotope abundance for the heavy isotope after the lights went off at 570 minutes after label swap (Figure 1). While nitrate assimilation is known to occur at slower rates in the dark compared to illuminated conditions (Aslam and Huffaker, 1982), this does not provide an obvious explanation for the decrease in amino acid $^{15}$N incorporation during the dark period. A possible explanation is the reported decrease in the amino acids pool sizes in the dark in both barley and Arabidopsis (Winter et al., 1993; Tschoep et al., 2009). This effect was most clearly demonstrated by glycine (Figure 1), which is also reported to be significantly reduced in abundance in the dark due to the cessation of photosynthesis in darkness (Winter et al., 1993; Tschoep et al., 2009). Also worth considering is that plant cells contain multiple pools of amino acids that differ in their metabolic activity (Holleman and Key, 1967), with large inert storage pools of many amino
acids being sequestered in the vacuole (Tohge et al., 2011). Therefore, this decrease in incorporation of whole tissue extracts during the dark period may represent both the cessation of N assimilation and a relative decrease in the size of metabolically active amino acid pools, which would have been more highly labelled relative to the metabolically inert pools. While this dynamic nature of amino acid pool labelling shows the complexity of the labelling process, the protein incorporation and turnover calculations using steady-state assumptions remain straightforward as long as the average $^{15}$N enrichment of utilized amino acid pools is 0.15 or greater in newly synthesised proteins.

The D1 protein of PSII is well documented as degrading quickly in leaf tissue and is therefore a good example to consider the applicability of our $^{15}$N labelling for the measurement of rapidly degraded proteins with this technique. Reports vary from 1-20 hrs in terms of half-life for this protein across different species (Wilson and Greenberg, 1993; Jansen et al., 1999; Booij-James et al., 2000). When calculated via regression, D1 was the fourth fastest turning over protein with a $K_d$ of 0.94 d$^{-1}$, or in other words a half-life of 15 hours, and is thus within the range of reported values for D1, albeit on the slower side. Because of the challenges that contribute to $^{15}$N lag and our desire to make accurate measurements of $K_d$ for all proteins, we consider whether other considerations in calculation would offer viable alternatives for rapid turnover proteins. We know that translation of D1 is strongly dependent on light intensity, with the translation rate being much faster in the light than the dark (Kim and Mayfield, 1997; Barnes and Mayfield, 2003). Our calculation of D1 $K_d$ incorporates data from both 12 hour and 24 hour time points, and this time series encompasses a long period of darkness. So to account for the light-induced variation in synthesis, we modified our calculation of $K_d$, using only data from the 12 hour time point. This results in an estimated half-life of 8.5 hours, which is well within the reported measurements mentioned above. However, this calculation does not account for the lag in label uptake and reduction. We further modified our calculation of D1 half-life, accounting for lag in label delivery based on the X-intercept data from the regressions so that we could allow for the lag in label incorporation. This provided a D1 half-life as short as 2.5 hours, which is comparable to some of the fastest observations in the literature (Aro et al., 1993; Tyystjarvi et al., 1994). We believe that the 2.5 hr half-life estimate is probably the most accurate value as it factors in the limitations of our system and is quite close to values reported by other methods. So while it can be complicated to obtain precise measurements of rapidly degraded leaf proteins using $^{15}$N, our dataset illustrates the power of $^{15}$N labelling and
mass spectrometry for obtaining accurate measurements of $K_d$ for hundreds of proteins, placing D1 in the top percentile.

**Label selection for protein turnover studies**

The ideal label would be one that is rapidly incorporated into amino acid pools with minimal lag time and that can accurately measure $K_d$ over a wide dynamic range (i.e. hours to weeks). In addition, a preferred label would be present in most peptides, and the organism being analysed would be auxotrophic for the label (Hinkson and Elias, 2011). Plants can synthesise all of the standard 20 amino acids, which would suggest that providing exogenous labelled amino acids may not work. One prior work using stable isotope labelling by amino acids (SILAC) in Arabidopsis cell culture confirmed that amino acid labelling would be problematic due to incomplete labelling (Gruhler et al., 2005) while another investigation of SILAC for proteomic applications in seedlings showed that the strategy may be viable, with high incorporations of labelled amino acids into proteins (Lewandowska et al., 2013). But even if this strategy is viable in some defined scenarios, given the high cost of SILAC reagents this strategy will be of limited use in hydroponic systems and would not scale well to measurement of older plants and larger species of agricultural importance. Given the autotrophic nature of plants, this would leave the elements in peptides that have multiple stable isotopes (S, H, C, N, O). Given the variable fashion in which different elemental labels are assimilated by plants there probably is no single best solution for all plant tissues and for all experimental conditions. As has already been established, deuterium at high levels is toxic (Kushner et al., 1999; Yang et al., 2010). Sulfur is present in only a fraction of peptides so is probably not a good choice as a label. Oxygen has been used as a proteomic tracer in bacteria (Bernlohr, 1972) but given the price of $^{18}\text{O}$ water this strategy may be cost prohibitive. Nitrogen is easy to apply in a controlled fashion as a salt, which is then taken up by root tissue, however, lag before delivery to aerial tissues limits measurements in the first few hours following labelling. It may be possible to shorten the lag by applying the label as reduced $\text{NH}_4^+$, but there are two shortcomings with this strategy. In many agricultural scenarios, nitrogen fertilizer may be provided in a reduced form such as urea or a $\text{NH}_4^+$, but this nitrogen is rapidly oxidized to nitrate via the action of bacteria in the soil (Skiba et al., 2011), also, different crop species have varying sensitivity to ammonium toxicity (Britto and Kronzucker, 2002). The final element to consider is carbon, which is challenging because the
label, $^{13}$CO$_2$ must be supplied as a gas and therefore is more difficult to regulate. However, given the rapid incorporation of this label into amino acids of illuminated leaves (Szecowka et al., 2013), it would be anticipated that the lag in robust labelling of proteins might be reduced to only minutes for aerial tissues. For more slowly degraded proteins, extended labelling periods may be required. While $^{13}$C conceivably could be used to measure slowly turning over proteins, this strategy would be expensive and difficult due to the challenges associated with a gas-phase label. A combination of labels, $^{13}$C for proteins that have fast turnover rates, and $^{15}$N for slower turning over proteins, is probably the optimal compromise.

**Reasons for rapid protein degradation**

Proteins could be rapidly degraded for one of two reasons, either the protein serves a regulatory role and rapid turnover is required to respond rapidly to some environmental perturbation, or the protein is damaged through its functioning. The canonical rapidly degraded protein, D1, is turned over due to light-induced damage in PSII. In addition to our observations of D1, we observed several other rapidly degraded proteins in our dataset with two of these proteins, THI1 and THIC, being involved in thiamine biosynthesis. In tobacco THI1 transcript abundance and polysome loading of this mRNA are high in the light and decrease in the dark (Tang et al., 2003), which would be consistent with our observations of daily regeneration of THI1 protein. Recent work of the yeast THI1 ortholog, THI4p, reported that the sulphur atom necessary for thiazole synthesis was transferred from an internal cysteine residue leaving behind a dehydroalanine residue so that this protein was classified as a suicide enzyme, due to the irreversible nature of this modification (Chatterjee et al., 2011). When the authors examined data on THI1 crystal structures, they concluded that a dehydroalanine residue was present in the analogous cysteine residue, suggesting this enzymatic mechanism may be conserved across eukaryotic taxa (Chatterjee et al., 2011). This would mean that organisms would need to synthesise a new copy of the THI1 protein for each newly synthesised thiamine molecule, which is surprising given the abundance of THI1 and the associated energetic costs. Even if THI1 is not a suicide enzyme, but instead is susceptible to consuming the internal cysteine residue during catalysis, it could still be expected that this enzyme would need to be recycled frequently. The other rapidly turning over protein involved in thiamine metabolism was THIC, which is the rate limiting step in thiamine biosynthesis (Bocobza et al., 2013). Interestingly, this protein is the only known
riboswitch in plants, whereby the THIC mRNA binds thiamine pyrophosphate (TPP), resulting in a shift to transcription of an alternative, less stable transcript that is rapidly degraded (Bocobza et al., 2013). Perhaps rapid turnover of the THIC protein represents an additional level of control.

Several enzymes involved in chlorophyll biosynthesis, belonging to the Mg$^{2+}$ branch of tetrapyrrole metabolism, were observed and many of these were turned over rapidly. Flux through this pathway is tightly regulated due to the toxic nature of the intermediates. Two of the fastest degrading proteins in our dataset were GUN5 and CHL27. We are not aware of any reports of protein turnover measurements for either of these gene products; however, GUN5 is known to function in retrograde signalling between the chloroplast and nucleus (Mochizuki et al., 2001). Another report established that GUN5 is regulated in a circadian manner by the clock gene TOC1 (Legnaioli et al., 2009). Rapid degradation for GUN5 would be consistent with its signalling role as well as its links with circadian oscillations. Other proteins that turned over rapidly in our dataset were POR and CHLM. While there are no other precise quantitative measurements of $K_d$ for these proteins in the literature, in one study from barley leaf discs the POR protein, as assessed through Western blotting, showed rapid degradation in illuminated leaf discs (Richter et al., 2010). In total, these observations suggest that protein turnover plays an important role in regulating the Mg$^{2+}$ branch of tetrapyrrole biosynthesis.

There were also differences within the compartmental components of photorespiration. Specifically, we observed that some peroxisomal-localized enzymes were significantly different from their mitochondrial and plastid-localized partners. Hydrogen peroxide produced by glycolate oxidase in peroxisomes could lead to its damage during high photorespiratory flux in the light, which may also affect neighbouring enzymes. Metabolic channelling of photorespiratory intermediates (NADH, glyoxylate, hydroxypyruvate) between these enzymes does occur, even when the peroxisomal membrane is disrupted, suggesting close proximity or protein complex formation (Heupel et al, 1991). The turnover of these peroxisomal enzymes of photorespiration is unlikely to be a result of bulk peroxisome turnover, as one of the major component of peroxisomes, catalase, has a $K_d$ of 0.06 d$^{-1}$, close to the cellular median.
Extension to non-steady state analysis of protein turnover

One obvious extension of these methodologies in intact plants will be the analysis of non-steady state systems such as the initial responses to biotic and abiotic stresses, as well as developmental transitions such as germination, senescence, tillering, and seed filling. These types of studies would help us understand the role of protein degradation in scenarios associated with agricultural productivity. Such analyses could be integrated with biochemical data that quantify the energetic requirements of growth, metabolism and cellular maintenance (Cheung et al., 2013; Poolman et al., 2013). This would allow us to measure the changing cost of protein turnover, and allow us to estimate the proportion of cellular energy that is expended on polypeptide synthesis and degradation in different scenarios. This information would greatly further our understanding of the energy budgeting of plants, enhancing strategies that attempt to rationally engineer the partitioning of resources between maintenance, growth and other energy consuming processes for optimal resource use efficiency in plants.

Conclusions:

By tracking the decay of unlabelled proteins following a switch to $^{15}$N-labelled media and using an assumption of constant $K_s$, we present the first large-scale LC-MS report of steady state $K_d$ values in leaves from intact plants. The data give insights into dynamics of protein complexes, and identifies a number of previously undocumented fast-turnover proteins, some of which are control points that regulate the metabolic response to environmental cues. This work serves as a foundation for future studies investigating the role of protein turnover in plant energy budgeting across different scenarios.

Methods:

Plant Growth

*Hordeum vulgare* (var. Baudin) seed was sterilized for five minutes with 6% (v/v) bleach and rinsed several times prior to placing seeds on wetted filter paper. After allowing four days for germination, seedlings were transferred to hydroponic tubs in media containing
3 mM KNO₃, 0.5 mM KH₂PO₄, 0.5 mM MgSO₄, 0.5 mM CaCl₂, 0.1 mM Fe-EDTA, 0.05 mM KCl, 0.025 mM H₂B₃O₃, 0.005 mM Na₂MoO₄, 0.002 mM MnSO₄, 0.002 mM ZnSO₄, and 0.0005 mM CuSO₄, adjusted to pH 5.9 with KOH. Roots were flooded and drained with nutrient solution every 30 min, according to the design of earlier work (Munns and James, 2003). Plants were grown for 25 days on a 12:12 hr light/dark schedule and a 23 ºC/18 ºC temperature regime, with 500 µmol m⁻² s⁻¹ light intensity and 65% relative humidity. Media was changed twice weekly. From days 21 to days 24, the nitrogen was checked and adjusted daily. After 25 days growth, the nitrogen salt in the nutrient solution was switched to K¹⁵NO₃.

**Amino Acid Analysis**

For amino acid analysis, plants were snap frozen immediately at label application and every 80 min thereafter for the first 12 h of labelling. Samples were ground in a mortar and pestle in liquid nitrogen. To 25 mg of ground tissue, 500 µL of pre-chilled (-20 ºC) metabolite extraction buffer (90% methanol (v/v) and 0.008 mg/mL ribitol) was added. Samples were heated to 65ºC and shaken at maximum speed for 30 minutes in a thermomixer. Tubes were then centrifuged at 20,000 x g and 60 µL of the supernatant dried in a Centrivap (Labconco) and stored at -80 ºC until analysis. To dried samples, 20 µL methoxyamine in pyridine (20 mg/mL) was added and shaken for 90 min at 37 ºC. Next, 30 µL MSTFA (Pierce) was added and shaken for 60 min at 750 rpm and incubated at 37 ºC. After 60 min of additional incubation without shaking, GC-MS analysis was conducted in a manner similar to previous work (Howell et al., 2009) with the following GC oven conditions: initial GC oven conditions were 70 ºC, held for 1 minute, increased to 76 ºC at 1 º/min, 76-325 ºC at 6 ºC/min, held at 325 ºC for 8 minutes, and then returned to 70 ºC for a total run time of 60 minutes. The ribitol spike was used as a retention time marker and samples aligned using Enhanced Chemstation Data Analysis version. E. 02.02.1431 (Agilent). Chromatographic peak areas were determined for amino acids and their ¹⁵N-labelled counterparts, making corrections for the natural abundance of heavy atomic isotopes that are mainly derived from ¹³C. Quantifier ions for amino acids and their ¹⁵N-labelled forms are provided in Supplementary Table 1.

**Proteomic Analysis**

For protein analysis, tissue samples from leaves were harvested immediately after label application and at 12, 24, 48, 72, 96, 144 and 192 h for assessment of peptide ¹⁵N
incorporation and protein turnover rates. Samples were snap frozen in liquid nitrogen and stored at -80°C until preparation for proteomic analysis. Frozen leaves from two samples at each time point were ground in a mortar and pestle sitting on dry ice and whole-cell protein extracted using a modified chloroform:methanol protocol (Wessel and Flugge, 1984). Briefly, 400 μl of extraction buffer (125mM Tris-HCL, 7% SDS, 10% β-mercaptoethanol, pH 7) was added to 250 mg of ground tissue, with samples then placed in ice on a rocking platform for 10 min, with intermittent inversion of the samples to facilitate thawing. Samples were then centrifuged at 10,000 x g for 5 min at 4°C with 200 μl of resulting supernatant transferred to a new eppendorf tube. To the supernatant, 800 μl of pre-cooled methanol (4°C), 200μl of pre-cooled chloroform (4°C) and 500 μl of pre-cooled ddH2O (4°C), were added and the sample vortexed. The solutions were then centrifuged at 10,000 x g for 5 minutes at 4°C in order to form an aqueous/organic phase separation, with precipitated protein present at the interface. The upper aqueous phase was removed and 500 μl of methanol was added before vortexing again. Samples were centrifuged at 9000 x g for 10 min at 4°C with the supernatant removed and discarded. To the remaining pellet, 1 ml of pre-cooled acetone (-20°C) was added and vortexed vigorously. Samples were incubated for 1 hour at -20°C and were then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded and the protein pellets left to air dry for approximately 15 minutes at room temperature.

To precipitated protein, sample buffer (2% SDS, 50 mM Tris (pH 8.5), 10 mM DTT) was added and incubated at 55°C for 30 minutes while gently shaking with an orbital mixer. Samples were then cooled to room temperature. Protein concentrations were determined using the Amido black protein assay (Schweikl et al., 1989). To 150 μg of re-solubilized protein, 0.5 M iodoacetamide was added to each sample to a final concentration of 25mM and incubated for 30 minutes at room temperature in the dark, with the reaction quenched by the addition of DTT till a final concentration of 30 mM. The protein concentrations of the samples were adjusted to 2.5 mg/ml by the addition of modified sample buffer (2% SDS, 50 mM Tris-HCl (pH 8.5), 20% glycerol), and 20 μl of sample was loaded onto three adjacent lanes of a precast AnyKD gel (Biorad). Proteins were electrophoresed at 300 V for 20 min. Following separation, gels were placed in gel fix solution (40% methanol, 10% acetic acid) for 60 min. Gel fix solution was then removed and replaced with three parts Coomasie stain (80 ml methanol, 0.6 g Coomassie powder, 9.4 ml 85% phosphoric acid and ddH2O to final volume of 320 ml) and one part ammonium persulfate buffer (50% (w/v)). Gels were left to stain overnight before being destained with ddH2O several times as required.
Gel samples were then cut into 12 fractions grouped by molecular weight, and each fraction sliced into ~1 mm cubes with a razor blade. Gel pieces were placed in microfuge tubes and destained in 1 ml 100 mM (NH₄) HCO₃/50% methanol for 10 minutes while being vigorously shaken at maximum with an orbital mixer for 10 minutes at room temperature. Supernatants were discarded and the destaining process repeated. Samples were then dehydrated using 1 ml of 10 mM (NH₄)HCO₃/50% acetonitrile for 2 min. Supernatant was removed and samples were further dehydrated with 100% acetonitrile for 30 s. Supernatant was removed and samples were dried in a Centrivap (Labconco) for 15 min. Trypsin (5 ng/μl in 10 mM (NH₄)HCO₃, 3% acetonitrile (v/v)) was added to each sample until gel pieces were covered. Additional 10 mM (NH₄)HCO₃ was added as necessary to keep gel pieces immersed. Samples were then incubated at 37 °C and left to digest overnight. Following overnight digestion, digested proteins were extracted using 700 μl of HPLC grade water and 1% TFA (v/v) by vortexing at room temperature in an orbital shaker for 10 min at high speed. Supernatant was transferred to a new 1.5 ml Eppendorf tube. An additional extraction was performed using 700 μl of 70% acetonitrile (v/v), 25% ddH₂O (v/v) and 5% TFA (v/v). The supernatant was removed and added to the first extraction and samples were then dried in the Centrivap.

**Mass Spectrometry**

Digested peptides were resuspended in 20 μl of 2% acetonitrile (v/v), 0.1% formic acid (v/v), and 5 μl of this resuspension was loaded onto a C18 high capacity nano LC chip (Agilent) in 98% Buffer A (0.1% (v/v) formic acid in Optima grade water (Fisher)) and 2% Buffer B (0.1% formic acid in Optima grade acetonitrile (Fisher)) using a 1200 series capillary pump (Agilent). Following loading, samples were eluted from the C18 column and into an inline 6550 Series QTOF mass spectrometer (Agilent) with an 1200 series nano pump (Agilent) using the following gradient: 2% B to 45% B in 26 minutes, 45% to 60% B in 3 minutes, 60% to 100% B in 1 minute. Fractions from a given sample were analysed in sequence and a blank was run between samples. The QTOF was operated in a data-dependent fashion with an MS spectrum collected prior to the three most abundant ions subjected to tandem mass spectrometry from doubly, triply, and higher charge states. Ions were dynamically excluded for 0.4 minutes following fragmentation. MS data were collected at eight spectra per second while MS/MS spectra were collected at three spectra per second with a minimum threshold of 10,000 counts and a target of 25,000 ions per MS/MS event.
**LC-MS Data Analysis**

Agilent .D files were converted to mzML using the Msconvert package (v. 2.2.2973) from the Proteowizard project, and mzML files subsequently converted to mascot generic files (MGF) using the mzxml2search tool from the Transproteomic pipeline v. 4.6.2 (TPP). MGF peak lists were searched against protein sequences released for barley ([ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/](ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/)) using Mascot 2.3 running on an in-house server, allowing for variable methionine oxidation, alkylation of cysteine, N-terminal acetylation of the protein, 100 ppm mass tolerance for the parent ion, and 0.5 Da tolerance on MS/MS peaks. Results were downloaded from the Mascot server as .dat files and then converted to pep.xml files using the ToPepXML tool in the TPP. Results from LC-MS/MS analysis were then assessed using the PeptideProphet tool with the accurate mass binning option selected and then processed further using ProteinProphet. Further details on calculation of protein turnover rates are provided in Supplementary Methods.

**Statistical Analysis of Organelles and Protein Complexes**

Proteins were grouped by known subcellular localization of the Arabidopsis homolog according to the SUBAcon call in SUBA3 database (suba.plantenergy.uwa.edu.au) and an analysis of variance was applied across compartments to identify organelles protein sets that varied significantly. For protein complexes, protein subunit outliers relative to their complex were determined by applying a Dixon Q-test (Dixon, 1950; Rorabacher, 1991). All analyses were conducted with R ([http://www.R-project.org](http://www.R-project.org)).

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**Supplemental Material**

**Figure S1.** Representative images of plant growth across the experiment

**Figure S2.** Leaf growth in mass over the course of the experiment
**Figure S3.** Normalized abundance of barley proteins by the top 3 ion approach

**Supplemental Methods.** Details of mass spectrometry methods and data analysis for protein turnover rate determination

**Table S1:** Information on fragment ions used to measure 14N and 15N forms of amino acids measured by GC-MS

**Table S2:** Information on barley proteins for which Kd was measured

**Table S3:** Peptides used to quantify proteins

**Table S4:** Relative abundance of major barley proteins

**References:**


Legnaioli T, Cuevas J, Mas P (2009) TOC1 functions as a molecular switch connecting the circadian clock with plant responses to drought. EMBO J 28: 3745-3757


Table 1. Rapid turnover protein $K_d$ values vs protein half-life from 12 hour measurements. Proteins marked NA in the predicted half-life column indicates that no peptides were observed from this protein in either of the 12 hour time points.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Annotation</th>
<th>$K_d$ (d$^{-1}$) all data</th>
<th>Predicted half-life in light (hrs)</th>
<th>$K_d$ (d$^{-1}$) alternative</th>
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<tbody>
<tr>
<td>MLOC_11312.1</td>
<td>THI1</td>
<td>1.65</td>
<td>1.8</td>
<td>1.21</td>
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<tr>
<td>MLOC_82117.2</td>
<td>Mg-chelatase (GUN5)</td>
<td>1.59</td>
<td>1.9</td>
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<td>AK359887</td>
<td>CRD1</td>
<td>1.1</td>
<td>2.4</td>
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<tr>
<td>MLOC_9279.1</td>
<td>D1: PSBA photosystem II</td>
<td>0.94</td>
<td>2.5</td>
<td>1.28</td>
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<tr>
<td>MLOC_43949.1</td>
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<td>0.64</td>
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<tr>
<td>AK250295.1, MLOC_19141.1, MLOC_71436.1</td>
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<td>AK356209, AK360524</td>
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<tr>
<td>MLOC_34830.1</td>
<td>disulfide isomerase (APR3)</td>
<td>0.33</td>
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Table 2. Turnover of protein complexes and outliers

<table>
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<tr>
<th>Protein Complex Name</th>
<th>No. barley proteins</th>
<th>No. quantified observations</th>
<th>Average $K_d$</th>
<th>STDEV</th>
<th>Proteins outside 2 STDEV</th>
</tr>
</thead>
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<tr>
<td>Mitochondrial ATP Synthase</td>
<td>8</td>
<td>72</td>
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<td>0.04</td>
<td>-</td>
</tr>
<tr>
<td>Cytosolic Ribosome 60S</td>
<td>24</td>
<td>272</td>
<td>0.04</td>
<td>0.04</td>
<td>-</td>
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<tr>
<td>Cytosolic Ribosome 40S</td>
<td>17</td>
<td>174</td>
<td>0.06</td>
<td>0.04</td>
<td>-</td>
</tr>
<tr>
<td>Chloroplast Ribosome 50S</td>
<td>22</td>
<td>414</td>
<td>0.08</td>
<td>0.06</td>
<td>3.5 STD: RPL33 (plastid encoded) ($K_d$=0.29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.2 STD: S18 (plastid encoded) ($K_d$=0.22)</td>
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<tr>
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<td>133</td>
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<tr>
<td>Chloroplast ATP Synthase PSII</td>
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<td>641</td>
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<td>-</td>
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<td>PSI</td>
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<td>0.04</td>
<td>0.02</td>
<td>2.2 STD-PSAP/CURT1A ($K_d$=0.09)</td>
</tr>
<tr>
<td>Calvin Cycle</td>
<td>8</td>
<td>767</td>
<td>0.11</td>
<td>0.11</td>
<td>2.4 STD: CP12 ($K_d$=0.37)</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** $^{15}$N enrichment ($^{15}$N enrich.) = H/(H+L) ± standard error (n=2 or 3) for the listed amino acids in 80 minute (min) intervals. A. $^{15}$N enrich. levels of amino acids in plants provided with $^{15}$N-label at 2.5 hours into the light period. B. RIA levels of amino acids in plants provided with $^{15}$N-label at 2.5 hours into the dark period. Light periods are marked in white and dark periods in grey.

**Figure 2.** Workflow for processing of mass spectrometry data. A. LC-MS datasets from all time points were compiled and a list of proteotypic peptides characterized using mass, retention time, charge state, and gel fraction number. Extracted ion chromatograms (EICs) were generated for isotopes of each peptide and the EICs used to calculate relative isotopic abundances at each time point and these values to calculate labelled and unlabelled populations via a non-negative least squares regression (NNLS). B. To reduce error in turnover calculations, a series of filters were applied to peptide level calculations. Peptide measurements were filtered first on the characteristics of measured isotopic ratios such as the number of measurable ratios and their distribution. These filters are described more thoroughly in Supplementary Methods. Measurements not meeting these criteria were discarded. Shown are representative data that were kept and discarded. As a second filter, we measured the value of the isotope one Dalton lighter (minus one peak) than the monoisotopic mass. If the ratio of the monoisotope to this minus one peak was less than 5:1, the measurement was discarded as this was a good indicator of overlapping and contaminating peptides. As a third filter, we assessed $^{15}$N enrichment ($^{15}$N enrich) for all peptides from a given time point and all peptides that fell outside two standard deviations of the distribution were removed as we found these peptides were of low quality. Shown in the figure are $^{15}$N enrichment levels for peptides at 24, 48, 72, and 96 hours. Shown on the left is total number of possible quantitation events across all experiments. Then listed beside each filter is the number of quantitation events that were removed by the respective filter with the final number of successful quantitation events listed below.
Figure 3. Example of protein turnover calculation. A. Top, the isotopic envelope represented as isotopic ratios for the peptide, ATLAQLGYEKLDIIGR, from the Fd-GOGAT (MLOC_70866.1), at 24, 48, 72, and 96 hours with all isotopes normalized to the monoisotopic value. Below, as determined by non-negative least squares (NNLS), the natural abundance (NA) form of the peptide, containing zero labelled $^{15}$N atoms, and the heavy (H) form of the peptide, containing one or more $^{15}$N atoms. The relative isotope abundance (RIA) can then be measured as NA/(NA+H). B. The inverse of the natural log (LN) of RIA for all time points for the protein are used to determine a degradation rate for the protein. C. The average $^{15}$N enrichment ($^{15}$N enrich) of labelled peptide populations at different time points through the course of the experiment.

Figure 4. A. Histogram of observed degradation rates. Values ranged over more than two orders of magnitude with a median $K_d$ of 0.08 d$^{-1}$. B. Distribution of degradation rates for different protein functional categories. Using best BLAST matches from Arabidopsis, barley proteins were sorted into categories based on searches of MapCave website (http://mapman.gabipd.org/web/guest/mapcave). The number of proteins in each category is marked to the right of each population. Abbreviations: AA met, amino acid metabolism; C met, carbon metabolism; Cell wall, cell wall metabolism; Co-factor, co-factor synthesis; Devpt., development and cellular organisation; DNA, DNA and RNA metabolism; Lipid, lipid metabolism; Misc, miscellaneous; Nucl, nucleotide metabolism, Photoresp, photorespiration; Photosyn, photosynthesis; Prot deg, protein degradation; Prot syn, protein synthesis and assembly; Redox, redox metabolism; Resp C-met, respiratory carbon metabolism; Resp Ox-P, respiratory oxidative phosphorylation; 2° met, secondary metabolism; Signal, signalling; Stress, stress response; C. Localizations were determined for Arabidopsis blast matches using the Suba website (http://suba.plantenergy.uwa.edu.au/) and organellar populations plotted. The number of observed proteins for each organelle is marked above.
Figure 5. Turnover of proteins in metabolic pathways in barley leaves A. Biosynthetic pathway for the co-factor thiamine pyrophosphate (TPP). Nicotinamide adenine dinucleotide (NAD⁺), cysteine (Cys), and glycine (Gly) are converted to 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (HET-P) via the rapidly degraded THI1. In the other branch of the pathway, another fast-turnover enzyme, THIC, catalyzes the conversion of S-adenosyl methionine (SAM), 5-aminomimidazole ribonucleotide (AIR), and hydroxymethylpyrimidine (HMP) to HMP-phosphate (HMP-P), which is then converted to HMP pyrophosphate (HMP-PP). HET-P and HMP-PP are then converted to thiamine-phosphate (Thiamine-P) and subsequently to thiamine pyrophosphate (TPP) for use as an enzymatic co-factor. B. The tetrapyrrole biosynthetic pathway. In the upper portion of the pathway five proteins: glutamyl tRNA synthetase (tRNAGlu syn), glutamate-1-semialdehyde aminomutase (GSA), porphobilinogen (PBG) deaminase, uroporphyrinogen (URO) decarboxylase, and coprogen oxidase) all had fairly typical degradation rates. In the Mg²⁺ branch of the pathway, leading to chlorophyll biosynthesis, two of the most rapidly degraded proteins in the dataset were observed, a Mg-chelatase subunit (GUN5) and Mg-protoporphyrin IX monomethyl ester cyclase (CRD1) as well as protochlorophyllide reductase (POR). Also observed in this branch were a (CHLM) protein from the Mg-protoporphyrin IX methyl transferase complex and another subunit of the magnesium chelatase complex (CHLI). One protein was classified as a heme binding protein (SOUL) and involved in tetrapyrrole metabolism but has no clearly assigned function. C. Photorespiratory pathway. The oxygenic reaction of Rubisco leads to formation of 2-phospho-glycolate which is converted to glycolate that this exported to the cytosol and enters peroxisomes where it is oxidised to glyoxylate and aminated to glycine by the fast-turnover glycolate oxidase and glutamate:glyoxylate amino transferase. After decarboxylation of glycine and synthesis of serine in mitochondria by the combined action of glycine decarboxylase (GDC) and serine hydroxymethyltransferase (SHMT), the serine re-enters peroxisomes where it is deaminated to hydroxyproline and reduced to glycerate by two rapidly degrading enzymes, serine glyoxylate aminotransferase (SGAT) and hydroxyproline reductase (HPR). Glycerate is then returned to the chloroplast where it is phosphorylated to re-enter the Calvin cycle.
A. Data Assembly

Compile LC-MS/MS Data

Data Extraction & RIA Calculation

B. Filtering Data

Assess Quality of Labeled Population

Check for Contaminants

$^{15}$N enrichment

Peptide #

Unfiltered: 401,296

-335,104

-14,342

Filtered and Measured: 45,448
A. Peptide: ATLAQLGYEKLDDIIGR

Envelopes

NNLS

B. Protein Regression

C. Average $^{15}$N incorporation

$^{15}$N enrich