ANALYSIS OF NITROGEN REALLOCATION FROM SENESCING BARLEY LEAVES: CHARACTERIZATION OF THE INFLUENCE OF A HIGH-GRAIN PROTEIN CONTENT LOCUS ON CHROMOSOME SIX, AND MOLECULAR CLONING AND HETEROLOGOUS EXPRESSION OF A SERINE CARBOXYPEPTIDASE

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Plant Science

MONTANA STATE UNIVERSITY
Bozeman, Montana

January 2007

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January 2007

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ABSTRACT

In cereals, senescence is a highly regulated and organized process in which the nutrients located in the leaves are translocated to the developing seeds. During this process multiple signaling cascades are activated either up-regulating or down-regulating senescence related genes. The majority of the nitrogen to be remobilized is located in the leaves in plastidial proteins, especially Rubisco. These proteins must be degraded before their components can be translocated to be reutilized in the seeds. The mechanism for plastidial protein degradation is largely unknown at this time. Proteases located in lytic vacuolar compartments, including carboxypeptidases, have been found to be up-regulated during senescence, which may indicate their involvement in the degradation of plastidial proteins. In a recent genomic study a serine carboxypeptidase, cp-mIII, was found to be up-regulated in senescing leaves. Further characterization and localization of this protease could lead to an increased understanding of the mechanisms of protein degradation within the leaves during senescence.

After the proteins are degraded they are transported to the developing seeds to be reincorporated into grain storage proteins. The amount of grain protein is noted to vary widely within a species depending on genotype and environmental conditions. Both high and low grain protein content (GPC) are economically important. Previously, a locus on chromosome 6 in barley was discovered that affects GPC. This locus has also been found to be homologous to chromosome 6B in wheat. To determine the effects of this locus, near isogenic lines (NIL) were generated from two varieties, one commonly grown high GPC variety and another known to have relatively low GPC. Several NIL were created where the high GPC allele(s) is isolated in the low GPC background and vice versa. Detailed physiological characterization of this germplasm indicated that the high-GPC trait in barley is associated with (a) earlier/accelerated leaf senescence and N remobilization; (b) enhanced availablity of amino nitrogen in kernels; (c) earlier grain total N accumulation; and (d) differences in whole-plant allocation and reallocation of nitrate. This analysis serves as a basis for the further, molecular analysis of the processes controlling (re)allocation of vegetative nitrogen to seeds in barley.

CHAPTER 1

EFFECTS OF A BARLEY (*HORDEUM VULGARE*) CHROMOSOME SIX GRAIN PROTEIN CONTENT LOCUS ON WHOLE-PLANT NITROGEN REALLOCATION UNDER TWO DIFFERENT FERTILIZATION REGIMES

Abstract

A large fraction of protein nitrogen harvested with crop seeds is derived from N remobilization from senescing vegetative plant parts, while a smaller fraction stems from *de novo* N assimilation occurring after anthesis. We contrasted near-isogenic barley (*Hordeum vulgare* L.) germplasm, varying in the allelic state of a major grain protein content (GPC) locus on chromosome six, controlling ~46% of the genetic variation in this important trait. Plant material was grown under two different N fertilization regimes, designated as low and high. Our analyses indicated that leaf N remobilization occurred earlier in high-GPC germplasm, as indicated by an earlier decrease of total leaf N, chlorophylls, soluble, and membrane proteins. At the same time, kernel amino acid levels were enhanced, while leaf amino acid levels were lower in high-GPC barleys, suggesting enhanced retranslocation of organic N to the developing sinks. As we also detected enhanced/longer availability of leaf nitrates in high-GPC varieties/lines, the GPC locus profoundly influences whole-plant nitrogen allocation and management. Results presented here, together with data from a recent transcriptomic analysis performed in our lab, make a substantial contribution to our understanding of whole-plant nitrogen storage, remobilization and retranslocation to developing sinks.

Introduction

Grain protein content (GPC) varies within a species, largely due to differences in genotype and environmental conditions. This variation has been studied in several cereals including wheat, barley, corn, and rice (Dembinski *et al.* 1995; Onodera *et al.* 2001; See *et al.* 2002; Distelfeld *et al.* 2004; Jukanti *et al.* in press). High GPC is important for nutritional value for both human and animal consumption and also for industries involving bread and pasta quality. In barley low GPC is commercially desirable in the malting industry (von Wettstein 2007). Increasing GPC by breeding efforts has proven to be difficult due to a consistent negative correlation between GPC and yield (Lawlor 2002). In both wheat and barley a genetic approach has been utilized where quantitative trait loci (QTL) for GPC were determined. A QTL on chromosome 6/6B, which (based on currently available mapping data) is homologous in these two species, and controls a large part of the genetic variability in GPC, has been identified (See *et al.* 2002; Mickelson *et al.* 2003; Distelfeld *et al.* 2004; Kade *et al.* 2005; Uauy *et al.* 2006*a,b*). In wheat, it has been demonstrated that the presence of a functional NAC transcription factor at this locus leads to earlier leaf senescence and high GPC, while low-GPC allele(s) are due to the presence of a mutated gene, or its absence (Uauy *et al.* 2006*b*).

Nitrogen is quantitatively the most important mineral nutrient in plants and often limits growth, yield, and quality (Fischer 2007). There are two principal sources of nitrogen available to seeds during development – organic nitrogen from vegetative plant parts, which is the source of the majority of nitrogen, and freshly assimilated nitrogen (Peoples and Dalling 1988). In photosynthetically active leaves, up to 75% of the organic

nitrogen is contained by soluble (stromal) and insoluble (thylakoidal) chloroplast proteins (Hörtensteiner and Feller 2002). Among these, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) alone accounts for ~50% of all plastidial nitrogen (Fischer 2007). During leaf senescence, multiple tightly controlled signaling cascades cause changes in gene expression, ultimately leading to the degradation of macromolecules and retranslocation of resulting nutrients to the developing seeds (Feller and Fischer 1994; Hopkins *et al.* 2007; Lim *et al.* 2007). Available data indicate that the degradation of plastidial proteins is caused or at least initiated by proteolytic enzymes present in the organelle itself. Additional proteases, which mostly accumulate in lytic vacuolar compartments during senescence, may have some role in the degradation of photosynthetic proteins as well (Hörtensteiner and Feller 2002). Interestingly, despite the fact that proteins are being degraded (releasing peptides and amino acids), accumulation of amino acids is not seen in senescing leaves of intact plants. This contrasts with studies utilizing detached leaves for senescence studies, in which enhanced proteolysis is accompanied by amino acid accumulation (Feller and Fischer 1994; Soudry *et al.* 2005). Together, these observations indicate amino acid retranslocation to developing sinks (seeds) in intact plants. In seeds, imported nitrogen is utilized for the synthesis of storage proteins including albumins, globulins and prolamins (termed “hordeins” in barley); hordeins are quantitatively the most important and responsible for 30-50% of total GPC (Qi *et al.* 2006). Total growth rates of developing grains as well as grain protein accumulation could be either source- or sink-controlled; experimental results published over the last thirty years have supported both hypotheses, depending on the species or

genotypes investigated (Krapp and Stitt 1995; Roitsch 1999; Lim *et al.* 2006; Barneix 2007).

From a physiological point of view, it appears reasonable to assume that contrasting different nitrogen metabolism-associated parameters in near-isogenic germplasm differing in GPC will identify processes, which contribute to grain protein accumulation. To explore this approach, we compared total leaf N, chlorophyll, protein, amino acid and nitrate levels as well as total kernel N and amino acid levels in near-isogenic barley lines differing in the allelic state of the major GPC locus on chromosome six. Experiments were performed at two different N fertilization levels prior to anthesis, to study the influence of the plant's N status on N retranslocation and kernel N accumulation. These analyses expand and complement a recent study from our laboratory, which contrasted the transcriptomes of the same lines (analyzed at the higher fertilization level), identifying several genes with potentially important functions in the control of the leaf senescence process (Jukanti *et al.* in press).

Material and Methods

Plant Material and Growth Conditions

Two barley (*Hordeum vulgare* L.) varieties, 'Karl' and 'Lewis,' were used as parents to create a GPC mapping population. 'Karl,' a six-rowed variety, was chosen due to its production of grain with consistently lower protein content compared to most other varieties. 'Lewis,' a two-rowed variety, was selected because it is a commonly grown barley and is known to have relatively high grain protein content (See *et al.* 2002;

Mickelson *et al.* 2003). Near-isogenic lines (BC_4F_2), denoted as '10_11', '49_43', '10_7' and '49_46' were created by backcrossing two recombinant inbred lines (from the original mapping population) containing the high-GPC locus on chromosome 6 with the low-GPC parent ('Karl'), followed by selection for molecular makers HVM74 and ABG458. Additional NIL (BC_4F_2; denoted '21_7', '44_11' and '44_27') were obtained after backcrossing low-GPC recombinant inbred lines with the high-GPC parent, 'Lewis'; details of all crosses are described in Jukanti *et al.* (in press). Homozygous BC_4F_2 plants were grown in 2005 at the Arthur Post Research Farm in Bozeman, MT in head rows, in a randomized block design. Flag leaves and ears from this experiment were collected at 14 and 21 days past anthesis (dpa), shock-frozen in liquid nitrogen and stored at -80 ºC until analysis for free amino nitrogen. BC_4F_3 seeds from these experiments were utilized for all subsequent experiments presented in this study. 'Karl', '10_11' and '49_43' used for the high-N treatment were planted on September 29, 2005, while the other varieties/lines ('Lewis', '21_7', '44_11') as well as 'Karl', '10_11' and '49_43' subjected to low N fertilization were planted on February 6, 2006 in a small randomized block design containing four ('Karl'/'10_11'/'49_43'; high N) or three (all other experiments) blocks per genotype in a greenhouse of the Montana State University Plant Growth Center. A 22/18 ºC day/night temperature regime was maintained in the greenhouse and days were extended to a 16 h photoperiod when required, using Son-Agro 430 W high-pressure sodium lamps (Philips, Somerset, NJ). Plants in potting soil were treated with 250 mL of Peter's Professional General Purpose fertilizer 20-20-20 (4 g L^{-1}, Scotts-Sierra Horticultural Products Company, Marysville, OH) per one-gallon pot

with three plants every alternate week until anthesis, starting at two weeks post germination (low N treatment). Plants subjected to high N treatment additionally received 100 mL 16 mM KNO_3 (Sigma-Aldrich, St. Louis, MO) per pot, twice per week from germination to anthesis. Plants in all experiments reached anthesis between 55 and 63 days past planting, and each shoot used for further experimentation was tagged with its exact anthesis date. Samples were harvested at 7, 14, 21, 28 and 35 dpa, immediately shock-frozen in liquid nitrogen and stored at -80 ºC until analysis. For each harvest time point, three ('Lewis'/'21_7'/'44_11', low and high N treatment; 'Karl'/'10_11'/'49_43', low N treatment) or four ('Karl'/'10_11'/'49_43', high N treatment) samples from a variety of plants consisting of flag leaves, second leaves (below the ear) or ears from 8-10 plants were collected.

Total Nitrogen and Soluble α-NH_2 Nitrogen

Frozen immature leaf and grain material was ground into a fine powder with a mortar and pestle under liquid nitrogen. Mature grain samples were milled into fine flour with a Perten mill (Perten Instruments, Inc; Springfield, IL). Mature leaf samples were ground to a fine powder with a commercially available coffee mill.

Total nitrogen was quantified through a combustion method using a LECO FP-528 (LECO Corp., St. Joseph, MO) nitrogen analyzer. Three or four biological replicates of developing and mature leaves and grains were analyzed. Soluble α-NH_2 nitrogen was extracted and assayed as described by Mickelson *et al.* (2003), using 2,4,6-trinitrobenzene sulfonic acid (TNBS). Three or four biological replicates of developing leaves and grains were assayed for each data point.

Soluble and Insoluble (membrane) Proteins

Proteins were extracted as described by Parrott *et al.* (2007) from ground leaves grown under low nitrogen conditions only. As extraction buffer, 25 mM Tris-HCl pH 7.5 with 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) was used. Samples were prepared with Laemmli (1970) sample buffer and analyzed on 13% (w/v) acrylamide gels in a Hoefer (San Francisco, CA) minigel apparatus. Each lane represents proteins extracted from three or four independent samples, with 8-10 leaves contributing to each sample to minimize the influence of leaf-to-leaf variation. Proteins loaded in each lane correspond to material extracted from 0.83 mg fresh weight for soluble proteins, and to 1.1 mg fresh weight for insoluble proteins. All proteins were visualized with Coomassie Brilliant Blue R-250.

Chlorophyll and Nitrate Quantification

Three or four biological replicates of developing leaves harvested from plants grown under high and low nitrogen conditions were extracted with 80% (v/v) acetone and analyzed spectrophotometrically at 649 and 665 nm as outlined by Feller and Fischer (1994). Three or four replicates of developing leaves were also analyzed for nitrates, using the nitrate reductase / sulphanilamide-N-naphthylethylenediamine method as described by Mickelson *et al.* (2003), using liquid nitrogen-ground leaves as starting material.

Results

To confirm the influence of the GPC locus under the selected experimental conditions, GPC was determined in the parental varieties ‘Karl’ and ‘Lewis’ as well as in their respective near-isogenic lines (NIL). Our data demonstrate a ~2% increase in GPC in NIL ‘10_11’ and ‘49_43’, containing the high-GPC locus in the low-GPC (‘Karl’) parental background under high N fertilization conditions (Fig. 1*a*). GPC was also increased in these lines under low N fertilization (Fig. 1*b*), albeit to a lesser degree. Similarly, GPC was lowered in near-isogenic lines, in which the low-GPC locus was crossed into the high-GPC parental background (‘21_7’, ‘44_11’; Fig. 1*c* and *d*). In this situation, observed differences between ‘Lewis’ and its NIL were somewhat higher under low N fertilization conditions. Overall, these data confirm the expected phenotype for all plant materials used. To understand if GPC differences are due to higher protein accumulation during the early or late grain developmental stages, we assayed the developing kernels of parents and corresponding NIL for total nitrogen content (Fig. 2). Samples are shown in weekly intervals from 7 to 35 dpa. Under both high- and low-nitrogen conditions for ‘Karl’ and its respective high-GPC NIL, earlier and higher accumulation of nitrogen is seen in the high-GPC lines (Fig. 2*a* and *b*). This trend is also seen under low nitrogen conditions where ‘Lewis’ displays earlier and higher accumulation of nitrogen than its corresponding low-GPC NIL (Fig. 2*d*). Somewhat surprisingly this trend is not observed for ‘Lewis’ and its low-GPC NIL under high nitrogen conditions (Fig. 2*c*).

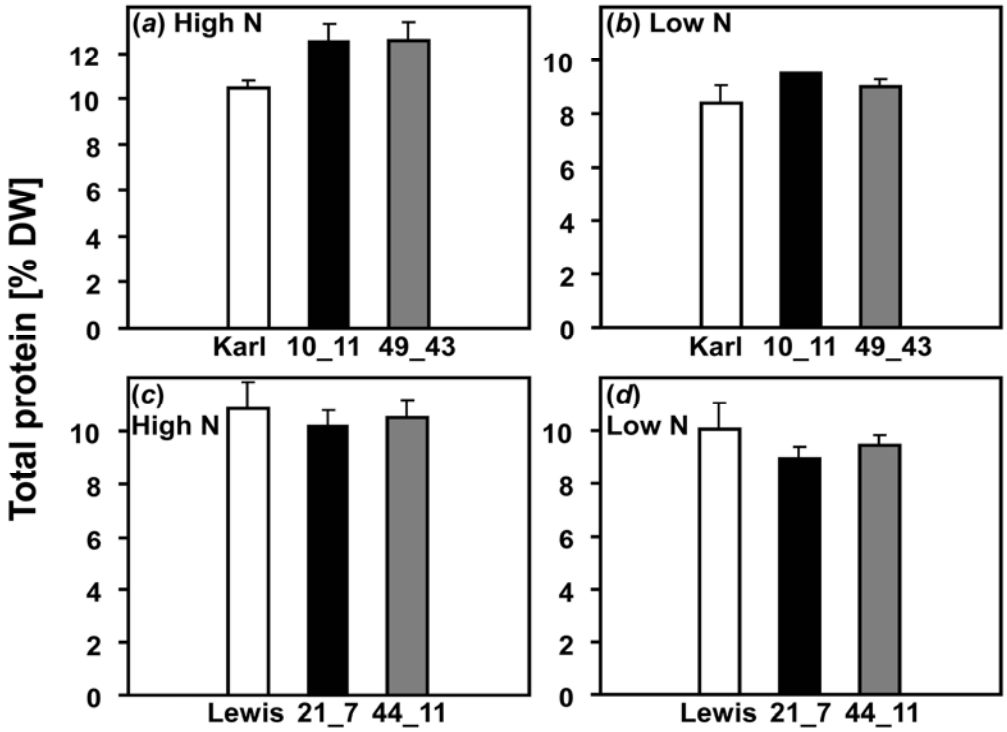


Figure 1. Comparison of total protein content in mature grains of germplasm used for this study. Low-GPC parent variety 'Karl' was compared with its high-GPC NIL '10_11' and '49_43'; and high-GPC parent variety 'Lewis' was compared with its low-GPC NIL '21_7--' and '44_11'. High N and low N refer to the two different N fertilization regimes utilized (see materials and methods). Means and standard deviations of at least three biological replicates are shown. DW, dry weight.

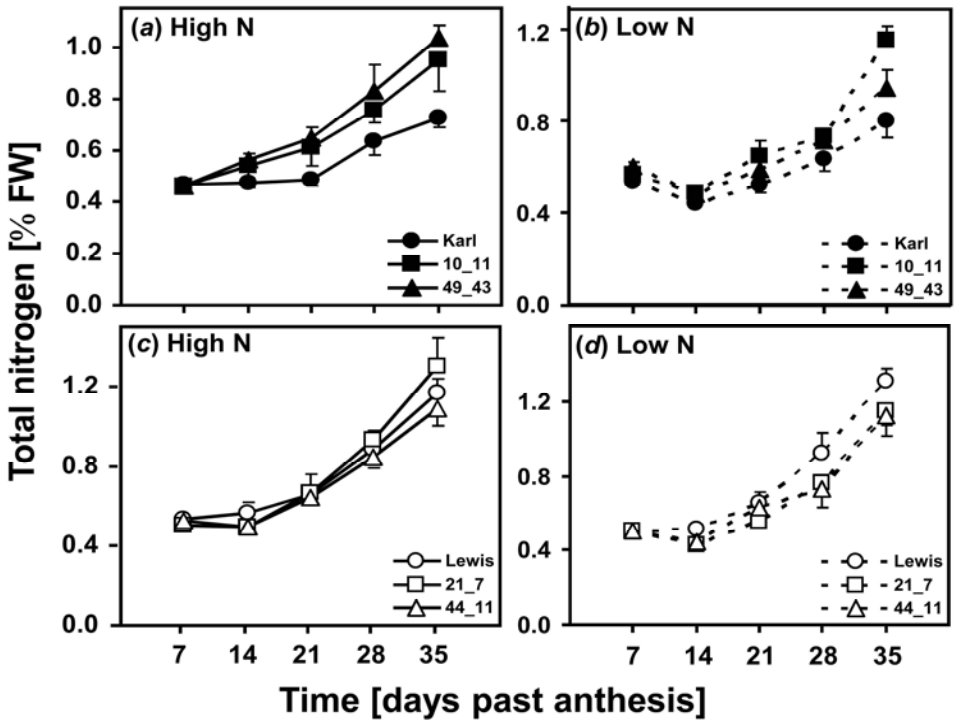


Figure 2. Total nitrogen in developing kernels of parental varieties and respective NIL under either high- or low-nitrogen experimental conditions. For details on germplasm see legend of Fig. 1. Means and standard deviations of at least three biological replicates are shown. FW, fresh weight.

Data in Fig. 2 indicate that high GPC in mature kernels is due to earlier protein accumulation during kernel development. As amino acids are the substrate for protein synthesis, their levels were analyzed in developing kernels of parents and respective NIL (Fig. 3). At earlier stages in development high-GPC lines display higher levels of free amino acids than low-GPC lines. These increased levels are seen in a range of 7 or 14 to 21 dpa, depending on genotypes and fertilization levels investigated (Fig. 3*a*-*d*). Free amino acid levels decrease over time in all varieties/lines, most likely due to their incorporation into proteins. To confirm these data we also analyzed amino acid levels in kernels of parents and several NIL grown in the field (Fig. 3*e* and *f*). The trends shown in

greenhouse experiments are repeated here, confirming a higher availability of free amino acids in developing kernels of high-GPC germplasm (especially at 14 dpa).

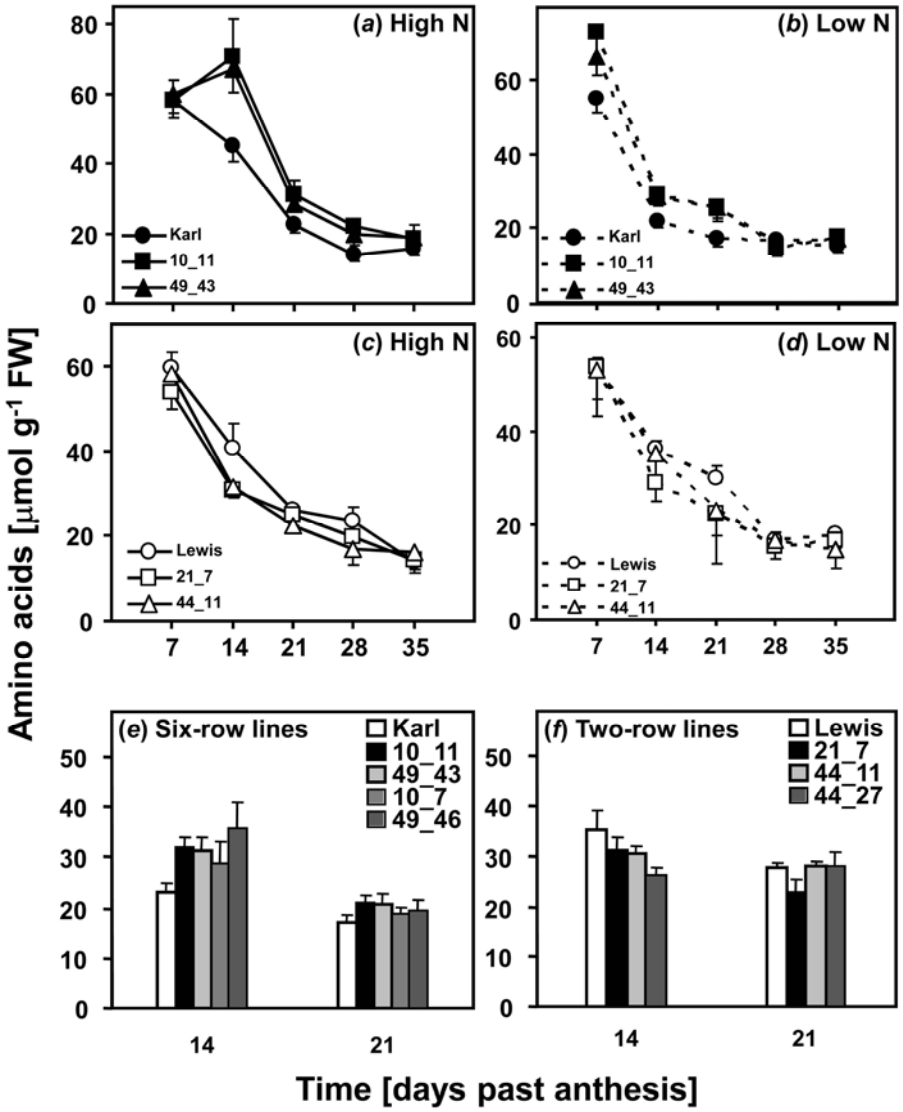


Figure 3. Amino acid content in developing kernels of parental varieties and respective NIL under either high- or low-nitrogen experimental conditions. The bottom two frames refer to field-grown material. For details on germplasm see legend of Fig. 1. Means and standard deviations of at least three biological replicates are shown. FW, fresh weight.

Most of the nitrogen utilized in grain protein development is remobilized from vegetative plant parts (Peoples and Dalling 1988; Feller and Fischer 1994). To determine the importance of leaf N remobilization for kernel protein accumulation, the amounts of nitrogen in mature (dry) flag and second leaves have been quantified (Fig. 4*a*-*h*). Under both high- and low-nitrogen conditions, the flag and second leaves of 'Lewis' showed somewhat lower levels of nitrogen than its low-GPC NIL, which may indicate more efficient nitrogen remobilization to the kernels (Fig. 4*c*,*d*,*g* and *h*). However, this trend was not observed among 'Karl' and its corresponding NIL under either high- or low-N conditions in flag or second leaves (Fig. 4*a*,*b*,*e* and *f*). This indicates that low-GPC lines are not impaired in leaf N remobilization, but may remobilize leaf nitrogen somewhat later. To examine the correlation between developmental nitrogen levels in the leaves and grain protein accumulation, amounts of nitrogen in developing flag and second leaves were measured (Fig. 5*a*-*h*). In these assays, nitrogen levels in high-GPC lines were typically, though not in every case, lower than in low-GPC lines; depending on the varieties/lines and fertilization regime, this trend was clearly observed starting at 21 dpa (Fig. 5*b*,*c*,*f* and *g*). As faster leaf N remobilization suggests earlier onset of leaf senescence, and as chlorophyll degradation is a highly visual hallmark of this process, we determined chlorophyll levels in flag and second leaves of all investigated germplasm (Fig. 6*a*-*h*). As suspected, chlorophylls are degraded more quickly in high-GPC lines although, as for total N, this is not seen in all situations. Differences in chlorophyll degradation are most clearly visible in flag leaves of 'Karl' and its high-GPC NIL under low N fertilization (Fig. 6*b*).

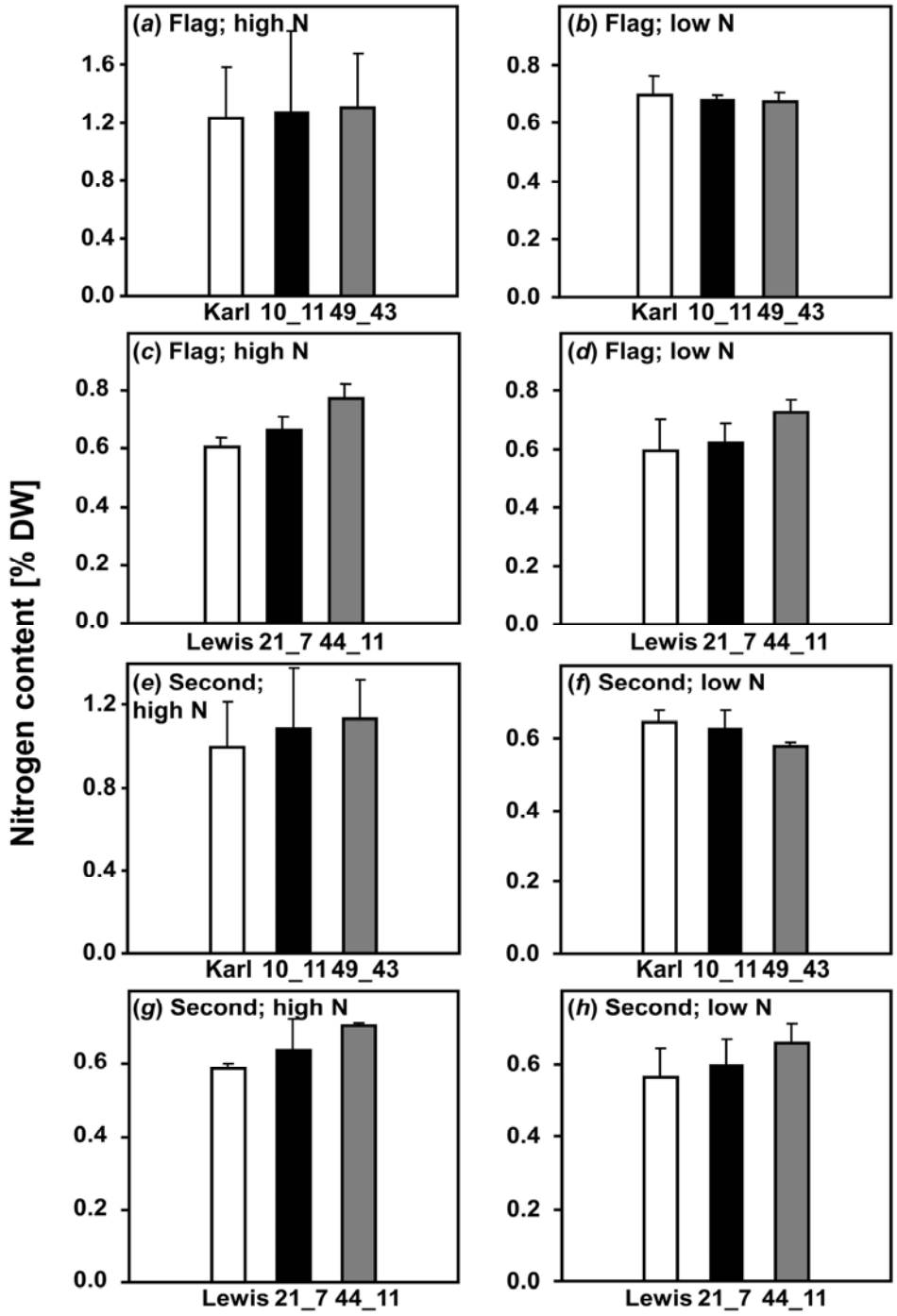


Figure 4. Total nitrogen in mature (fully senesced) flag and second leaves of parental varieties and respective NIL under either high- or low-nitrogen experimental conditions. For details on germplasm see legend of Fig. 1. Means and standard deviations of at least three biological replicates are shown. DW, dry weight.

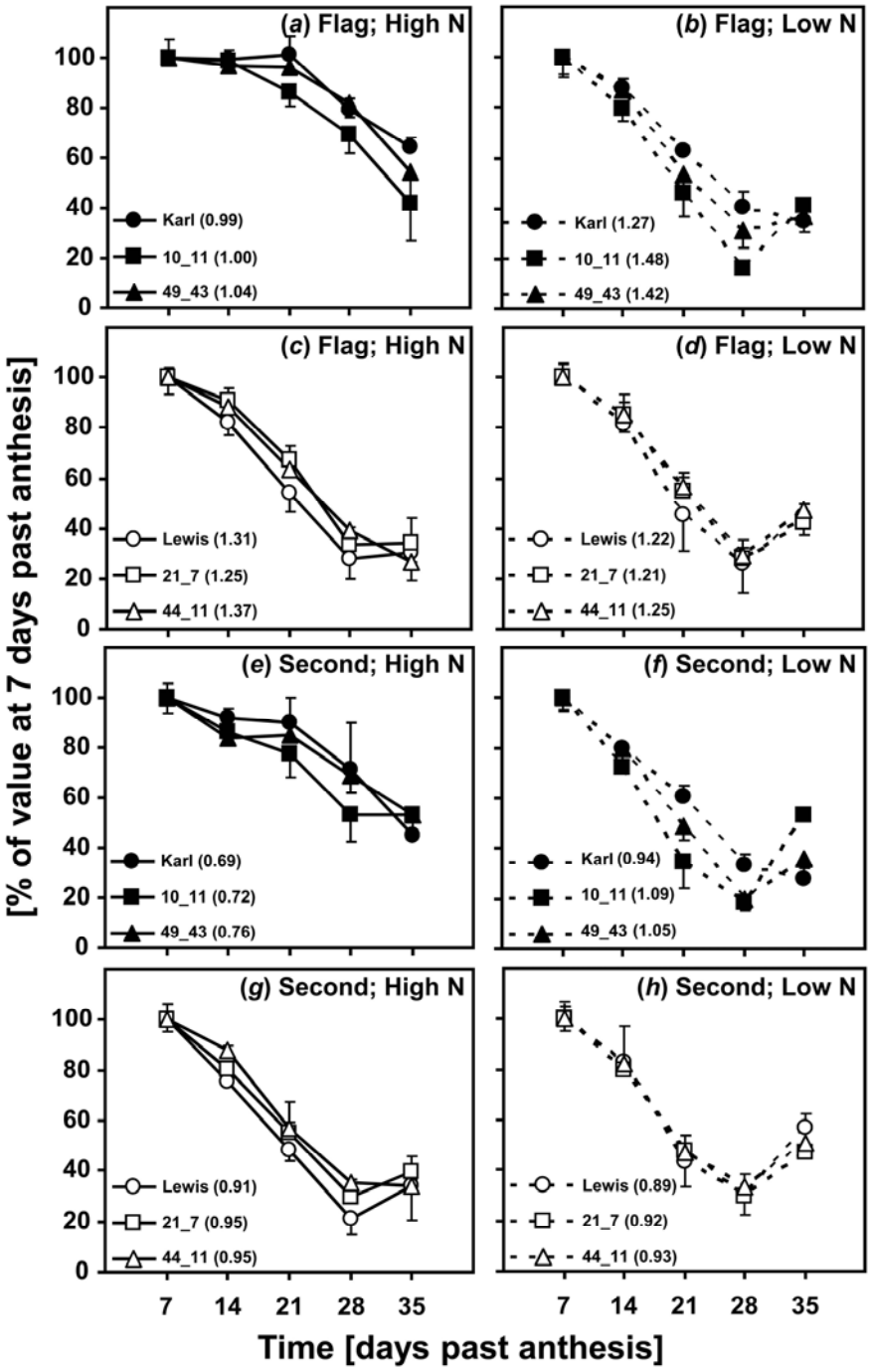


Figure 5. Total nitrogen in senescing flag and second leaves of parental varieties and respective NIL under either high- or low-nitrogen experimental conditions. Relative values (with N contents at 7 days past anthesis = 100%) are given. Data printed in the panels represent absolute values (N content in % of fresh weight) at 7 days past anthesis. For details on germplasm see legend of Fig. 1. Every data point represents the mean and standard deviation of at least three biological replicates.

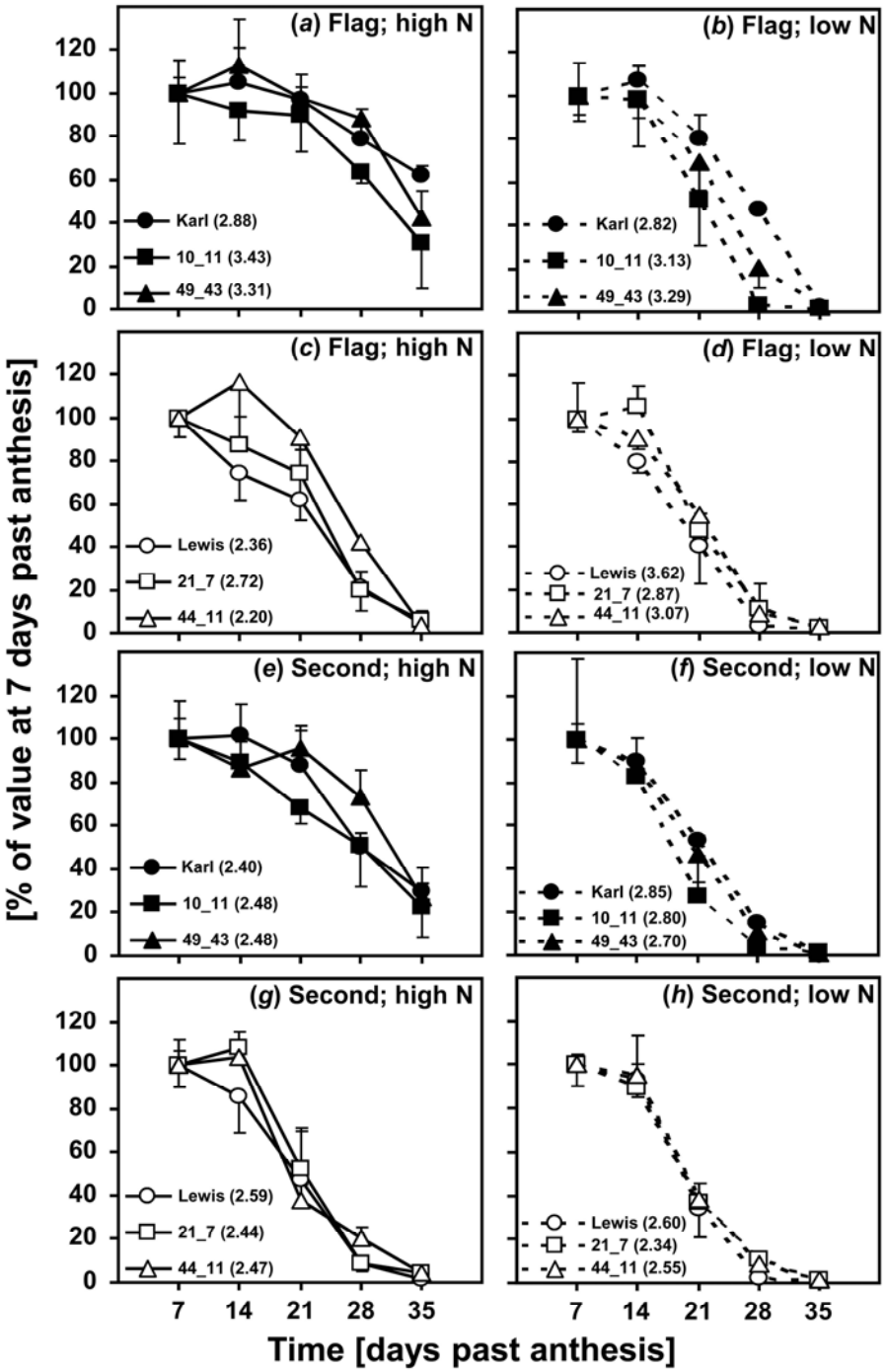


Figure 6. Chlorophyll content in senescing flag and second leaves of parental varieties and respective NIL under either high- or low-nitrogen experimental conditions. Relative values (with chlorophyll contents at 7 days past anthesis = 100%) are given. Data printed in the panels represent absolute values (chlorophyll content in mg g-1 fresh weight) at 7 days past anthesis. For details on germplasm see legend of Fig. 1. Every data point represents the mean and standard deviation of at least three biological replicates.

Around 60% of total protein in photosynthetic cells is contained in soluble proteins and 40% by insoluble proteins (Peoples and Dalling 1988). Of all the plastidial proteins degraded during senescence, around 50% of nitrogen comes from ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Fischer 2007). To compare levels of proteins over time between high- and low-GPC lines, soluble proteins were extracted and visualized by SDS-PAGE in senescing flag and second leaves of low N-fertilized plant material (Fig. 7*a*-*d*). Large (LS) and small subunits (SS) of Rubisco are indicated in the figure. As suspected, proteins are degraded somewhat earlier in high-GPC lines. This is seen in the flag leaves of 'Karl' and its corresponding NIL, especially 10_11 (compare protein levels at 28 dpa; Fig. 7*a*), and to a lesser degree in 'Lewis' and its NIL (compare protein levels at 21 dpa; Fig. 7*c*). Overall lower protein levels are seen in the second leaves (especially for 'Karl' and its NIL), but the same trends shown in flag leaves still persist (Fig. 7*b* and *d*). Insoluble proteins are also thought to be a source of N during senescence (Tang *et al.* 2005) and thus were extracted and visualized by SDS-PAGE in developing flag and second leaves (Fig. 8*a*-*d*). Depending on the variety(ies)/line(s) compared, subtle differences in the velocities of membrane protein degradation were observed, again with somewhat higher protein stability in low-GPC germplasm.

Amino acids are typically the main transport form of organic nitrogen from sources to sinks (Mae 2004). Amino acid levels for developing flag and second leaves were determined (Fig. 9*a*-*h*). Under high-nitrogen conditions 'Lewis' shows lower levels of amino acids compared to its low-GPC NIL in both flag and second leaves (Fig. 9*c* and *g*), and the same trend is observed in flag leaves at low N fertilization levels (Fig. 9*d*).

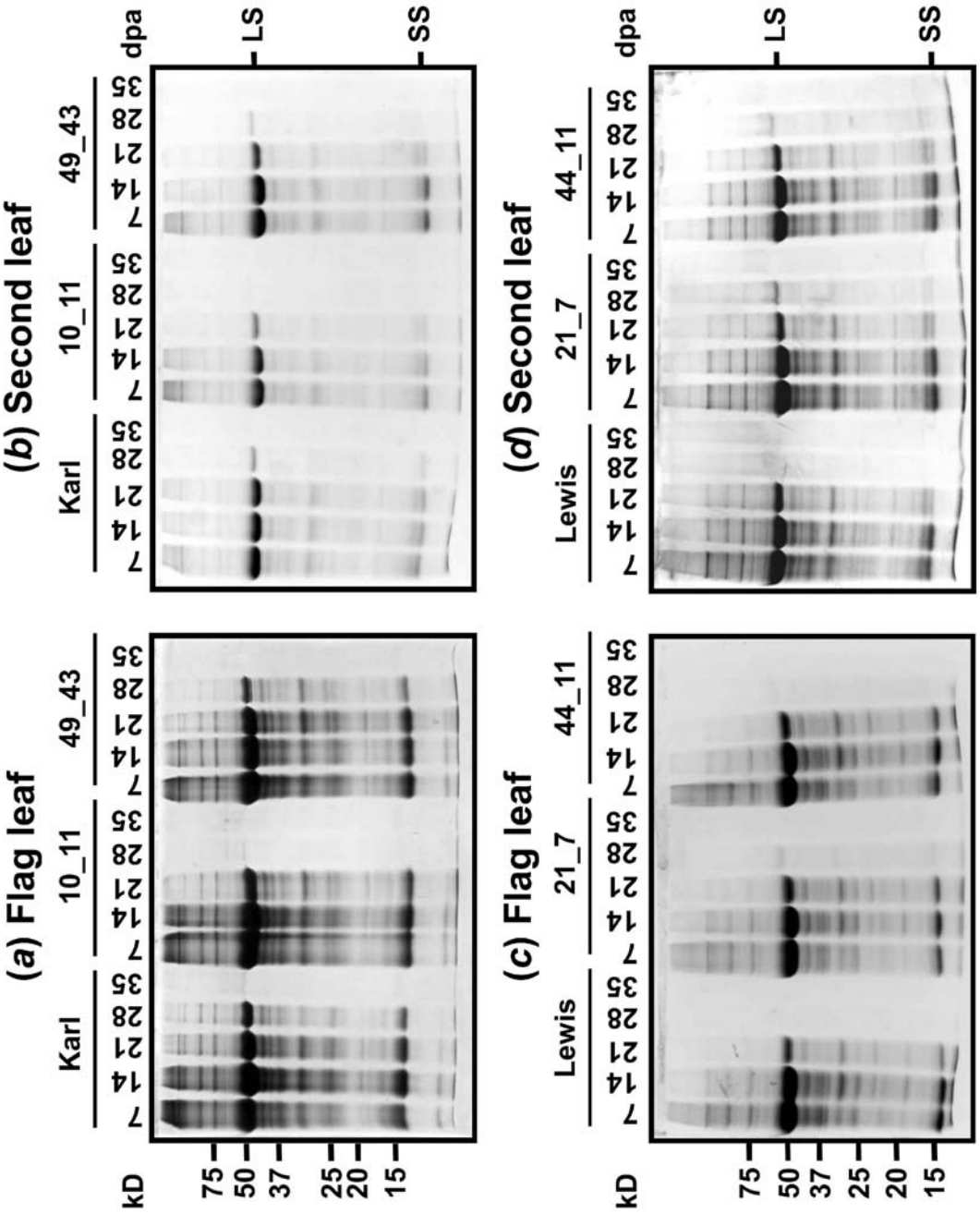


Figure 7. SDS-PAGE analysis of soluble proteins in senescing flag and second leaves of parental varieties and respective NIL under low-nitrogen experimental conditions. For details on germplasm see legend of Fig. 1. Each lane represents proteins mixed from three or four biological replicates, with 8-10 leaves contributing to each sample. Proteins separated in each lane correspond to material extracted from 0.83 mg leaf fresh weight. The positions of the large (LS) and small (SS) subunits of Rubisco are indicated on the right side of the panels.

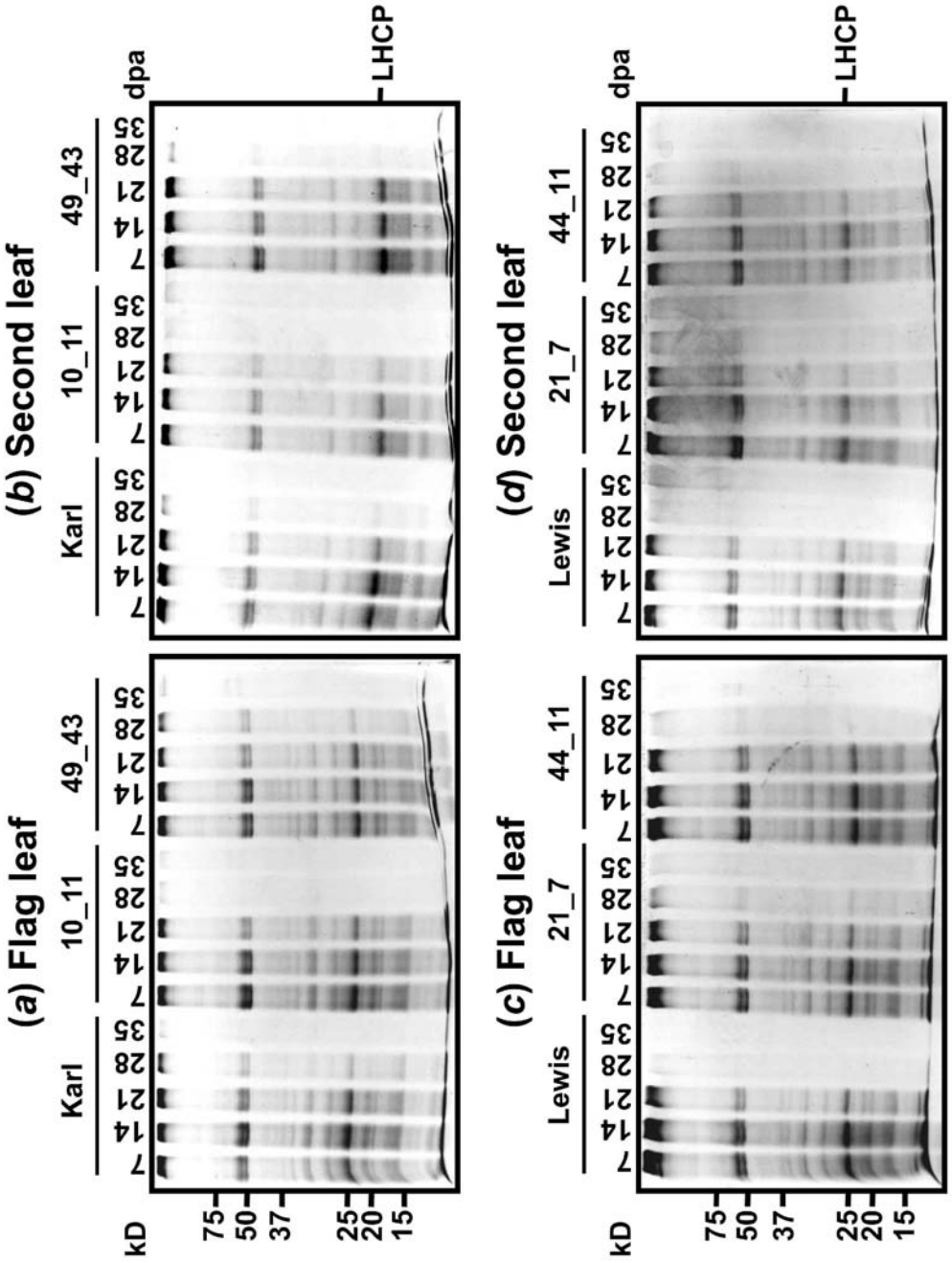


Figure 8. SDS-PAGE analysis of membrane proteins in senescing flag and second leaves of parental varieties and respective NIL under low-nitrogen experimental conditions. For details on germplasm see legend of Fig. 1. Each lane represents proteins mixed from three or four biological replicates, with 8-10 leaves contributing to each sample. Proteins separated in each lane correspond to material extracted from 1.1 mg leaf fresh weight. The position of LHCP (light-harvesting chlorophyll-binding protein) is indicated on the right side of the panels.

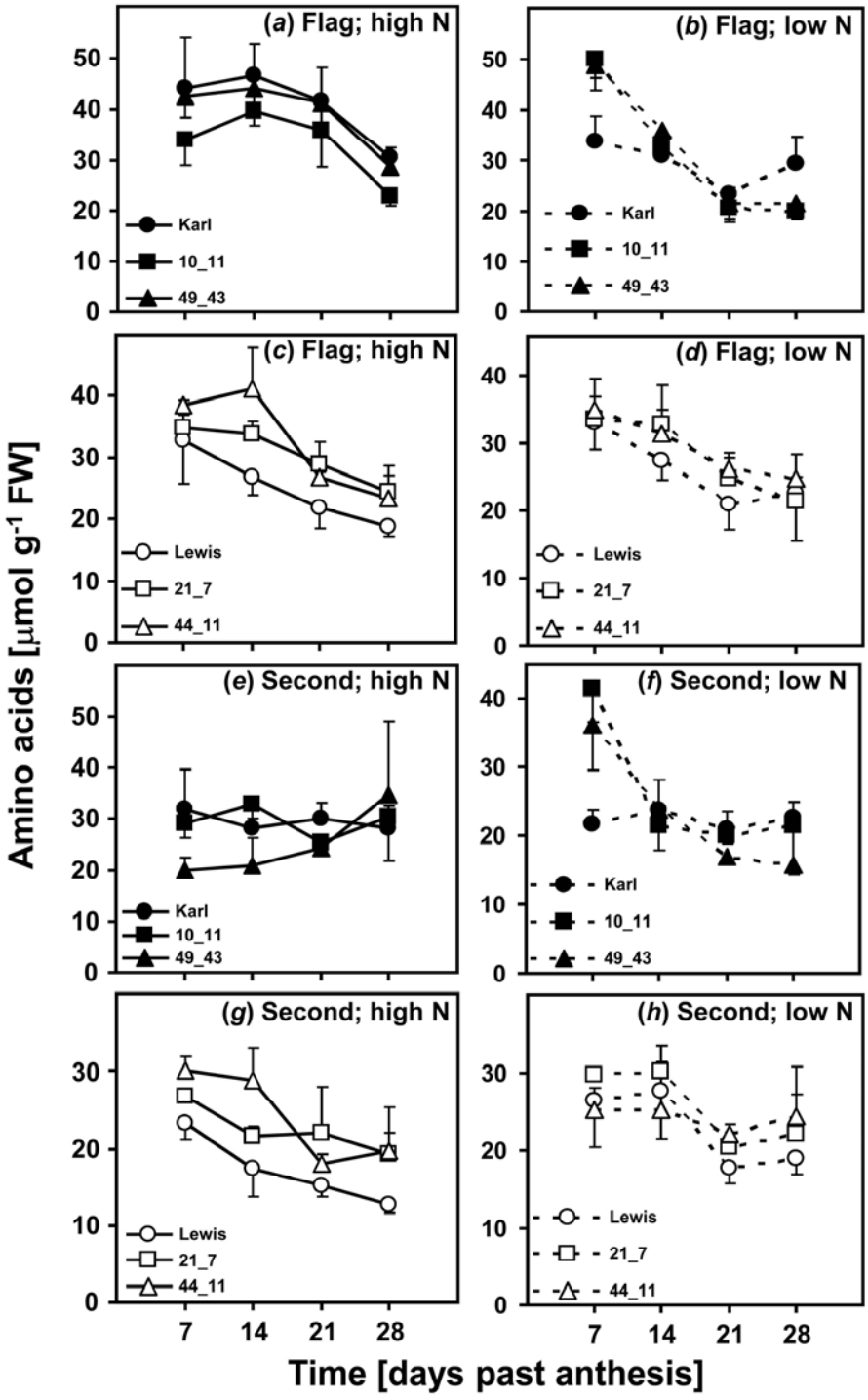


Figure 9. Amino acid content in senescing flag and second leaves of parental varieties and respective NIL under either high- or low-nitrogen conditions. For details on germplasm see legend of Fig. 1. Every data point represents the mean and standard deviation of at least three biological replicates. FW, fresh weight.

These differences are not observed in six-row barley ('Karl' and its near-isogenic lines; Fig. 9*a*,*b*,*e* and *f*). Therefore, in 'Lewis', higher amino acid levels in developing kernels are correlated with lower levels in senescing leaves, suggesting enhanced N remobilization. To confirm these data, we also analyzed field-grown material at 14 and 21 dpa (Fig. 10*a*-*d*). Again, lower levels of amino acids are seen in 'Lewis' (high-GPC variety) for both flag and second leaves compared to its low-GPC NIL (Fig. 10*b* and *d*), but no significant differences are seen between 'Karl' and its high-GPC NIL in flag or second leaves (Fig. 10*a* and *c*).

The most important uptake and storage form of inorganic nitrogen for most plants is nitrate (Miller *et al.* 2007); therefore, we compared nitrate levels in senescing flag and second leaves of high- and low-GPC germplasm (Fig. 11*a*-*h*). Under low-nitrogen conditions overall nitrate levels were low and comparable among all of the lines, except at 7 dpa where nitrate levels are higher in high-GPC lines (Fig. 11*b*,*d*,*f* and *h*). However, after high-N fertilization prior to anthesis, nitrate levels in 'Karl' flag and second leaves were depleted at 14 dpa, whereas levels increased or remained stable up to 21 dpa in its high-GPC NIL (Fig. 11*a* and *e*). A somewhat extended presence of nitrate was also observed in 'Lewis' as compared to its low-GPC NIL, although not as clearly (Fig. 11*c* and *g*). As fertilization was stopped in both (low- and high-N) experiments at anthesis, our data suggest important differences in the management of inorganic (in addition to organic, as outlined above) nitrogen between near-isogenic low- and high-GPC germplasm. Furthermore, our data indicate a high variability in leaf nitrate contents (see error bars in Fig. 11). This represents genuine biological variation in the investigated

plant material, as extractions for most data points were performed twice, with similar results.

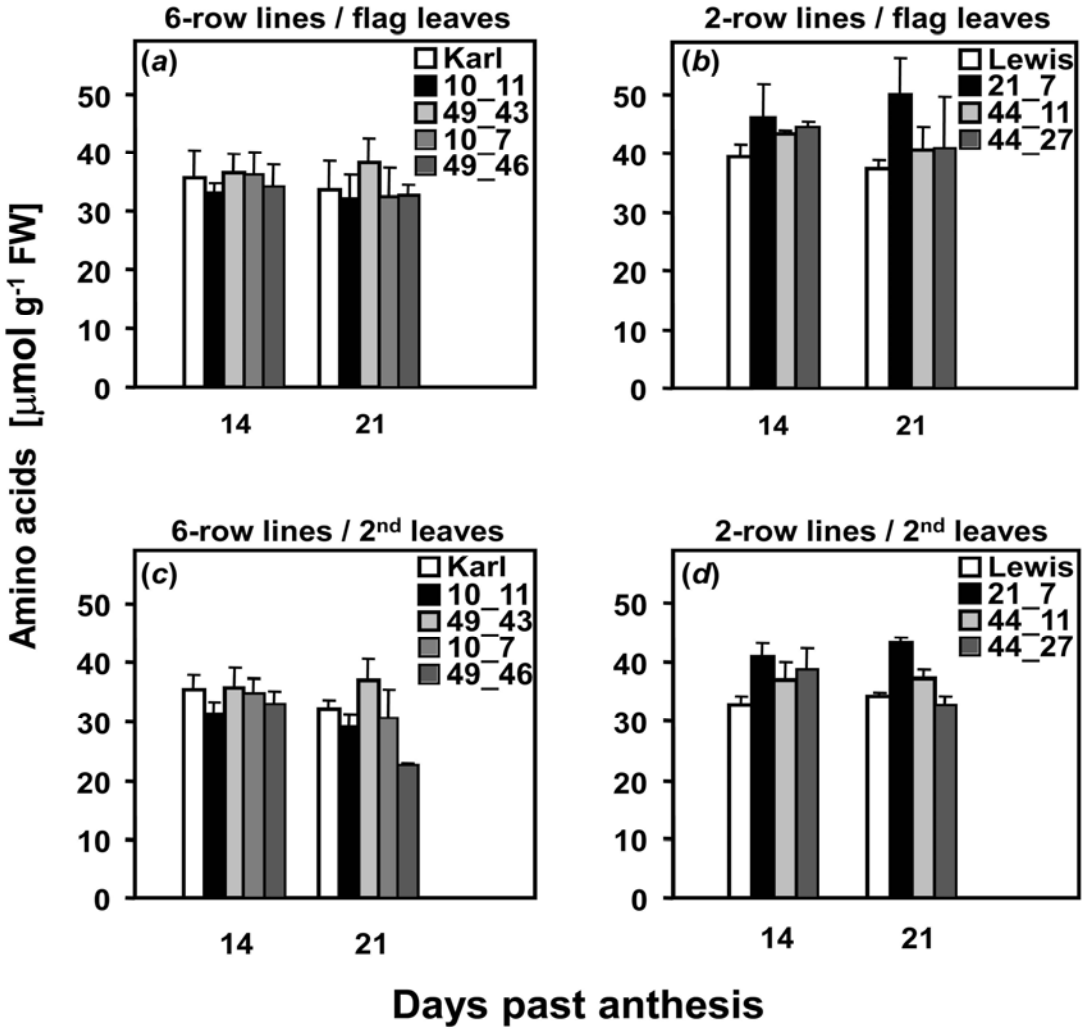


Figure 10. Amino acid content in developing flag and second leaves of parental varieties and respective NIL from field-grown material. For details on germplasm see legend of Fig. 1. Every data point represents the mean and standard deviation of at least three biological replicates. FW, fresh weight.

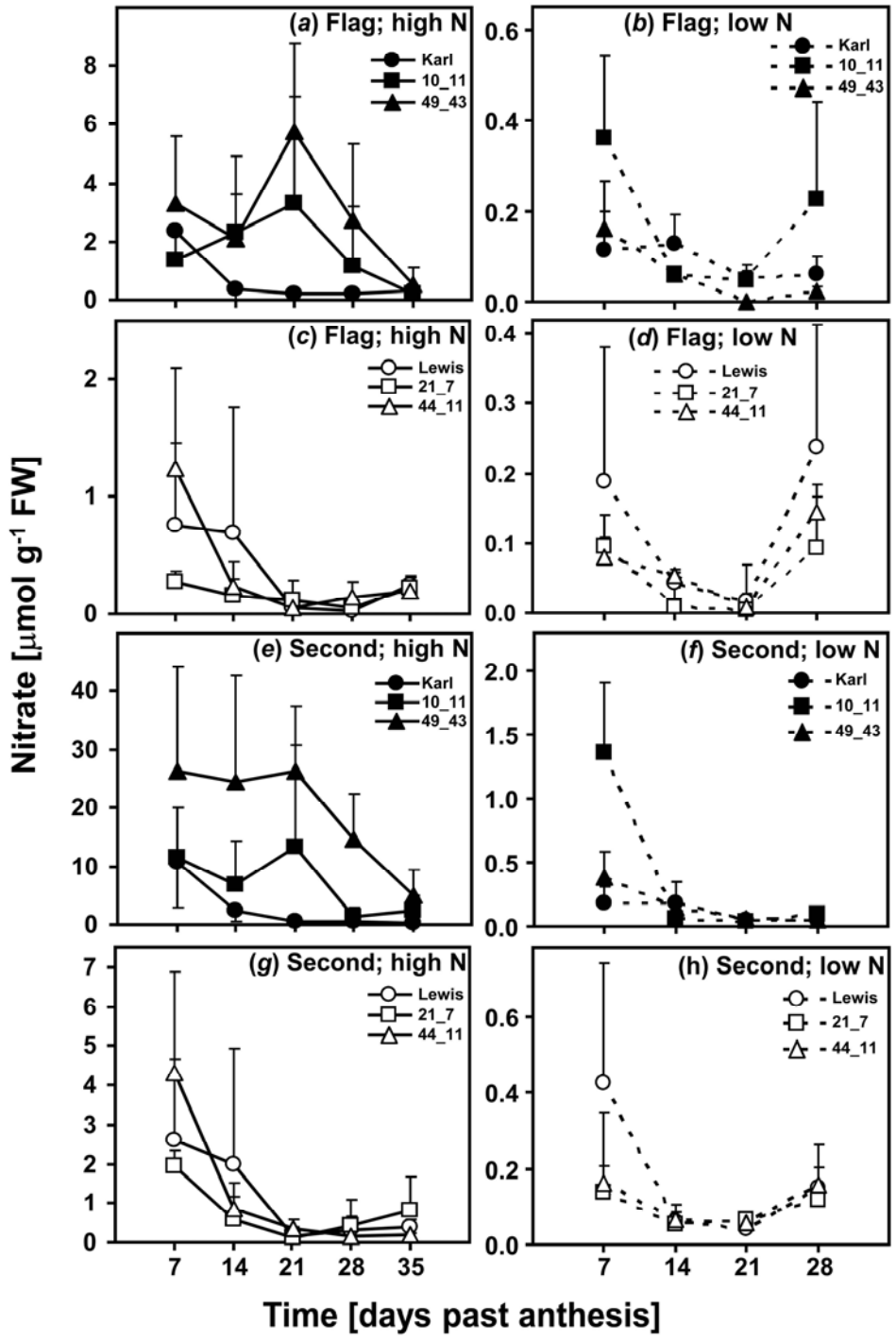


Figure 11. Nitrate levels in developing flag and second leaves of parental varieties and respective NIL under either high- or low-nitrogen conditions. For details on germplasm see legend of Fig. 1. Every data point represents the mean and standard deviation of at least three biological replicates. FW, fresh weight.

Discussion

In this manuscript, we utilized a genetic approach to further our understanding of whole-plant nitrogen reallocation. Previous experiments have led to the identification of a major GPC locus on barley chromosome 6. This locus controls ~46% of the genetic variability in the GPC trait, but somewhat unexpectedly did not co-localize with yield QTL, or with other QTL relevant for plant N metabolism (See *et al.* 2002; Mickelson *et al.* 2003, Yang *et al.* 2004). Based on the mapping population developed for these previous studies, near-isogenic lines were created where the high-GPC allele(s) were isolated in the low-GPC background and vice versa. The resulting lines showed the expected phenotype (Fig. 1; Jukanti *et al.* in press), with up to 2% differences in grain protein concentration. Most grain protein nitrogen in cereals is derived from its remobilization and retranslocation from senescing vegetative plant parts (Feller and Fischer 1994). However, the molecular and cellular details of nitrogen reallocation, including leaf protein degradation and N transport and import into developing seeds, are only incompletely understood. From a physiological point of view, near-isogenic germplasm varying in a major GPC locus represents an ideal tool to analyze these processes. In such germplasm, differences between NIL and their respective parents are directly or indirectly related to nitrogen reallocation to the developing grains.

Taken together, our data suggest an earlier and/or higher availability of organic nitrogen during grain development in high-GPC germplasm, leading to an earlier accumulation of grain protein, most likely in the form of storage proteins (Figs. 2-11; Jukanti *et al.* in press). This interpretation is supported by the upregulation of storage

protein genes, of genes involved in protein folding (peptidyl-prolyl cis/trans isomerases or cyclophilins, protein disulfide isomerases, numerous heat shock proteins), and of genes involved in nitrogen transport and metabolism including several aminotransferases and an asparagine synthetase in developing kernels of the high-GPC NIL '10_11' as compared to its low-GPC parent, 'Karl' (Jukanti *et al.* in press). From a basic point of view, this enhanced nitrogen availability may be due either to increased sink or source strength; furthermore, our data would be compatible with earlier leaf senescence and nitrogen remobilization, with enhanced post-anthesis nitrogen uptake/assimilation, or a combination of these two possibilities. Total nitrogen levels in fully senesced leaves of both low- and high-GPC germplasm indicate that low-GPC lines are not impeded in nitrogen remobilization (Fig. 4). On the other hand, total nitrogen and chlorophyll data (Figs. 5 and 6) in senescing leaves indicate earlier leaf senescence and nitrogen remobilization, with some variability depending on genetic background and nitrogen nutrition levels. Assays of soluble and membrane protein levels by SDS-PAGE (Figs. 7 and 8) identified Rubisco and, to a lesser degree, other soluble and thylakoid proteins as sources of (earlier) remobilized nitrogen. Interestingly, while kernel amino acid levels are enhanced in high-GPC germplasm (at 14 to 21 dpa; Fig. 3), there is a trend to lower leaf amino acid levels in these same lines (Figs. 9 and 10), suggesting enhanced organic N transport to the sinks. Although this interpretation would need direct confirmation with (phloem) transport analyses, it is again compatible with our previous transcriptomic results. These data indicated upregulation of numerous (both plastidial and extraplastidial) leaf proteases, some leaf membrane transporters, as well as genes

potentially involved in import of organic nitrogen from the apoplast in developing seeds of high-GPC germplasm, mostly at 21 dpa (Jukanti et al. in press). Together, data from the previous study and this manuscript confirm that our experimental system allows substantial progress in this area, and identify a number of candidate genes potentially involved in both leaf protein degradation and long-distance N retranslocation in cereals. It should also be noted that our data are largely compatible with analyses in wheat, in which the homologous locus on chromosome 6B was associated with earlier leaf senescence and N reallocation (Uauy *et al.* 2006*a*,*b*).

To test the possibility that *de novo* nitrogen assimilation is also (in addition to increased nitrogen remobilization) responsible for earlier/higher nitrogen availability in developing kernels of high-GPC germplasm, we analyzed flag and second leaf nitrate levels (Fig. 11). Since nitrate is considered phloem-immobile, and developing kernels are fed by the phloem, it was unnecessary to extend these experiments to grains (Feller and Fischer 1994; Marschner 1995). As expected, nitrate levels were largely negligible in plants kept on the low-nitrogen regime. In contrast, substantial amounts of nitrate were detected in high-N treated plants at 7 dpa. Intriguingly, nitrate levels in senescing flag and second leaves of high-GPC germplasm were maintained or even increased during this phase (especially in the six-row genetic background). This contrasted sharply with the low-GPC parent, 'Karl', in which leaf nitrate became undetectable by 14 dpa. While the source of this nitrate is unclear, due to the high solubility of the nitrate ion and the fact that nitrogen (including direct nitrate) fertilization was stopped at anthesis, it is unlikely that it was primarily derived from soil uptake. More likely, this nitrate was retranslocated

from stems and/or roots, assimilated in leaves, and contributed to some degree to enhanced grain N availability. This interpretation is again supported by our transcriptomic data, which show upregulation of all genes commonly associated with nitrate assimilation and amino acid synthesis, including nitrate reductase, nitrite reductase and GS/GOGAT cycle, in flag leaves of high-GPC line '10_11' as compared to 'Karl'. Additionally, a putative nitrate transporter (contig23379_at on the barley Affymetrix DNA microarray; see Close *et al.* 2004) was among the most strongly upregulated genes at 21 dpa in this experiment. Together, these data indicate that the GPC locus on barley chromosome 6 does not only influence leaf senescence and reallocation of organic nitrogen, but also bears on the plant's handling of its inorganic nitrogen stores. At this point, no corresponding information is available from wheat. Unfortunately, our experimental system was not easily amenable to root analyses, comparing (at different time points) root nitrate storage and uptake.

In summary, the GPC locus on barley chromosome 6 profoundly influences the plant's storage and allocation of both organic and inorganic nitrogen. Data presented in this and earlier studies (both from this and other labs) indicate that further molecular and cellular analysis of the experimental system presented here will considerably enhance our understanding of whole-plant nitrogen metabolism, transport and storage.

CHAPTER 2

SERINE CARBOXYPEPTIDASE CP-MIII IN SENESCING BARLEY LEAVES: MOLECULAR CLONING AND PRODUCTION OF A POLYCLONAL ANTIBODY

Abstract

Senescence is a highly ordered and regulated process where nutrients are moved from the leaves into the developing kernels. The majority of nitrogen remobilized from senescing leaves is contained by plastidial proteins, especially Rubisco. These proteins must be degraded before the nitrogen can be transported and reused in the seeds. Molecular details of this process are largely unknown. As previous data from our laboratory indicated an involvement of serine carboxypeptidases in this process, we analyzed one enzyme of this type, whose gene is strongly upregulated in senescing leaves, in more detail. Serine carboxypeptidase cp-mIII was cloned from senescing leaves, subcloned into an *E. coli* protein expression system, purified, and a polyclonal anti-cp-mIII antibody was raised. Characterization of this antibody indicates that it is both avid and specific. This research has therefore produced a valuable tool for the further cellular and biochemical characterization of cp-mIII, as needed to evaluate its role in the degradation of leaf proteins during senescence.

Introduction

During the process of leaf senescence in annual plants, multiple tightly controlled signaling cascades cause changes in gene expression, ultimately leading to the

degradation of macromolecules, and retranslocation of resulting nutrients to the developing seeds (Hopkins et al., 2007; Feller and Fischer 1994). The majority of the N accumulating in seeds is from vegetative plant parts, especially leaves (Feller and Fischer, 1994). In photosynthetically active leaves, up to 75% of the organic nitrogen is contained by soluble (stromal) and insoluble (thylakoidal) chloroplast proteins (Hörtensteiner and Feller, 2002). These proteins, especially Rubisco which alone accounts for ~50% of all plastidial nitrogen, must be degraded before remobilization of N in the form of peptides and amino acids can occur (Fischer, 2007). Available data indicate that the degradation of plastidial proteins is caused or at least initiated by proteolytic enzymes present in the organelle itself. Additional proteases found mostly in lytic vacuolar compartments during senescence may also have some role in the degradation of photosynthetic proteins (Hörtensteiner and Feller, 2002). These include carboxypeptidases, several of which have previously been shown to be located in vacuolar compartments. A correlation between one or several carboxypeptidases and leaf N remobilization has been found (Yang et al., 2004). In a recent transcriptomic study, 50 protease genes, including the serine carboxypeptidase cp-mIII (malt carboxypeptidase III), were found to be up-regulated during senescence (Parrott et al., 2007).

Serine carboxypeptidases (SCPs) are a large family of protein hydrolyzing enzymes that have been implicated in a variety of roles including protein degradation, wound responses, C-terminal cleavage, and xenobiotic metabolism (Feng and Xue, 2006). SCPs are exopeptidases which cut only near the C-terminus of polypeptide chains. Specifically, at acidic pH values, they catalyze the hydrolysis of the C-terminal peptide

bond in proteins or peptides. The catalytic triad involved in this reaction is a Ser-Asp-His which leads to the classification of SCPs as part of the S10 family, Clan SC, of serine peptidases (http://merops.sanger.ac.uk/). Enzymes from this family have many similar structural features including the presence of a signal sequence for intracellular trafficking and/or secretion, several N-linked glycosylation sites, and four evolutionarily conserved regions involved in substrate binding and catalysis. However the many differences between members of the family lead to further classification into three groups annotated as I, II, and III based on sequence similarity and characterized by a central catalytic domain called the α/β hydrolase fold. Groups I and II consist of two polypeptide chains, whereas group III contains a single polypeptide chain and is found mostly in yeast, plants and filamentous fungi. Group II usually contains around 480 amino acid residues and group I and III usually contain around 420-430 amino acid residues (Feng and Xue, 2006). SCPs are also separated into two more groups by substrate preference – C and D. C carboxypeptidases have high affinity for hydrophobic C-terminal amino acid residues and D carboxypeptidases for basic C-terminal residues (http://merops.sanger.ac.uk/).

Carboxypeptidase cp-mIII (*Hordeum vulgare* L.) in its full length form is a single N-terminally blocked polypeptide chain around 508 amino acids long (http://www.ncbi.nlm.nih.gov, accession number P21529). The mature form (cp-mIIIm), 399 amino acids long, was predicted using similar sequences through BLAST comparison in the MEROPS database (MER01760). The gene responsible for its production is known as CBP3 or CXP;3. Carboxypeptidase cp-mIII is formed as a pro-peptide which must be cleaved to induce its proteolytic activity. Because cp-mIII itself

has no endopeptidase activity, it is dependent on other enzymes for its maturation. An aspartic protease with similar sequence and specificity to yeast proteinase A, which participates in the processing of the yeast serine carboxypeptidase Y, was found in barley. This aspartic protease may have regulatory function for cp-mIII and a possible role in processing cp-mIII propeptides (Degan et al., 1994).

In seeds, significant expression of cp-mIII has been found throughout the 5-day germination period mainly in the aleurone, along with smaller amounts in the embryo, with a maximal level at 3 days. It has also been found in the roots and shoots of growing seedlings and in senescing leaves (http://www.ebi.uniprot.org/). In senescing leaf material, it is found to be strongly upregulated at 4- and 8-days after the onset of senescence with almost no expression in non-senescing control leaf material (Parrott et al., 2007). The sequence of cp-mIII from barley (*Hordeum vulgare* L.) is 97% identical to the corresponding carboxypeptidase III of wheat (cp-wIII) and is also 26% identical with the two peptide chains of malt carboxypeptidase I, 34% identical with carboxypeptidase II and 34% with the single peptide chain of carboxypeptidase Y (Sorensen et al., 1989). Both the N-terminus and the C-terminus of cp-wIII have amino acid extensions with no homology to cp-mIII. The first residues of the N-terminal extension most likely make up a signal peptide and the next 50 possibly a pro-peptide. This indicates that wheat and potentially barley cp-mIII are synthesized as inactive zymogens (Sorensen et al., 1989). The 16 amino acid C-terminal extension may indicate that the enzyme is subject to proteolysis after synthesis. According to the Uniprot database cp-mIII has a potential signal peptide from amino acids 1-19, a propeptide 61 amino acids long from 20-80, and

a propeptide from 492-508, 17 amino acids long. The protease itself is from 81-491, 411 amino acids long (http://www.ebi.uniprot.org/). Secreted hydrolases are often formed as inactive precursor forms, zymogens, activated upon reaching final destination by removal of parts of the zymogen polypeptide. This is to inhibit destructive hydrolytic ability at the site of synthesis, guide folding, and also contain the molecular address which direct them to the correct compartment (Ramos and Winther, 1996). As previous work in our laboratory indicated that barley cp-mIII may be functionally involved in senescence-associated protein degradation, the primary goal of this work was the production of a polyclonal antibody usable for further *in vitro* and *in vivo* enzyme characterization.

Materials and Methods

Plant Material Used For Gene Cloning and Antibody Evaluation

Barley seeds (*Hordeum vulgare* cv. 'Harrington') were grown and treated as described by Parrott et al. (2005). Briefly, barley seeds (*H. vulgare* L., cv. ''Harrington'') were germinated and grown on potting soil in a greenhouse of the Montana State University (Bozeman, MT) Plant Growth Center for 7 days, with a 22/18 C day/night temperature cycle. When necessary, days were extended to a 16-hour photoperiod, using Son-Agro 430 W high-pressure sodium lamps (Philips, Somerset, NJ). After this time, plants were transferred to a growth chamber (Conviron, Winnipeg, Manitoba, Canada) with a 22/

16 C day/night temperature cycle and a 16 h photoperiod with a light intensity of 200 μE m^{-2} s^{-1}. Secondary (fully mature) leaves of some plants were treated for 5 s with a steam-

heated (95^{O}C) hypodermic needle at the base of the leaf to interrupt the phloem. Untreated control leaves were compared to leaves where the phloem was completely interrupted (girdled) or where two opposing phloem interruptions, each spanning half the lamina, were separated by ~1 cm (shift girdled; Feller and Fischer, 1994). Leaves were harvested at 0 days (immediately after girdling), and 4, 8 and 12 days after girdling. They were immediately frozen in liquid nitrogen, and stored at -80 C until extraction and analysis.

Carboxypeptidase cp-mIII Cloning

RNA was purified from 8-day girdled samples (which have previously been shown to be high in cp-mIII transcripts; Parrott et al. 2005) using the RNeasy Plant mini kit (Qiagen, Valencia, CA, USA) as described by Parrott et al. (2007). cDNA was subsequently synthesized using oligo dT primers included with the Retroscript reverse transcriptase kit, where a 75ºC heat denaturation, 1 µL Reverse Transcriptase, 44ºC incubation for 1 hour, and 95ºC ten minute termination was used (Ambion, Austin, TX, USA). A first round of PCR amplification using the primers cp-mIIIF (5' gaa acc gtc ctc cgc tcc 3') and cp-mIIIR (5'tct tct cct ttt ttg gtc ggt 3') yielded the complete cp-mIII sequence corresponding to the previously published sequence (http://www.ncbi.nlm.nih.gov, accession number P21529). The PCR, using Pfu turbo polymerase (Stratagene, La Jolla, CA, USA), was run at 95 ºC for 2 minutes, with 30 cycles of 95 ºC for 30 sec, 52 ºC for 30 sec, and 72 ºC for 2 minutes, and a final temperature of 72 ºC for 10 minutes. This amplicon was gel-purified using the Gene Clean III kit (MP Biomedicals, LLC Solon, OH) and ligated into the pDrive cloning

vector using a PCR cloning kit (Qiagen, Valencia, CA, USA) following the supplied procedure. Cell transformation into Qiagen EZ competent cells was then completed according to the manufacturer's instructions. Cells were subjected to blue/white selection, and plasmids with inserts were purified using the Wizard Plus Mini-Prep kit (Promega, Madison, WI, USA). Purified plasmids were checked for appropriate insert size after double digestion with *BamH1* and *Hind III* (Takara, Otsu, Shiga, Japan). Inserts on one clone were verified by sequencing.

Carboxypeptidase Protein Expression

The mature form of cp-mIII (MER01760) was determined using the MEROPS peptidase database computer (http://merops.anger.ac.uk). Primers were designed for the mature form with restriction endonuclease sites for *Bam HI* and *HindIII* (Takara, Otsu, Shiga, Japan) designed into the primers such that the PCR product would be properly orientated 5' to 3' and in frame (cp-mIIImF; 5'att aat gga tcc gac ctc ggc cac cac 3', and cp-mIIImR; 5'aga gca aag ctt cta ccc ctg ggt gaa 3'). The PCR, using Pfu turbo polymerase (Stratagene, La Jolla, CA, USA), was run at 95 ºC for 2 minutes, with 30 cycles of 95 ºC for 30 sec, 55.7 ºC for 30 sec, and 72 ºC for 2 minutes, and a final temperature of 72 ºC for 10 minutes. PCR amplification from the cp-mIII insert containing clone (previous paragraph) yielded the correct product, as checked by size. The PCR product was purified by phenol chloroform extraction (Ausubel et al., 1999), digested with *BamHI* and *HindIII*, and the resulting product was ligated into pQE80-L expression vector (Qiagen, Valencia, CA, USA) digested with the same restriction enzymes. Cell transformations were completed into the XL-10 Gold cell line according to

the manufacturer's protocol and the resulting colonies from LB agar antibiotic plates (ampicillin^{20}) were grown in LB/ampicillin^{20} and lysed with the Wizard Plus mini-prep kit (Promega, Madison, WI, USA) and checked for appropriate size by digesting with BamH1 and Hind III (Takara, Otsu, Shiga, Japan), running over an agarose gel and comparing the actual size of the product with predicted size. Cultures containing appropriately sized products were then grown in LB/ampicillin^{20} and lysed with a Wizard Plus midi-prep kit (Promega, Madison, WI, USA) according to protocol. The product was checked for appropriate size as previously described, and verified by sequencing. The plasmid was introduced into the BL21 CodonPlus (DE3)-RIL, cell line (Stratagene, La Jolla, CA, USA) according to the manufacturers instructions and resulting colonies from LB agar antibiotic plates (chloramphenicol^{34} and ampicillin^{75}) were grown in LB media containing chloramphenicol^{34} and ampicillin^{75} at either 25 ^{o}C or 37^{o}C in a shaking incubator at 250 rpm. Growth was continued to an OD_{550} of 0.4-0.6. At this point, the cells were induced with 150 μL of 100 mM IPTG, grown for around six hours and harvested.

Protein Purification and Antibody Production

During expression, a His tag was added to the protein from the pQE80-L vector. The cells were lysed using the CellLytic B (Sigma, St. Louis, MO, USA) protocol. The cell lysate was then purified by affinity chromatography on Ni^{++} (Sigma, St. Louis, MO, USA) resin according to the manufacturer's instructions. Fractions containing protein were collected, formulated with Laemmli (1970) sample buffer and further purified by size separation on 13% (w/v) acrylamide SDS-PAGE gels in a Hoefer (San Francisco,

CA) minigel apparatus. The band corresponding to the mature form of cp-mIII (based on molecular weight) was excised. The protein was extracted from the gel in two volumes of elution buffer (10mM Tris-HCl, 50mM NaCl, 1mM EDTA) by mixing on a rotating table overnight. The resulting protein was concentrated in a Centricon centrifugal concentration device (Amicon, Bedford, MA, USA) with a molecular weight cutoff of 10 kD. The concentrated protein was sent to Alpha Diagnostic Intl. Inc (San Antonio, TX) for antibody preparation. Previously described techniques (Fischer et al. 1999) were utilized to prepare nitrocellulose membranes containing either E. coli-expressed protein, or plant protein, for evaluating antibody fractions.

Results

In Fig. 12 the full length sequence of cp-mIII (Genbank accession number P21529) is shown, and the length of the mature (active) protein as derived from the MEROPS database (http://merops.sanger.ac.uk) is indicated. The start (ATG) and stop (TGA) codons, along with the primers used for both full-length and expression cloning into pQE80-L, are shown. The mature form of malt carboxypeptidase III (cp-mIIIm) was successfully cloned and expressed as shown in Fig. 13 where *E. coli* extracts are separated before and after protein induction with IPTG. Partial purification of cp-mIII through a Ni^{++} affinity column (see materials and methods) is demonstrated in Fig. 14 with some remaining bands of sizes not matching that of the target protein. These bands, at least those which are below the MW of mature cp-mIII, are either unrelated proteins (contaminants), fragments from degradation of the cp-mIII protein in *E. coli*, or chain-



Figure 12. Cp-mIII cDNA sequence. The full sequence of cp-mIII is denoted by black type with primers used for sequencing in blue. The mature length sequence is denoted by red with blue primers (used for expression cloning) containing restriction sites in orange. Start and stop codons are underlined and labeled in green.

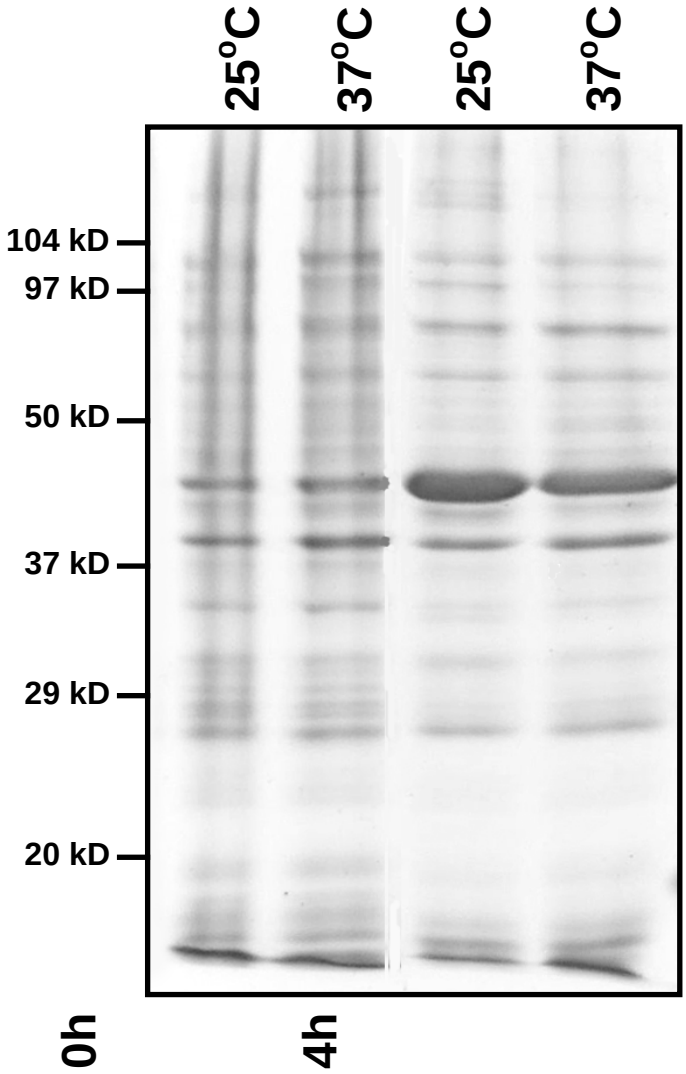


Figure 13. *E. coli* expressing cp-mIII m before and 4 hours after induction. Shown at both 25**o**C and 37**o**C for both time points.

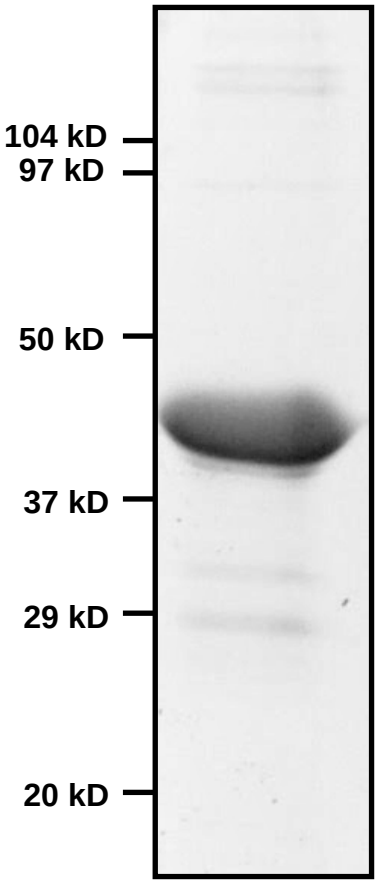


Figure 14. Partially purified cp-mIII m (by affinity chromatography on Ni++ resin), as analyzed by SDS-PAGE.

interruption synthesis products which are a common problem when expressing eukaryotic proteins in *E. coli*. Further purification of cp-mIIIm was therefore carried out by cutting the appropriately sized band out of a gel (Fig. 15), resulting in the elimination of potential contaminants and the preparation of pure protein. Polyclonal antibodies were raised and have been shown to bind to pure cp-mIII m at varying concentrations (Fig. 16).

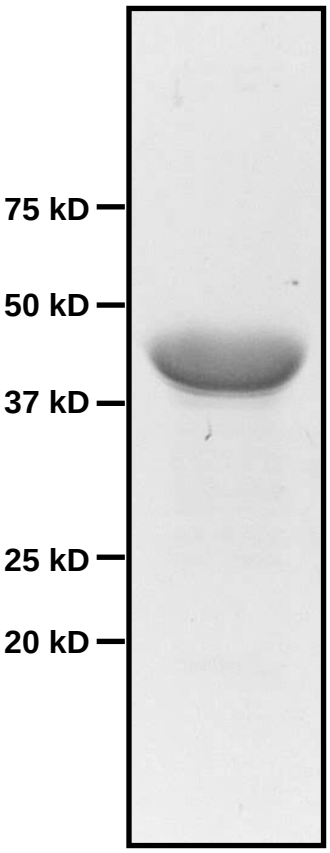


Figure 15. Purified cp-mIII m (after affinity chromatography and gel purification), as analyzed by SDS-PAGE.

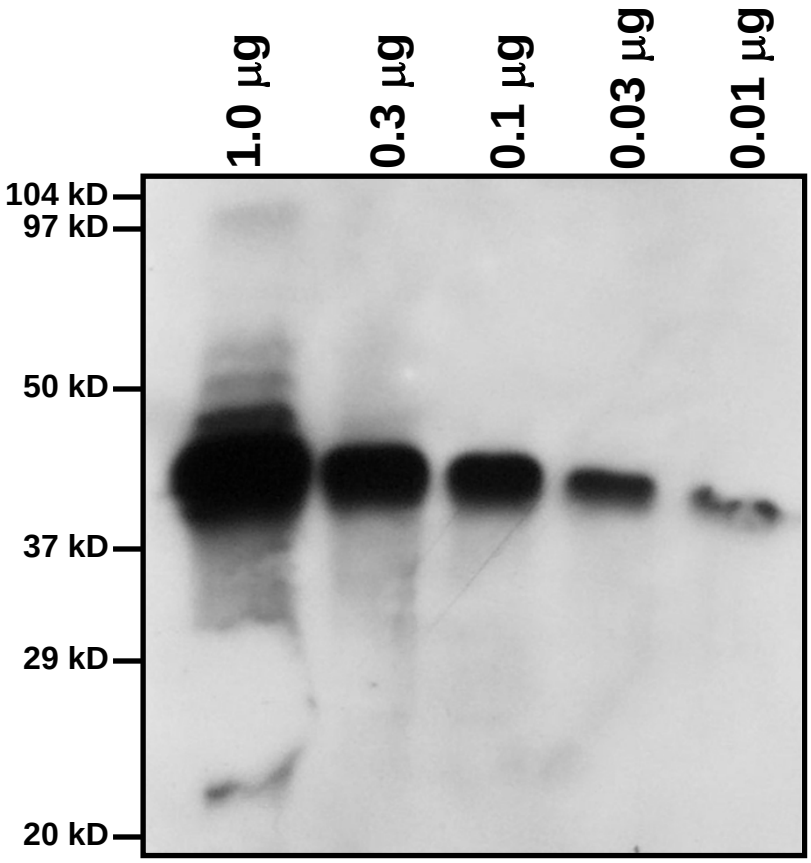


Figure 16. Immunoblot analysis of pure cp-mIII (from 0.01 to 1.0 mg of pure protein) with anti-cp-mIII m polyclonal antibody.

Antibodies were further tested against crude protein from *E. coli* (after IPTG induction, Fig. 17). In these experiments, the strongest band is seen with immune serum at the expected size of ~47 kD (predicted weight of the cloned protein: 46.6 kD). No bands were observed with pre-immune serum. The antibody was then tested against plant material (Fig. 18). Two additional bands, with molecular weights of ~40 and ~75 kD were detected. Conspicuously, the strongest bands were seen with plant proteins

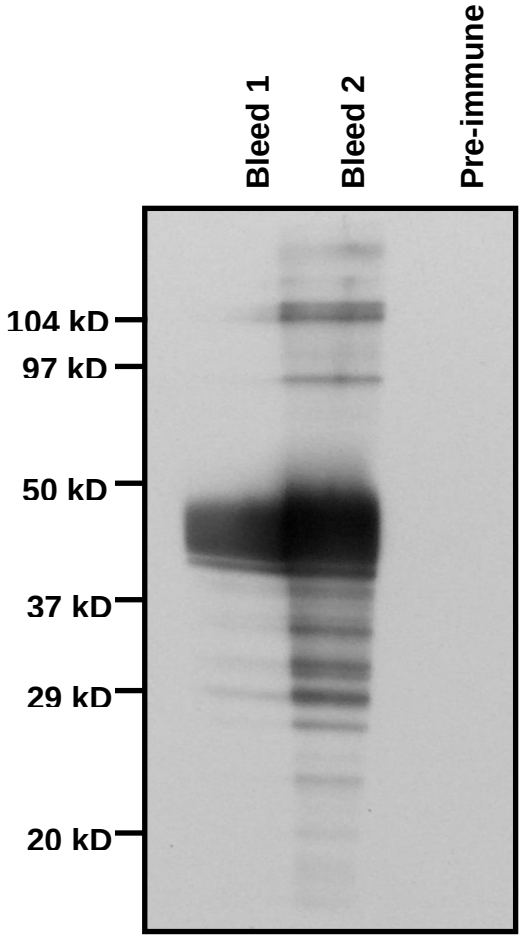


Figure 17. Evaluation of anti-cp-mIII m polyclonal antibody. Bleed1, 2 and pre-immune serum from rabbit #1 were analyzed against crude cp-mIII m protein expressed in *E. coli*. Primary and secondary antibodies were diluted to 1:10,000. The film was exposed for 1 second.

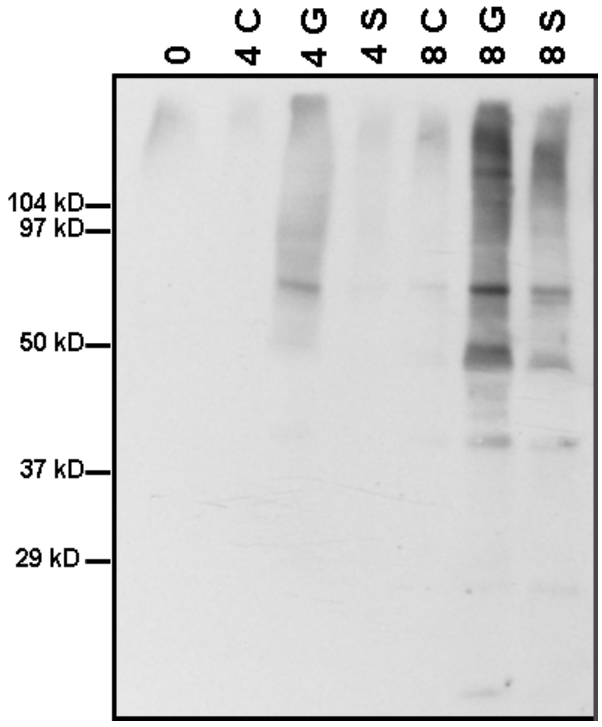


Figure 18. Immunoblot analysis of plant proteins with anti-cpmIII m antibody. Soluble leaf proteins were extracted from control (0 days) leaves, and from leaves after 4 and 8 days. Leaves were either untreated (C, control), girdled (G) or shift-girdled (S), as described under Materials and Methods.

extracted from 8-day girdled leaves, in which previous experiments (Parrott et al. 2005, 2007) indicated highest transcript levels. Among these, the band detected at ~47 kD may correspond to mature cp-mIII protein, while the two additional bands at 40 and 75 kD could be products of posttranslational modifications, including glycosylation and limited proteolysis. However, cross-reactivity of the antibody with other (non-cp-mIII) plant proteins cannot be excluded at this point.

Discussion

It is unclear whether the degradation of major chloroplast proteins during leaf senescence takes place in the intact organelle or in vacuolar compartments. Some data indicate that at least the initial proteolytic steps are catalyzed by plastidial proteases (Feller and Fischer 1994). However, there is also evidence for an involvement of additional proteolytic enzymes, which accumulate mostly in lytic vacuolar compartments. It has been shown that isolated mature chloroplasts do not have the ability to degrade chlorophyll, which suggests that degradation is dependent on extraplastidial components. Some evidence implies that entire chloroplasts, or chloroplast vesicles, are enveloped by lytic vacuoles preceding their degradation. Other data seem to indicate plastidial degradation by extraplastidial enzymes after the loss of membrane integrity (Hörtensteiner and Feller, 2002).

Carboxypeptidases are known as vacuolar enzymes that are upregulated during senescence. In a recent genomic study a serine carboxypeptidase (cp-mIII), which is also found in germinating seeds, was found to be upregulated in girdled barley leaves after 8

days and also in naturally senescing leaves (Parrott et al. 2005, 2007). These data suggest that this protease may be involved in extraplastidial degradation of barley photosynthetic proteins. However, as proteases are notorious for being regulated at the posttranscriptional and/or posttranslational level (Fischer 2007), the main purpose of this research was to obtain an avid, specific polyclonal antibody against this protein, allowing its thorough biochemical and cellular characterization. Data shown in Figs. 12-18 indicate that this goal was achieved. Further studies will investigate if high transcript and protein levels overlap both spatially and temporally as suggested by Fig. 18 and previous results (Parrott et al. 2005, 2007). Preliminary data (not shown) indicate that the protein expressed in *E. coli* is not active, suggesting that posttranslational modifications that occur *in vivo* are not performed in *E. coli*. Immunoaffinity purification of the enzyme from plant material could allow its biochemical characterization, including activity against artificial substrates and purified plant proteins. Further experimentation will also include localization studies to ascertain the subcellular location of cp-mIII using microscopy, and possibly cell fractionation studies and analysis of fractions by immunoblotting. This antibody will also be used to analyze the results of either transient or stable gene silencing studies aimed at directly establishing the function of this protein *in vivo*. Generally speaking, there is a severe lack of biochemical and functional information on plant proteases, and this antibody will help to improve this situation for one enzyme potentially important during leaf senescence.

CHAPTER 3

CONCLUSIONS

A quantitative trait locus (QTL) on chromosome 6 in barley, delineated by molecular markers HVM74 and ABG458, was found to affect GPC (See *et al.* 2002; Mickelson *et al.* 2003). To study the effects of this locus two parents were chosen, Lewis, a commonly grown two-row high-GPC variety, and Karl, a six-row low-GPC variety. Several near isogenic lines (NIL) were created where the high GPC allele(s) are isolated in the low GPC background and vice versa. The resulting lines showed an up to 20% difference in GPC. The majority of the nitrogen utilized in the developing grains is remobilized from vegetative plant parts, especially from the plastidial proteins located in the leaves (Feller and Fischer 1994). It is unclear at this time how these processes affect grain protein accumulation. In this study several physiological aspects were investigated to determine their effects on grain protein accumulation. In the developing and mature grains, levels of nitrogen and amino acids were quantified. In developing leaves through senescence to maturity, levels of nitrogen, amino acids, chlorophyll, soluble and insoluble proteins, and nitrates were analyzed. Several trends appeared in the data as effects from the high or low GPC locus. An earlier and/or higher availability of organic nitrogen during grain development was seen in high-GPC lines, leading to an earlier accumulation of grain protein. This trend was further established by the total nitrogen and chlorophyll data in senescing leaves where earlier leaf senescence and nitrogen remobilization are seen. These data are similar to analyses in wheat, in which the

homologous locus on chromosome 6B was associated with earlier leaf senescence and N reallocation (Uauy *et al.* 2006*a,b*). The transcriptomic data from a recent study in our lab also verify these results. In the developing kernels of the high-GPC line '10_11' compared to its low-GPC parent, 'Karl,' upregulation of storage protein genes, genes involved in protein folding (peptidyl-prolyl cis/trans isomerases or cyclophilins, protein disulfide isomerases, numerous heat shock proteins), and of genes involved in nitrogen transport and metabolism were seen (Jukanti *et al.* in press). Leaf nitrate levels were found to be enhanced in high-GPC germplasm at 14 to 28 dpa, probably due to retranslocation from stems and/or roots, providing some contribution to enhanced grain N availability. In addition kernel amino acid levels are higher in high-GPC grain (at 14 to 21 dpa), and there is a corresponding trend to lower leaf amino acid levels in these same lines, suggesting increased organic N transport. These data again are compatible with our previous transcriptomic results, where upregulation of several (both plastidial and extraplastidial) leaf proteases and some leaf membrane transporters are seen (Jukanti *et al.* in press).

One of the upregulated proteases, cp-mIII, has been shown previously in our lab (Yang et al. 2004) to be associated with leaf N remobilization. A second goal of this study was to further characterize this protein by obtaining an avid polyclonal antibody. This was carried out successfully through cloning and expression of the protein in *E. coli*, and generation of an antibody. As expected from transcriptomic analysis (Parrott et al. 2007), the antibody reacts most strongly with protein bands from senescing leaves. This

confirms that we have obtained a tool which will allow progress in the area of senescence-associated proteolysis.

Together, data presented in this thesis shed new light on the physiological basis of grain protein accumulation in barley, setting the stage for further molecular analysis. Furthermore, we have created a useful tool for the characterization of one possible player in the remobilization of leaf proteins, serine carboxypeptidase cp-mIII.

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1992) Phenol extraction and ethanol precipitation of DNA. In 'Short Protocols in Molecular Biology, 4th edition'. Unit 2.1 A p. 2-3. (John Wiley and Sons, Inc: New York.)

Barneix AJ (2007) Physiology and biochemistry of source-regulated protein accumulation in the wheat grain. *Journal of Plant Physiology* **164**, 581-590. doi: 10.1016/j.jplph.2006.03.009

Close TJ, Wanamaker SI, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP (2004) A new resource for cereal genomics: 22K barley GeneChip comes of age. *Plant Physiology* **134**, 960-968. doi: 10.1104/pp.103.034462

Degan FD, Rocher A, Cameron-Mills V, Wettstein DV (1994) The expression of serine carboxypeptidases during maturation and germination of the barley grain. *Proceedings of the National Academy of Sciences USA* **91**, 8209-8213.

Dembinski E, Bany S, Raczynska-Bojanowska K (1995) Asparagine and glutamine in the leaves of high and low protein maize. *Acta Physiologiae Plantarum* **17**, 361-365.

Distelfeld A, Uauy C, Olmos S, Schlatter AR, Dubcovsky J, Fahima T (2004) Microcolinearity between a 2-cM region encompassing the grain protein content locus Gpc-6B1 on wheat chromosome 6B and a 350-kb region on rice chromosome 2. *Functional & Integrated Genomics* **4**, 59-66. doi: 10.1007/s10142-003-0097-3

Feller U, Fischer A (1994) Nitrogen metabolism in senescing leaves. *Critical Reviews in Plant Sciences* **13**, 241-273.

Feng Y, Xue Q (2005) The serine carboxypeptidase like gene family of rice (*Oryza sativa* L. ssp. *japonica*). *Functional and Integrative Genomics,* **6**, 14-24. doi:10.1007/s10142-005-0131-8

Fischer AM (2007) Nutrient remobilization during leaf senescence. In 'Senescence processes in plants'. (Ed S Gan) pp. 87-107. (Blackwell Publishing, Ltd.: Oxford, UK.)

Hopkins M, Taylor C, Liu Z, Ma F, McNamara L, Wang T-W, Thompson JE (2007) Regulation and execution of molecular disassembly and catabolism during senescence. *New Phytologist* **175**, 201-214. doi: 210.1111/j.1469-8137.2007.02118.x

Hörtensteiner S, Feller U (2002) Nitrogen metabolism and remobilization during senescence. *Journal of Experimental Botany* **53**, 927-937.

Jukanti AK, Heidlebaugh NM, Parrott DL, Fischer IA, McInnerney K, Fischer AM (2007 or 2008) Comparative transcriptome profiling of near-isogenic barley (*Hordeum vulgare*) lines differing in the allelic state of a major grain protein content locus identifies genes with possible roles in leaf senescence and nitrogen reallocation. *New Phytologist*, in press.

Kade M, Barneix AJ, Olmos S, Dubcovsky J (2005) Nitrogen uptake and remobilization in tetraploid 'Langdon' durum wheat and a recombinant substitution line with the high grain protein gene Gpc-B1. *Plant Breeding* **124**, 343-349.

Krapp A, Stitt M (1995) An evaluation of direct and indirect mechanisms for the "sink-regulation" of photosynthesis in spinach: Changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady-state transcript levels after cold-girdling source leaves. *Planta* **195**, 313-323.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lawlor DW (2002) Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *Journal of Experimental Botany* **53**, 773-787.

Lim JD, Cho J-I, Park Y-I, Hahn T-R, Choi S-B, Jeon J-S (2006) Sucrose transport from source to sink seeds in rice. *Physiologia Plantarum* **126**, 572-584. doi: 10.1111/j.1399-3054.2006.00654.x

Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. *Annual Review of Plant Biology* **58**, 115-136. doi: 10.1146/annurev.arplant.57.032905.105316

Mae T (2004) Leaf senescence and nitrogen metabolism. In 'Plant cell death processes'. (Ed LD Noodén) pp. 157-168. (Elsevier Academic Press: Amsterdam.)

Marschner H (1995) Mineral nutrition of higher plants, 2nd edition. pp. 889 (Academic Press: San Diego.)

Mickelson S, See D, Meyer FD, Garner JP, Foster CR, Blake TK, Fischer AM (2003) Mapping of QTL associated with nitrogen storage and remobilization in barley (*Hordeum vulgare* L.) leaves. *Journal of Experimental Botany* **54**, 801-812. doi: 10.1093/jxb/erg084

Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM (2007) Nitrate transport and signalling. *Journal of Experimental Botany* **58**, 2297-2306. doi:2210.1093/jxb/erm2066

Onodera Y, Suzuki A, Wu C-Y, Washida H, Takaiwa F (2001) A rice functional transcriptional activator, RISBZ1, responsible for endosperm-specific expression of storage protein genes through GCN4 motif. *Journal of Biological Chemistry* **276**, 14139-14152. doi: 10.1074/jbc.M007405200

Parrott DL, McInnerney K, Feller U, Fischer AM (2007) Steam-girdling of barley (*Hordeum vulgare*) leaves leads to carbohydrate accumulation and accelerated leaf senescence, facilitating transcriptomic analysis of senescence-associated genes. *New Phytologist* **176**, 56-69. doi: 10.1111/j.1469-8137.2007.02158.x

Peoples MB, Dalling MJ (1988) The interplay between proteolysis and amino acid metabolism during senescence and nitrogen reallocation. In 'Senescence and aging in plants'. (Eds LD Noodén, AC Leopold) pp. 181-217. (Academic Press: San Diego.)

Qi JC, Zhang GP, Zhou MX (2006) Protein and hordein content in barley seeds as affected by nitrogen level and their relationship to beta-amylase activity. *Journal of Cereal Science* **43**, 102-107. doi:10.1016/j.jcs.2005.08.005

Ramos and Winther (1996) Exchange of regions of the carboxypeptidase Y propeptide - Sequence specificity and function in folding in vivo. *European Journal of Biochemistry* **242** (1): 29-35.

Roitsch T (1999) Source-sink regulation by sugar and stress. *Current Opinion in Plant Biology* **2**, 198-206.

See D, Kanazin V, Kephart K, Blake T (2002) Mapping genes controlling variation in barley grain protein concentration. *Crop Science* **42**, 680-685.

Sorensen SB, Svendsen I, Breddam K(1989) Primary structure of carboxypeptidase III from malted barley. *Carlsberg Research Communication* **54**, 193-202.

Soudry E, Ulitzur S, Gepstein S (2005) Accumulation and remobilization of amino acids during senescence of detached and attached leaves: *in planta* analysis of tryptophan levels by recombinant luminescent bacteria. *Journal of Experimental Botany* **56**, 695-702. doi:10.1093/jxb/eri054

Tang Y, Wen X, Lu C (2005) Differential changes in degradation of chlorophyll–protein complexes of photosystem I and photosystem II during flag leaf senescence of rice. *Plant Physiology and Biochemistry* **43**, 193-201. doi:10.1016/j.plaphy.2004.12.009

Uauy C, Brevis JC, Dubcovsky J (2006*a*) The high grain protein content gene Gpc-B1 accelerates senescence and has pleiotropic effects on protein content in wheat. *Journal of Experimental Botany* **57**, 2785–2794. doi:2710.1093/jxb/erl2047

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006*b*) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* **314**, 1298-1301. doi: 10.1126/science.1133649

Von Wettstein D (2007) From analysis of mutants to genetic engineering. *Annual Review of Plant Biology* **58**, 1-19. doi: 10.1146/annurev.arplant.58.032806.104003

Yang L, Mickelson S, See D, Blake TK, Fischer AM (2004) Genetic analysis of the function of major leaf proteases in barley (*Hordeum vulgare* L.) nitrogen remobilization. *Journal of Experimental Botany* **55**, 2607-2616. doi: 10.1093/jxb/erh267