1 Proteomic comparison of near-isogenic barley (*Hordeum vulgare* L.)
2 germplasm differing in the allelic state of a major senescence QTL identifies
3 numerous proteins involved in plant pathogen defense

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ABSTRACT

Senescence is the last developmental phase of plant tissues, organs and, in the case of monocarpic senescence, entire plants. In monocarpic crops such as barley, it leads to massive remobilization of nitrogen and other nutrients to developing seeds. To further investigate this process, a proteomic comparison of flag leaves of near-isogenic late- and early-senescent barley germplasm was performed. Protein samples at 14 and 21 days past anthesis were analyzed using both two-dimensional gel-based and label-free quantitative mass spectrometry-based ('shotgun') proteomic techniques. This approach identified >9,000 barley proteins, and one-third of them were quantified. Analysis focused on proteins that were significantly ($p < 0.05$; difference ≥ 1.5 -fold) upregulated in early-senescent line '10_11' as compared to late-senescent variety 'Karl', as these may be functionally important for senescence. Proteins in this group included family 1 pathogenesis-related proteins, intracellular and membrane receptors or co-receptors (NBS-LRRs, LRR-RLKs), enzymes involved in attacking pathogen cell walls (glucanases), enzymes with possible roles in cuticle modification, and enzymes involved in DNA repair. Additionally, proteases and elements of the ubiquitin-proteasome system were upregulated in line '10_11', suggesting involvement of nitrogen remobilization and regulatory processes. Overall, the proteomic data highlight a correlation between early senescence and upregulated defense functions. This correlation emerges more clearly from the current proteomic data than from a previously performed transcriptomic comparison of 'Karl' and '10_11'. Our findings stress the value of studying biological systems at both the transcript and protein levels, and point to the importance of pathogen defense functions during developmental leaf senescence.

Keywords:

2D-DIGE proteomics

Barley (*Hordeum vulgare* L.)

Defense

Plant-pathogen interaction

Senescence

Shotgun proteomics

1. Introduction

Senescence is the last life phase of plant tissues, organs and, in the case of monocarpic species, entire plants. Senescence is optimized for nutrient (particularly nitrogen) remobilization to surviving organs of perennial or seeds of annual plants (Feller and Fischer, 1994; Distelfeld et al., 2014). It therefore relies on the activities of proteases, lipases, nucleases, and membrane transport proteins. During nutrient remobilization plants may become more susceptible to opportunistic pathogens (Quirino et al., 2000), at least partially explaining why many genes typically associated with plant-pathogen interactions are also induced during developmental senescence (Hanfrey et al., 1996; Guo and Gan, 2012). Additionally, pathogen infection leads to the induction of *Senescence-Associated Genes* (SAGs)¹ (Espinoza et al., 2007), indicating significant overlap, at the molecular level, between processes controlling senescence and plant defense against pathogens.

We have previously analyzed barley (*Hordeum vulgare* L.) germplasm varying in senescence timing, namely the late-senescent variety ‘Karl’ and a near-isogenic early-senescent line (termed ‘10_11’). The early-senescence phenotype of line ‘10_11’ is characterized by accelerated degradation of major flag leaf proteins including Rubisco, faster leaf chlorophyll degradation, enhanced amino acid levels and earlier storage protein accumulation in developing grains, and by higher protein concentration in mature kernels (Jukanti and Fischer, 2008; Jukanti et al., 2008; Heidlebaugh et al., 2008). ‘Karl’ and ‘10_11’ flag leaf and kernel transcriptomes were compared at 14 and 21 days past anthesis (dpa) using the Affymetrix Barley1 GeneChip (described in Close et al., 2004). Transcript levels of numerous protease genes and SAGs, but also of numerous genes coding for disease resistance and pathogenesis-related proteins were found to be upregulated in line ‘10_11’ as compared to ‘Karl’ flag leaves, especially at the later date (21 dpa; Jukanti et al., 2008).

¹*Abbreviations:* 2D-DIGE, Two-dimensional difference in-gel electrophoresis; BAP, B-cell receptor-associated protein; CNGC, cyclic nucleotide gated ion channel; CyDyes, N-hydroxysuccinimide ester cyanine dyes; dpa, days past anthesis; DTT, dithiothreitol; IEF, isoelectric focusing; LC-MS, liquid chromatographic-mass spectrometric analysis; LRR, leucine-rich repeat; LRR-RLK, leucine-rich repeat receptor-like protein kinase; NBS, nucleotide binding site; NBS-LRR, nucleotide binding site-leucine-rich repeat gene or protein; SAG, *senescence-associated gene*; UPS, ubiquitin-proteasome system.

Variety ‘Karl’ and near-isogenic line ‘10_11’ vary in the allelic state of a chromosome six locus which encompasses the *HvNAM-1* transcription factor gene (Distelfeld et al., 2008, 2014). The role of NAC transcription factors in senescence regulation is now well-established (Podzimska-Sroka et al., 2015). However, some *NAC* genes are also involved in plant adaptation to biotic and abiotic stresses (Al-Daoud and Cameron, 2011; Chen et al., 2014; McGrann et al., 2015a), suggesting possible roles in crosstalk between senescence regulation and plant pathogen defense. In barley, *HvNAM-1* expression is strongly induced in flag leaves after anthesis (Distelfeld et al., 2014). Differences between ‘Karl’ and ‘10_11’ flag leaves identified in previous as well as the present studies from our laboratory therefore depend on the accelerated-senescence phenotype of line ‘10_11’, as controlled by *HvNAM-1*.

Several studies in both model and crop species have analyzed the leaf senescence transcriptome in detail (e.g., Breeze et al., 2011), but much less is known about protein-level changes (Kim et al., 2016). Correlation between mRNA and protein levels can be low due to a variety of posttranscriptional factors such as RNA and protein stability (Haider and Pal, 2013), suggesting that a more complete understanding of biological processes will be achieved by combining transcriptomic and proteomic analyses. To investigate how closely mRNA-level differences between flag leaves of our early- and late-senescing germplasm are correlated to protein-level regulation, the same plant material was compared using two proteomic techniques. These included two-dimensional difference in-gel electrophoresis (2D-DIGE) and label-free quantitative mass spectrometry-based (‘shotgun’) proteomics, both of which comprise a final liquid chromatographic-mass spectrometric analysis (LC-MS). The 2D-DIGE method provides reliable and accurate quantification of the most abundant proteins (Lilley and Friedman, 2004), while advances in shotgun proteomics allow increased numbers of identifications with reliable quantification by means of precursor ion intensities/peak areas of both highly and less abundant proteins (Wienkoop and Weckwerth, 2006).

2. Materials and methods

2.1 Plant material

Near-isogenic barley (*Hordeum vulgare* L.) germplasm used in this study has previously been described and used for physiological and transcriptomic analysis of leaf and whole-plant senescence (Heidlebaugh et al., 2008; Jukanti and Fischer, 2008; Jukanti et al., 2008). Line ‘10_11’ is characterized by earlier senescence and higher grain protein concentration as compared to variety ‘Karl’, due to allelic variation at a chromosome 6H locus controlling these traits (see introduction). Seeds were planted on 1 February 2013 in a small randomized block design containing four blocks each of variety ‘Karl’ and line ‘10_11’ (eight blocks total) in a glasshouse of the Montana State University Plant Growth Center. A 22 °C / 18 °C day / night temperature regime was maintained in the glasshouse, and days were extended to a 16 h photoperiod when required, using Son-Agro 430-W high-pressure sodium lamps (Philips, Somerset, NJ, USA). Plants in potting soil were fertilized with 250 mL Peter’s Professional General Purpose fertilizer (4 g L⁻¹; Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) per 4-Liter pot with three plants every alternate week until anthesis, starting at two weeks past germination. When plants reached anthesis, each shoot used for further experimentation was labeled with its exact anthesis date. Flag leaves (leaf blades only) harvested at 14 and 21 dpa were flash-frozen in liquid nitrogen and stored at -80 °C until protein extraction. For each harvest time point, four to six ‘Karl’ and four to six ‘10_11’ samples were collected from the four blocks of the experiment, with each sample consisting of flag leaves from 8 to 10 plant shoots. Three samples of ‘Karl’ flag leaves (at 14 and 21 dpa) and three samples of ‘10_11’ flag leaves (at 14 and 21 dpa) were randomly picked from the material stored at -80 °C for proteomic analyses.

2.2 Sample preparation for DIGE analysis

Frozen flag leaves were ground for 1 min in liquid nitrogen with a mortar and pestle. Aliquots of 200 mg of leaf powder were suspended in 2 mL of extraction buffer (25 mM Tris/HCl pH 7.5

with 1.0 % (w/v) polyvinylpolypyrrolidone and 0.1% (v/v) β -mercaptoethanol). In order to block proteolysis, 20 μ L of protease inhibitor cocktail (Sigma, St. Louis, MO, USA, product P8849; containing 4-(2-aminoethyl)benzenesulfonyl fluoride, phosphoramidon, pepstatin A, bestatin and E-64) was added to each sample. Soluble proteins were concentrated by precipitation with cold acetone (5 volumes), after which they were resuspended for 1 h in suspension buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM dithiothreitol (DTT), 0.002% (w/v) bromophenol blue) without DTT. Particulates were removed by centrifugation at 24,700g for 20 min followed by careful removal of supernatant and storage at -80 °C. Protein concentration was determined with the Bradford Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Samples were diluted to concentrations of 2 mg mL⁻¹ with suspension buffer (without DTT) and were used as stocks for experiments. Protein quality was checked by 1D SDS-PAGE.

2.3 2D-DIGE

The protocol was used as previously described (Maaty et al., 2012a) and is briefly documented here. Minimal labeling of lysine side chains with N-hydroxysuccinimide ester cyanine dyes (CyDyes; Cy2, Cy3, Cy5; GE Healthcare Bio-Sciences Corp., Piscataway, NY, USA) was performed according to the manufacturer's protocol. Briefly, 50 μ g protein from each biological replicate was reacted with 400 pmol of CyDye (either Cy3 or Cy5; dissolved in 99.8% dimethylformamide; Sigma). The internal standard was created from pooled samples and labeled with Cy2. Reactions were quenched by the addition of 1 μ L of a 10 mM lysine solution.

Isoelectric focusing (IEF) used pre-cast IPG strips (pH 3-11, nonlinear, 24 cm length; GE Healthcare). Fluorescent-dye labeled samples were combined (e.g. 50 μ g 'Karl' Cy3, 50 μ g '10_11' Cy5, and 50 μ g internal standard Cy2) and brought to a volume of 450 μ L with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer pH 3-11 NL, 40 mM DTT, 0.002% (w/v) bromophenol blue). Gels for protein identification were loaded with 400 μ g protein. IEF was carried out with an IPGPhor II (GE Healthcare) at 20 °C, with a maximum of 50 mA/strip. IEF strips were then equilibrated for 15 min with 50 mM Tris/HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 20% (w/v) SDS and 0.007% (w/v) bromophenol blue with 65 mM DTT, followed by 15 min with iodoacetamide (153 mM). The second-dimension SDS-PAGE

was performed in a Dalt II (GE Healthcare) using 1 mm thick, 24 cm, 13% polyacrylamide gels, at constant current.

Gels were scanned using a Typhoon Trio Imager (GE Healthcare) at 100 μ m resolution. Images were analyzed for changes in protein abundance using Progenesis SameSpots software version 3.0.2 (Nonlinear Dynamics Ltd., Durham, NC, USA). Gels used for protein identification were stained with Coomassie GelCode Blue Safe (Thermo Scientific Inc., Rockford, IL, USA), destained in water, and then stored at 4 °C in 10% acetic acid for visible spot excision.

2.4 Protein spot identification and data analysis

Protein spots of interest were excised from the gels, washed in 100 mM (NH₄)HCO₃ (200 μ L), in-gel reduced and alkylated, followed by digestion with porcine trypsin (Promega Corp., Madison WI, USA) as described (Maaty et al., 2012a). LC-MS analysis was performed using an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS fitted to an Agilent 1100 Cap LC and ChipCube (Agilent Technologies, Santa Clara, CA, USA).

The data analysis pipeline consisted of the input from raw data files from the Agilent 6520 instrument in profile mode. These files were processed initially using the OpenMS software (Junker et al., 2012; Weisser et al., 2013) using the FileConverter script to generate molecular graphic format (mgf) files. A complete barley protein sequence file from <http://www.uniprot.org>, containing 52,289 protein entries, was used with SearchGUI (Vaudel et al., 2011) for protein identification. A set of reversed-decoy sequences were also appended to the target library and used in false discovery rate estimation. The final target-decoy library was queried using the following search parameters: up to two missed cleavages, precursor charges +2; +3; +4; precursor ion mass tolerance 20 ppm, fragmentation mass tolerance of 0.5 Da, and three variable post-translational modifications including (1) acetylation, + 42.04 Da, Cys/N-terminus; (2) oxidation, +15.99 Da, Cys/Met; (3) phosphorylation, + 79.97 Da, Tyr. Identification of b and y fragment ions and peptides was achieved by using X!Tandem (Muth et al., 2010) and OMSSA (Barsnes et al., 2009) search engines. The resulting files were in both .xml and .omx format (one file per sample, per search engine) and were directly loaded into PeptideShaker software (Vaudel et al., 2015). Searched data were filtered using a minimum false discovery rate setting of 1.0%

(at the protein level) and subsequently interpreted manually. To confidently identify proteins the contribution of two or more significant peptides was required (significance based on PeptideShaker software) (Vaudel et al., 2015).

2.5 Sample preparation for shotgun proteomics

Shotgun protein samples were acquired as described above for 2D-DIGE sample preparation. Stock samples of 2 mg mL⁻¹ (determined by Bradford analysis) were used to obtain 50 µg of each treated protein sample that were then transferred into two separate Eppendorf tubes, one for tryptic digest and the second for 1D gel electrophoresis. Protein digestion was performed in 50 mM (NH₄)HCO₃ with Trypsin Gold (Promega Corp., Madison WI, USA) at a 50:1 ratio (soluble protein / trypsin) followed by an overnight incubation at 37 °C. Residual undigested protein was removed by filtration using 3 K OMEGA Spin Columns (Pall Life Sciences, Ann Arbor, MI, USA) (24,700g, 5 min) prior to mass spectroscopic analysis of peptides. Three biological replicates from each of the two lines ('Karl' and '10_11') and time points (14 and 21 dpa) were used and prepared for MS experiments, totaling 12 samples.

2.6 LC-MS/MS for shotgun mass spectrometric analysis

LC-MS/MS analysis was performed using a Bruker maXis Ultra High Resolution microTOF-Q (Bruker Corporation, Fremont, CA 94538, USA) mass spectrometer fitted to an UltiMate[®] 3000 Nano LC (Thermo Scientific Inc., Rockford, IL 61101). Samples were trapped and desalted on a ProntoSIL Eurobond capillary column (125 mm x 4 mm ID; packed with ProntoSIL 120-5-EuroBOND-C18; 5 µm particles, Bischoff). The peptides were then eluted and loaded onto the analytical Synergi[™] 4 µm Fusion-RP 80 Å column (100 mm x 2 mm ID, packed with C18 particles, 4 µm particles, 40 nL volume, Phenomenex Inc.) in 5% acetonitrile / 0.1% formic acid delivered by an auxiliary pump at 4 µL min⁻¹. Peptides were eluted from the analytical column at 600 nL min⁻¹ using a 5 to 90% acetonitrile gradient over 120 min. Data-dependent acquisition of collision-induced dissociation tandem mass spectrometry was performed in Auto (MS/MS) mode over a mass range of 200 to 2,200 at 24,300 m/z-s. The acquired mass spectra were processed by

Compass DataAnalysis 4.0 software (Bruker Daltonics) and exported as raw data files for analysis.

2.7 Peptide identification and data analysis

The data analysis pipeline utilized a two-tiered search system. Initially, raw data were searched against a target-decoy library, assigned peptide identifications, filtered by false discovery rate, and exported by a minimum coverage. Separately, this output was matched to a dataset that was composed of normalized/aligned mass lists to obtain precursor ion intensities that corresponded to abundance of each peptide/protein in a run. For quality management of experimental data, a theoretical peptide target library was generated by performing an *in silico* tryptic digest on the protein sequence entries available in the current *Hordeum vulgare* L. UniProt database (<http://www.uniprot.org>; 52,289 sequences) and used for comparison. Experimental MS data files were converted using OpenMS FileConverter scripts (Junker et al., 2012; Weisser et al., 2013) into two separate file formats: (1) mgf; (2) MZxml for quantification in later analysis. Analyses were performed on two distinct sets of biological replicates depending on time point (14 or 21 dpa). Each set was composed of six samples; three from variety ‘Karl’, and three from line ‘10_11’. Database searching and peptide identification were performed as described for 2D-DIGE analysis. Protein hits were categorized into one of three groups. A protein was ‘identified’ when two or more peptides were assigned. Members of this group possessing three or more peptides were designated ‘quantifiable’. Potentially ‘quantifiable’ group members and their precursor ion information (*m/z* and retention time) were exported to a new mass library from assigned experimental data values (experimental mass library). Quantifiable proteins with a fold-change of ≥ 1.5 and a *p* value < 0.05 were additionally categorized as having ‘significantly different abundance’.

2.8 Label-free peptide quantification

Data files (MZxml) obtained in parallel were separately loaded into MZmine 2, a program designed for processing, visualizing, and analyzing MS-based molecular profile data (Pluskal et al., 2010). Analyses were performed simultaneously on each set of biological replicates. Feature

detection was based on centroid data using a minimum intensity cut-off of 1,500. The remaining mass lists were assigned retention times in seconds with the following limits: minimum intensity 2,500; timespan of 0.1 min; m/z tolerance of 20 ppm. Resulting chromatograms were normalized by retention time (0.01 m/z unit tolerance; 20% relative retention time tolerance; minimum intensity 5,000), then aligned to one another (0.01 m/z unit tolerance; 20% retention time tolerance; weighted equally; same charge state required), and finally gap-filled (50% intensity tolerance; 0.01 m/z unit tolerance; 20% retention time tolerance). The resulting mass list contained aligned features that could further be assigned using the previously generated experimental mass library (search criteria considered: 0.01 m/z unit tolerance; 20% retention time tolerance). The resulting assignments were filtered to only include entries in which three or more unique peptide precursor ions were present in every biological replicate. To limit false-positives and false-negatives resulting from differences in ionization the three peptides with most similar ion intensity were selected for quantification (Schulze and Usadel, 2010).

2.9 Quantification, statistics, and protein functional analysis

Protein abundances were determined for each sample by averaging tryptic peptide ion intensities of three peptides from three biological replicates. Fold-change ratios were determined by dividing the abundance values against one another (line '10_11' vs. variety 'Karl'). A Student's t -test was performed and p values are reported for each quantified ratio. Statistical analysis was performed using a combination of software packages including Microsoft Excel (Microsoft Corporation), XLStats (version 2013.5.01; Addinsoft, New York, NY, USA) and R (Ihaka and Gentleman, 1996).

In order to obtain Gene Ontology (The Gene Ontology Consortium, 2015) and functional information, each protein of interest was analyzed using the UniProt database (<http://www.uniprot.org>) (The UniProt Consortium, 2015) and a protein sequence homology search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (NCBI Resource Coordinators, 2015). When appropriate, the Phyre2 protein fold recognition server (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) (Kelley et al., 2015), the rice kinase database <http://ricephylogenomics.ucdavis.edu/kinase/> (Jung et al., 2010), the MEROPS protease database (<http://merops.sanger.ac.uk>) (Rawlings et al., 2014) and the carbohydrate-

active enzymes database (<http://www.cazy.org/>) (Lombard et al., 2014) were also used. MS data have been deposited at the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) using the PRIDE partner repository and can be accessed via the ProteomeXchange FTP directory (<ftp://massive.ucsd.edu>) (Jones et al., 2008). The dataset identifier is PXD002307.

3. Results

3.1 Proteomic comparison of early- and late-senescing barley germplasm

Late-senescing barley variety ‘Karl’ and early-senescing near-isogenic line ‘10_11’ have previously been compared using physiological and transcriptomic approaches (see introduction). In the present study, this project has been extended to capture protein-level regulatory processes, by comparing ‘Karl’ and ‘10_11’ flag leaf proteomes at 14 and 21 dpa.

Using 2D-DIGE analysis, 332 (14 dpa) and 323 (21 dpa) protein spots were present in both ‘Karl’ and ‘10_11’ (Fig. 1). Rubisco large subunits were conspicuous in 14-dpa (Fig. 1 A, B) but not in 21-dpa samples (Fig. 1 C, D). This contrasts with data obtained by 1D SDS-PAGE (appendix A, Fig. A1; see also Jukanti and Fischer, 2008), which indicate the presence of this abundant protein in both 14- and 21-dpa flag leaves. The difference may be due to decreased solubility of Rubisco with advancing leaf senescence (Moreno et al., 2008), allowing it to be solubilized with SDS, but not with urea/thiourea used for 2D-DIGE sample preparation. As the well-documented degradation of Rubisco during senescence (Feller et al., 2008) was not the focus of this study, we did not pursue this point further. Mass spectrometry-based protein identification from 2D-DIGE analysis focused on proteins which were of significantly (p value <0.05 ; ≥ 1.5 -fold) higher abundance in early-senescing line ‘10_11’ as compared to ‘Karl’ at either 14 dpa (7 proteins) or 21 dpa (10 proteins; highlighted with arrows in Fig. 1). Such proteins may be important for senescence regulation and/or execution.

Shotgun proteomics allowed the identification and quantification of a large number of additional proteins. Proteins with two or more peptides contributing to their characterization were considered as ‘identified’ (appendix A, Table A1). Proteins for which three or more peptides were identified met the criterion of ‘quantifiable’ (Schulze and Usadel, 2010) (appendix

A, Table A2). Samples obtained at 14 and 21 dpa generated 1,176 and 1,839 quantifiable proteins from 5,055 and 5,338 identifications, respectively (Table 1). Based on the total number of predicted barley proteins (52,289 entries, retrieved from <http://www.uniprot.org>), this corresponds to a proteome coverage of ~10% at both time points. Most quantified proteins, at both 14 and 21 dpa, did not show a significant difference in abundance between ‘Karl’ and ‘10_11’ flag leaves (Table 1, Fig. 2). Nine proteins were more abundant in line ‘10_11’, while 56 were less abundant compared to ‘Karl’ at 14 dpa (Table 1, Fig. 2A). A total of 347 proteins had different abundances at the later time point, with 49 having higher abundance and 298 having lower abundance in ‘10_11’ as compared to ‘Karl’ (Table 1, Fig. 2B). As net protein degradation is one of the hallmarks of leaf senescence, these results are in agreement with previous physiological and transcriptomic analyses establishing the early-senescence phenotype of line ‘10_11’.

3.2 Overview of identified proteins

All proteins identified by shotgun experiments (from two or more tryptic peptides) at 14 or 21 dpa, both from variety ‘Karl’ and from line ‘10_11’, were analyzed for the presence of conserved protein domains. Numerous proteins from both sampling time points contained transmembrane helices, signal domains, coiled coils, repeat domains or zinc fingers (Fig. 3). A series of additional domains were less frequent. Most domains occurred with similar frequencies at both sampling time points, but some (including ankyrin repeats and redox-active centers) were notably less frequent at 21 dpa, while others (including homeobox domains and LIM domains) were more abundant at the later time point.

All identified proteins were also subjected to gene ontology analysis (The Gene Ontology Consortium, 2015) (Fig. 4). Most proteins classified under ‘biological process’ occurred at comparable frequencies in 14- and 21-dpa samples, but ‘detoxification’ was assigned to 60 proteins at 14 dpa (none at 21 days), while the more specific ‘cellular oxidant detoxification’ (defined as removing or reducing superoxide radicals or hydrogen peroxide; 22 proteins) was identified only at the later time point, suggesting that this function may become more important with increasing leaf age.

3.3 Overview of quantified proteins

All proteins quantified by shotgun experiments (from three or more tryptic peptides), and proteins which were found to be higher-abundance in line ‘10_11’ by 2D-DIGE, were analyzed for the presence of conserved protein domains. Coiled coils, transmembrane helices and signal domains were most numerous, both in proteins whose abundances were unchanged between ‘Karl’ and ‘10_11’, and in proteins with significantly different abundances (Fig. 5A, B). A number of domains occurring at lower frequencies were only found in proteins present at similar levels in ‘Karl’ and ‘10_11’ (particularly at 14 dpa), but coiled coils, transmembrane helices, zinc fingers, WD repeats, leucine-rich repeats and transit peptides were well-represented among proteins with significant differences in abundance between early- and late-senescing barley, particularly at 21 dpa.

To further explore functions associated with quantified proteins, gene ontology analysis was utilized. Table 1 and our previous transcript-level study (Jukanti et al., 2008) indicated more differences between ‘Karl’ and ‘10_11’ flag leaves at 21 than at 14 dpa. This fact was emphasized when separating quantified proteins by gene ontology/biological process (Fig. 6A, B). Considerably more proteins classified under ‘response to stimulus’, ‘biological regulation’, ‘localization’ and ‘cellular component organization’ were found at lower abundance in line ‘10_11’ as compared to ‘Karl’ at 21 than at 14 dpa (Fig. 6A, B). Proteins found at higher levels in late-senescing line ‘10_11’ were also more frequent at 21 than at 14 dpa, particularly under ‘biological regulation’, ‘localization’, and in several of the (overall) less-frequent categories.

The gene ontology class termed ‘cellular metabolic process’ provided additional useful information (Fig. 7A, B). Downregulation of many metabolic functions in flag leaves of early-senescing line ‘10_11’ as compared to late-senescing line ‘Karl’ at 21 dpa is particularly visible in this analysis, especially if the ratio of proteins with low abundance in line ‘10_11’ to all quantified proteins is considered (Fig. 7B). In the ‘photosynthesis’ class, a majority of quantified proteins (8 out of a total of 12) were found at lower abundance in line ‘10_11’ at 21 dpa, and in ‘cofactor metabolic processes’, all 5 quantified proteins were downregulated in line ‘10_11’. Despite this general trend, data in Fig. 7B also identify several categories with substantial numbers of proteins found at higher levels in line ‘10_11’ as compared to ‘Karl’ at 21 dpa, particularly in the macromolecule, nitrogen compound, aromatic compound, heterocycle,

phosphorus and organic acid metabolism classes. As proteins which (against the overall trend towards lower abundance in the early-senescing line) are present at higher levels in line '10_11' may be functionally important for the leaf senescence process, these were analyzed in greater detail.

3.4 Proteins which are present at higher levels in line '10_11' as compared to 'Karl'

Proteins with significantly higher levels in early-senescing line '10_11' as compared to 'Karl', at either 14 or 21 dpa, and based on both shotgun and 2D-DIGE analyses, were further analyzed using the web-based tools indicated under materials and methods. Using this approach, they were assigned to six functional categories (Table 2), described in the following sections.

3.4.1 Proteins with (putative) roles in plant pathogen defense

The largest group, composed of 25 members, consisted of proteins with clear or putative roles in plant pathogen defense. This group included two family 1 pathogenesis-related proteins (F2DMI6, Q05968), three nucleotide-binding site leucine-rich repeat (NBS-LRR)/disease resistance proteins (M0Z6M8, F2DAV7, A9UKM2), three glucanases (O64938, M0YUE3, F2DLS4; β -1,3-glucanases have been classified as family 2 pathogenesis-related proteins) (Roulin et al., 1997; Van Loon et al., 2006), a ribosome-inactivating protein (P22244), a peroxidase (F2DVY5), and three leucine-rich repeat receptor-like protein kinases (LRR-RLKs) which may be involved in the detection of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) (F2DLM6, F2CYK3, F2DKJ2) (Table 2).

Several additional, functionally diverse proteins may also be involved in plant-pathogen interactions. The protein which was most abundant in line '10_11' as compared to 'Karl' (with a ratio of 23.9; F2DS71) is similar to quinone reductases. A wheat quinone reductase plays a role in the interaction between this crop and powdery mildew (Greenshields et al., 2005). Proteins involved in lipid metabolism and signaling (F2E0G6, F2DEY4, M0W8C6, M0XVD2) may function in cuticle layer modification to prevent pathogen entry (Tang et al., 2007). Genes coding for proteins related to M0W4D3, a putative calcium-permeable cation channel, are

upregulated in response to both abiotic and biotic stress (Hou et al., 2014) and could be involved in calcium signaling. Numerous *Arabidopsis* ABC transporter C family members (related to M0VRP3) have been demonstrated to be induced after pathogen treatment, and an ABC transporter G family member has been demonstrated to be important for plant resistance to powdery mildew (Stein et al., 2006). Such transporters could be involved in the export of toxic metabolites to attempted fungal pathogen invasion sites (Stein et al., 2006). Tyrosine monooxygenases/hydroxylases (related to M0XBQ5) are involved in catecholamine metabolism, converting tyrosine to L-DOPA, a precursor of defense compounds whose synthesis is activated by fungal elicitors, attempted fungal infection, viruses and bacteria (Kulma and Szopa, 2007). Intriguingly, a barley cyclic nucleotide gated ion channel (CNGC4) which is 90% identical to F2EHG6 (336 amino acid overlap) has been implicated in both plant pathogen defense and regulation of (dark-induced) senescence (Rostoks et al., 2006; Zhang et al., 2009; McGrann et al., 2015b). *Arabidopsis* CNGC2 and CNGC4 have also been shown to function in plant pathogen defense (Ma and Berkowitz, 2007, 2010), and CNGC2 is also involved in senescence regulation (Ma et al., 2010). Protein M0UVB4 contains an SCP-like extracellular domain, and is therefore related to pathogenesis-related protein 1 (Van Loon et al., 2006). A role for an ABA 8'-hydroxylase (Q0PD05) in abscisic acid catabolism, leading to upregulation of defense compound synthesis, has previously been described (Mialoundama et al., 2009). Knockdown of two *Arabidopsis* pentatricopeptide genes has been found to lead to more severe bacterial and fungal disease symptoms (Park et al., 2014), and a related protein (F2EGM4) was ~5-fold more abundant in line '10_11' than in 'Karl' at 14 dpa.

3.4.2 Protein synthesis, folding and degradation

This group, with 14 members, included three ribosomal proteins (F2CT33, F2CRD7, F2CTV4), chaperonins (F2DB07, M0VYQ7, M0VN45), a (putative) prolyl-tRNA synthetase (F2CTM7) and an elongation factor (F2EE16) (Table 2). Functions involved in protein degradation include elements of the ubiquitin-proteasome system (UPS; F2DPJ3, M0WZ82, F2CTE6, F2DQ10), a subtilisin-like (serine-type) protease (F2DFK1) and a tripeptidyl peptidase (M0YRC6). *De novo* protein synthesis has been shown to be required during plant senescence (Thompson et al., 2004), and proteolysis has long been acknowledged as an important factor in

senescence-associated nitrogen remobilization (Feller et al., 2008; Distelfeld et al., 2014) as well as for the regulation of the senescence process (Fischer, 2012).

3.4.3 DNA/chromatin synthesis and repair

This group included four members (listed in Table 2). Interestingly, bacterial, fungal and oomycete plant pathogens have been found to induce double strand DNA breaks in plant DNA (Song and Bent, 2014); prevention and repair of DNA damage may therefore be part of the observed upregulation of pathogen defense responses in line ‘10_11’.

3.4.4 Primary metabolism

Four proteins involved in primary plant metabolism were found to be higher-abundance in ‘10_11’ leaves (Table 2), including a citrate synthase, an aminomethyltransferase, a sulfite reductase and a glutamine synthetase. Citrate synthase (F2EIG7) has been found by other authors to be upregulated at the transcript level during senescence (Gregersen and Holm, 2007), and could be involved in preparing/modifying carbon skeletons for nitrogen re-assimilation and export from the leaf.

3.4.5 Hormone metabolism

Three proteins directly associated with plant hormone metabolism (an ABA 8'-hydroxylase, already mentioned in section 3.4.1; Q0PD05), a protein involved in ethylene signaling (CTR1; F2E5Y1), and a putative auxin efflux carrier (F2CZK2) were present at higher levels in 21-dpa ‘10_11’ as compared to ‘Karl’ flag leaves. Roles for ethylene and abscisic acid in senescence regulation are well-established, with both factors involved in promoting the process (Fischer, 2012).

3.4.6 Other functions

Additional proteins that were upregulated in line ‘10_11’ include two putative B cell receptor-associated proteins (BAPs; F2DBM9, M0XNJ9) (Table 2). BAP31 is an integral membrane protein of the endoplasmic reticulum, and functions as an escorting factor in the sorting of newly synthesized proteins in the ER (Wang et al., 2008). Intriguingly, BAP31 has also been demonstrated to be involved in the initiation of apoptosis in mice (Namba et al., 2013), and both the hypersensitive response and the final stage of leaf senescence end in cell death.

Annotation of 24 additional proteins which are significantly upregulated in line ‘10_11’ as compared to ‘Karl’ did not assign them to any of the functional groups discussed above, such as senescence regulation or plant-pathogen interaction. Annotation of these proteins is shown in appendix A, Table A3.

3.4.7 Comparison of transcriptomic and proteomic analysis

Late-senescing barley variety ‘Karl’ and early-senescing line ‘10_11’ have previously been compared using a transcriptomic approach (Jukanti et al., 2008), allowing the comparison of new proteomic with existing transcriptomic data. Ten genes were demonstrated to be upregulated at both transcript and protein levels (albeit not always at the same time point); in two cases, downregulation was found for transcripts, while proteins were higher-abundance in line ‘10_11’ than in variety ‘Karl’ (summarized in Table 3). For 24 proteins occurring at higher levels in line ‘10_11, at either 14 or 21 dpa, no probes representing the corresponding genes are present on the Affymetrix Barley1 GeneChip used by Jukanti et al. (2008). While such probes can be found for an additional 39 proteins, the corresponding genes were not significantly upregulated in line ‘10_11’ (data not shown). However, for 31 of the more abundant proteins without directly corresponding transcript-level data (lacking probes, or not regulated), genes with identical or related functions were upregulated. For many biological processes identified here, differential regulation occurs at both the transcript and protein levels but, as has been found in other studies, regulation at the transcript level is not always a good indicator for protein-level regulation (Haider and Pal, 2013).

4. Discussion

Senescence is a controlled developmental process that has a substantial impact on the yield and quality of cereal crops (Distelfeld et al., 2014). This study focused on the identification and quantification of global changes in the proteome of barley (*Hordeum vulgare* L.) flag leaves during senescence. The selected strategy is distinct because both 2D-DIGE and shotgun proteomic techniques were employed, and data can be compared with our previous transcriptomic comparison of ‘Karl’ and ‘10_11’ flag leaves at 14 and 21 dpa (Jukanti et al., 2008). In total, 9,258 proteins were identified (corresponding to ~18% coverage of the barley proteome) and one third of them were quantified in this study. Compared to other recent crop proteomic studies, these numbers are high, underlining the value of using shotgun in addition to 2D-DIGE techniques.

There was a considerably larger number of proteins with a significant difference in abundance at 21 as compared to 14 dpa (Table 1, Fig. 2). More proteins were down- than upregulated in line ‘10_11’ as compared to ‘Karl’ at both time points (Table 1, Fig. 2). Based on the previously established fact that line ‘10_11’ senescences faster than variety ‘Karl’ and that protein degradation and N remobilization are hallmarks of senescence (Feller and Fischer, 1994), this result may be expected. However, it is reasonable to argue that proteins which do not follow this trend, i.e. which are more abundant in the early-senescing line, may be functionally important for the senescence process. Based on an overview of all identified (Figs. 3 and 4) and particularly of all quantified proteins (Figs. 5-7), in-depth analysis has therefore focused on those proteins which are more abundant in line ‘10_11’ flag leaves, at either 14 or 21 dpa (Tables 2 and 3).

The largest group of proteins associated with the early-senescence phenotype of line ‘10_11’ in this study consists of those with likely or putative functions in plant pathogen defense. These include classical pathogenesis-related proteins, intracellular and membrane receptors or co-receptors (NBS-LRRs, LRR-RLKs), enzymes involved in attacking pathogen cell walls (glucanases), enzymes with possible roles in cuticle modification, and enzymes involved in DNA repair (see results and Table 2). Several or numerous mRNAs belonging to all these functional groups, with the exception of DNA repair, were also identified as upregulated in our previous transcriptomic comparison of ‘Karl’ and ‘10_11’ flag leaves at 14 and 21 dpa (section 3.4.7),

indicating regulation of defense-related functions at both the transcript and protein levels. It should be borne in mind, however, that some of the defense-related proteins identified may function in a more generalized stress response rather than just pathogen defense. As an example, it has been demonstrated that β -1,3-glucanase activity is significantly induced by drought (Lee et al., 2008).

Induction of defense-related genes during developmental senescence has previously been observed (Hanfrey et al., 1996; Guo and Gan, 2012). At the same time, *Senescence-Associated Genes* (SAGs) have been shown to be induced during plant defense against various pathogens (Espinoza et al., 2007), and some genes and proteins have been demonstrated to be involved in both senescence regulation and plant pathogen defense (Robatzek and Somssich, 2002; Kwon et al., 2009; Ma et al., 2010). The hypothesis has been forwarded that senescing plant organs are susceptible to opportunistic infections (Quirino et al., 2000); upregulation of defense-related genes and proteins may therefore have evolved as part of the developmental senescence program to protect plant nutrients during their remobilization to surviving organs of perennial or seeds of annual plants.

Variety ‘Karl’ and line ‘10_11’, which is near-isogenic to ‘Karl’, differ in the allelic state of a chromosome six locus containing the *HvNAM-1* gene (see introduction). The role of several NAC transcription factors in senescence regulation is now well-established (Podzimska-Sroka et al., 2015), but *NAC* genes are also important for plant adaptation to biotic stresses (Al-Daoud and Cameron, 2011; McGrann et al., 2015a). Upregulation of the genes and proteins described in Jukanti et al. (2008) and in the present study therefore likely depend on allelic variation in the coding or control sequences of *HvNAM-1* (Distelfeld et al., 2008), but it cannot be excluded that linked genes also have some influence on the presented data.

Besides defense-related proteins, a second functional group identified as upregulated in line ‘10_11’ consists of proteins involved in protein synthesis and protein degradation. This group contains three ribosomal proteins, two chaperonins, a putative prolyl-tRNA synthetase and a putative elongation factor (Table 2). While overall/net proteolysis is predominant during senescence, it has long been acknowledged that senescence is associated with changes in gene expression, and with the synthesis of new proteins (Thompson et al., 2004). Enzymes and proteins involved in protein degradation include several elements of the ubiquitin-proteasome system (UPS), a family S8 serine protease and a tripeptidyl peptidase (Table 2). The low number

of significantly regulated proteases may be surprising compared to transcriptomic analyses of the senescence process including our own (Parrott et al., 2007; Jukanti et al., 2008). Additional proteases were found to be upregulated in this study, but with p values >0.05 . These included a calpain (family C2A cysteine peptidase), an additional (family S53) tripeptidyl peptidase, two family A1 aspartic peptidases or related proteins, and two metallopeptidases from families M41 and M48, suggesting that biologically relevant information was eliminated by the statistical threshold applied. Considering that peptidases, particularly cysteine and serine peptidases, are typically activated from precursor proteins through limited proteolysis (Van der Hoorn, 2008), it will become important to apply additional proteomic methods such as activity-based protein profiling (Maaty et al., 2012b) to fully characterize senescence-associated proteolysis and nitrogen remobilization.

While the UPS is not involved in bulk protein degradation during leaf senescence, several lines of evidence indicate that it contributes to selective hydrolysis of proteins important for senescence regulation (Fischer, 2012). The UPS is also involved in the regulation of defense responses, and some elements of the UPS may have functions in both defense and senescence regulation (e.g., Zhou et al., 2015). Data presented in Table 2, together with previous transcriptomic results indicating ~13-fold upregulation of a polyubiquitin transcript in 21-dpa ‘10_11’ leaves (Jukanti et al., 2008), may serve as a basis for the characterization of UPS function at the intersection of plant senescence regulation and plant-pathogen interaction.

Among the additional proteins identified as upregulated in line ‘10_11’, a citrate synthase and two proteins involved in hormone signaling and transport are conspicuous. The latter include CTR1 (involved in ethylene signal transduction) and a putative auxin efflux carrier. Upregulation of a citrate synthase gene has been found in a transcriptomic analysis of wheat flag leaf senescence (Gregersen and Holm, 2007); citrate synthase may be involved in organic and amino acid metabolism needed for synthesis of nitrogen transport forms. Senescence regulation by hormones has been studied for decades; ethylene is considered among the prominent senescence-accelerating factors and is also involved in plant pathogen responses, while the role of auxins in senescence is less well-defined (Fischer, 2012). Additional biochemical and cell biological analyses will be needed to delineate the function of the identified hormone-associated proteins, but their differential regulation in the experimental system used here will provide an important starting point for such studies.

Data from the present proteome-based analysis demonstrate that defense functions are upregulated in line '10_11' as compared to 'Karl', more clearly so than our previous transcriptomic study. A series of genes coding for proteins with putative or confirmed roles in defense are upregulated in Jukanti et al. (2008), but they are less conspicuous, as functions associated with cell signaling/regulatory processes, protein remobilization, and nutrient metabolism and transport are more prevalent in the previous (transcriptomic) than in the current (proteomic) analysis. It is well-accepted that important regulation occurs posttranscriptionally; studying biological systems and processes at both the RNA and protein levels is therefore particularly valuable (Haider and Pal, 2013). While biologically relevant information may have been eliminated by the statistics applied here (as discussed above for proteases), the fact that defense-related functions are more conspicuous at the protein than at the RNA level points to the particular importance of such functions during leaf senescence.

Overall, this study demonstrates the value of complementing well-established 2D-DIGE with more recently developed shotgun proteomic approaches. Together with transcriptomic data, results presented here provide a more complete picture of barley leaf senescence, as influenced by the NAC transcription factor *HvNAM-1*, and point to the importance of defense functions during developmental leaf senescence.

Author contributions

KEM performed most of the experiments, analyzed data and wrote a first draft of the manuscript. JKM collected shotgun and 2D-DIGE mass spectrometry data. WSM helped KEM with 2D-DIGE sample preparation, data collection and data analysis. BDR and PAG provided dyes for 2D-DIGE and critically read the manuscript. BB directed mass spectrometry experiments (data collection and analysis) and helped with manuscript writing. AMF directed the study and finalized the manuscript with feedback from all authors.

Acknowledgments

This work was supported by the U.S. National Institute of Food and Agriculture (NIFA), Hatch project 0192396 and by the Montana Agricultural Experiment Station (MAES). The Proteomics,

Metabolomics, and Mass Spectrometry facility at Montana State University received support from the Murdock Charitable Trust and National Institutes of Health (NIH) 5P20RR02437 of the Centers of Biomedical Research Excellence (CoBRE) program. Funding sources had no role in study design.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/xxx>.

Fig. A.1. One-dimensional SDS-PAGE gels of ‘Karl’ and ‘10_11’ flag leaf proteins at 14 (panel A) and 21 days past anthesis (panel B). The large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are conspicuous at both time points.

Table A.1. All barley proteins identified in this study.

Table A.2. Quantifiable protein groups with significant differences between ‘Karl’ and ‘10_11’.

Table A.3. Proteins which are significantly ($p < 0.05$) upregulated in line ‘10_11’ as compared to ‘Karl’, and which do not belong to one of the major functional categories.

Figure legends

Fig. 1. Representative 2D gels showing total soluble proteins from barley flag leaves. Late-senescing variety ‘Karl’ (A, C) and early-senescing line ‘10_11’ (B, D) flag leaf proteomes at 14 (A, B) and 21 days past anthesis (dpa) (C, D) are shown. Exactly 332 and 323 spots were common in all samples imaged with CyDye-based labeling in 14- and 21-dpa samples, respectively. Protein spots with significantly higher abundance in line ‘10_11’ as compared to ‘Karl’ (based on three replicates) at either 14 or 21 dpa are identified on the gels. Rubisco large (R_L) and small subunits (R_{ss}) are highlighted in panels A and B.

Fig. 2. Shotgun proteomic analysis of late-senescing barley variety ‘Karl’ and early-senescing line ‘10_11’ flag leaves. Protein abundances, based on ion intensities of three peptides across three replicates, were compared at 14 (A) and 21 days past anthesis (B). Proteins that are not significantly different in abundance are shown with small grey symbols; proteins that are significantly (p value <0.05 ; difference ≥ 1.5 -fold) more or less abundant in line ‘10_11’ as compared to ‘Karl’ are shown with large symbols.

Fig. 3. Protein domains in identified proteins. Proteins identified in ‘Karl’ and ‘10_11’ flag leaf samples at 14 (filled columns) and 21 days past anthesis (open columns) are shown.

Fig. 4. Analysis of identified proteins by gene ontology/biological process. Proteins identified in ‘Karl’ and ‘10_11’ flag leaf samples at 14 (filled columns) and 21 days past anthesis (open columns) are shown.

Fig. 5. Protein domains in quantifiable proteins at 14 (A) and 21 days past anthesis (B). Proteins without significant differences in abundance between variety ‘Karl’ and line ‘10_11’ (black), proteins which are significantly less abundant (p value <0.05 ; ≥ 1.5 -fold) in line ‘10_11’ (dark grey), and proteins which are significantly more abundant (p value <0.05 ; ≥ 1.5 -fold) in line ‘10_11’ (light grey) are shown. Data for higher-abundance proteins include those identified by 2D-DIGE.

Fig. 6. Analysis of quantifiable proteins by gene ontology/biological process at 14 (A) and 21 days past anthesis (B). Proteins without significant differences in abundance between variety ‘Karl’ and line ‘10_11’ (black), proteins which are significantly less abundant (p value <0.05 ; ≥ 1.5 -fold) in line ‘10_11’ (dark grey), and proteins which are significantly more abundant (p value <0.05 ; ≥ 1.5 -fold) in line ‘10_11’ (light grey) are shown. Data for higher-abundance proteins include those identified by 2D-DIGE.

Fig. 7. Analysis of quantifiable proteins by gene ontology/cellular metabolic process at 14 (A) and 21 days past anthesis (B). Proteins without significant differences in abundance between variety ‘Karl’ and line ‘10_11’ (black), proteins which are significantly less abundant (p value

<0.05; ≥ 1.5 -fold) in line '10_11' (dark grey), and proteins which are significantly more abundant (p value <0.05; ≥ 1.5 -fold) in line '10_11' (light grey) are shown. Data for higher-abundance proteins include those identified by 2D-DIGE.

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Table 1
Shotgun proteomics data summary.

Protein category	Sample time point (dpa)	
	14	21
	Number of proteins	
Identified (≥ 2 peptides)	5055	5338
Quantifiable (≥ 3 peptides)	1176	1839
Significantly different between ‘Karl’ and ‘10_11’ (<i>p</i> value <0.05 ; ≥ 1.5 -fold)	65	347
Significantly <i>higher</i> abundance in ‘10_11’ vs. ‘Karl’ (<i>p</i> value <0.05 ; ≥ 1.5 -fold)	9	49
Significantly <i>lower</i> abundance in ‘10_11’ vs. ‘Karl’ (<i>p</i> value <0.05 ; ≥ 1.5 -fold)	56	298

dpa, days past anthesis

Table 2

Proteins which are significantly ($p < 0.05$) upregulated in line '10_11' vs. variety 'Karl' at 14 or 21 dpa, as determined by both 2D-DIGE and shotgun analysis

ID ^a	Name/gene ontology/homology	Time point (dpa)	Ratio 10_11/Karl
Pathogen defense/putative pathogen defense			
F2DMI6	100% identical to 'PR-1a pathogenesis related protein' [<i>Hordeum vulgare</i>] ^b	14	2.2
Q05968	Pathogenesis-related protein 1 ^a ; 100% identical to 'pathogenesis-related protein 1, precursor' [<i>Hordeum vulgare</i>] ^b	14	1.8
M0Z6M8	ADP binding ^a ; 75% identical to 'disease resistance protein RPM1' [<i>Aegilops tauschii</i>] ^b	21	3.9
F2DAV7	ADP binding ^a ; 45% identical to 'putative disease resistance protein RGA3' [<i>Aegilops tauschii</i>] ^b	21	6.7
A9UKM2	NBS-LRR resistance-like protein ^a ; ADP binding ^a ; 100% identical to 'NBS-LRR resistance-like protein' [<i>Hordeum vulgare</i>] ^b	21	3.4
O64938	β -1,3-glucanase 2 ^a ; 100% identical to ' β -1,3-glucanase 2' [<i>Hordeum vulgare</i>] ^b ; glycoside hydrolase family 17 / GH17 ^f	14	2.4
M0YUE3	Glucan endo-1,3- β -glucosidase GII [<i>Hordeum vulgare</i>] ^b ; glycoside hydrolase family 17 / GH17 ^f	14	2.3
F2DLS4	99% identical to ' β -D-glucan exohydrolase isoenzyme Exol' [<i>Hordeum vulgare</i>] ^b	21	9.7
P22244	Protein synthesis inhibitor I ^a ; defense response to fungus ^a ; 100% identical to 'protein synthesis inhibitor I/ribosome-inactivating protein I/rRNA N-glycosidase chain A' [<i>Hordeum vulgare</i>] ^b	21	1.5
F2DVY5	Peroxidase ^a ; 70% identical to 'peroxidase 52 isoform 1' [<i>Zea mays</i>] ^b	14	1.8
F2DLM6	80% identical to 'predicted: probable LRR receptor-like serine/threonine protein kinase At1g14390-like' [<i>Brachypodium distachyon</i>] ^b ; 70% identical to Os06g38970, an LRR-VI non-RD kinase ^e	21	2.5
F2CYK3	87% identical to 'somatic embryogenesis receptor kinase 1' (SERK1) [<i>Triticum urartu</i>] ^b ; 71% identical to Os04g38480, an LRR-II RD kinase ^e	14	4.0
F2DKJ2	94% identical to 'tyrosine-sulfated glycopeptide receptor 1' [<i>Aegilops tauschii</i>] ^b ; 71% identical to Os02g05960, an LRR-Xb RD kinase ^e	21	2.4
F2DS71	Oxidoreductase activity ^a ; zinc ion binding ^a ; 50% identical to 'NADPH:quinone reductase' [<i>Sphingobacterium</i> sp.] ^b	21	23.9
F2E0G6	95% identical to 'long-chain fatty acid-CoA ligase 4' [<i>Aegilops tauschii</i>] ^b	21	9.3
F2DEY4	Integral component of membrane ^a ; 66% identical to 'no exine formation 1 isoform 1' [<i>Theobroma cacao</i>] ^b	21	3.8
M0W8C6	85% identical to 'GDSL esterase/lipase' [<i>Aegilops tauschii</i>] ^b	21	3.3
M0XVD2	Phospholipid-transporting ATPase ^a ; 95% identical to 'phospholipid-transporting ATPase 1' [<i>Aegilops tauschii</i>] ^b	21	2.1
M0W4D3	Integral component of membrane ^a ; 48% identical to the only characterized DUF221 protein, At4g22120, a stress-inducible calcium-permeable cation channel ^b	21	6.4
M0VRP3	92% identical to 'ABC transporter C family member 10' [<i>Aegilops tauschii</i>] ^b	21	3.4
M0XBQ5	88% identical to 'tyrosine N-monooxygenase/hydroxylase' [<i>Aegilops tauschii</i> , <i>Triticum urartu</i>] ^b	21	3.0
F2EHG6	93% identical to 'predicted: cyclic nucleotide-gated ion channel 4-like' [<i>Brachypodium distachyon</i>] ^b	21	2.4
M0UVB4	Plasmodesma ^a ; zinc ion binding ^a ; 96% identical to 'sacsin' [<i>Triticum urartu</i>] ^b	21	2.2
Q0PD05	ABA 8'-hydroxylase ^a ; 100% identical to 'ABA 8'-hydroxylase 1' [<i>Hordeum vulgare</i> subsp. <i>vulgare</i>] ^b	21	1.8
F2EGM4	83% identical to 'predicted: pentatricopeptide repeat-containing protein, mitochondrial-like' [<i>Brachypodium distachyon</i>] ^b	14	4.8
Protein synthesis, folding and degradation			
F2CT33	87% identical to 'predicted: 50S ribosomal protein L4, chloroplastic' [<i>Brachypodium distachyon</i>] ^b	14	1.7
F2CRD7	98% identical to 'predicted: 60S ribosomal protein L4-like isoform 1' [<i>Acyrtosiphon pisum</i>] ^b	21	3.0
F2CTV4	87% identical to '30S ribosomal protein S1, chloroplastic', [<i>Triticum urartu</i>] ^b	21	2.1
F2DB07	96% identical to '10 kDa chaperonin' [<i>Aegilops tauschii</i>] ^b	14	2.4
M0VYQ7	Chaperone; modeled with 100% confidence to template c2kglA ^d	21	8.8
M0VN45	86% identical to 'luminal-binding protein 5' [<i>Aegilops tauschii</i>] ^b . Modeled with 100% confidence to template c3d2fC, chaperone/heat shock protein ^d	21	1.6
F2CTM7	Prolyl tRNA aminoacylation ^a ; 88% identical to 'putative prolyl-tRNA synthetase' [<i>Oryza sativa</i> Japonica Group] ^b	21	4.7
F2EE16	79% identical to 'elongation factor 3' [<i>Cryptococcus neoformans</i>] ^b	21	2.3
F2DPJ3	88% identical to 'ubiquitin carboxyl-terminal hydrolase 19' [<i>Triticum urartu</i>] ^b	14	1.5

M0WZ82	Ubiquitinyl hydrolase activity ^a ; 91% identical to 'inactive ubiquitin carboxyl-terminal hydrolase 54' [<i>Triticum urartu</i>] ^b	21	2.6
F2CTE6	Protein ubiquitination ^a ; 86% identical to 'U-box domain-containing protein 44-like' [<i>Brachypodium distachyon</i>] ^b	21	1.6
F2DQ10	94% identical to 'predicted: 26S proteasome non-ATPase regulatory subunit 2 homolog A-like' [<i>Brachypodium distachyon</i>] ^b	21	1.5
F2DFK1	72% identical to 'subtilisin-like protease' [<i>Aegilops tauschii</i>] ^b ; MEROPS ^c classification S08.A28, 'senescence-associated subtilisin protease'	14	3.5
M0YRC6	93% identical to 'tripeptidyl-peptidase 2' [<i>Aegilops tauschii</i>] ^b	21	6.7
DNA/chromatin synthesis and repair			
F2DD24	DNA binding ^a ; DNA repair ^a ; 91% identical to 'DNA polymerase λ ' [<i>Triticum urartu</i>] ^b	14	3.6
F2EFZ8	DNA repair ^a ; 86% identical to 'BREAST CANCER SUSCEPTIBILITY 1-like protein' [<i>Aegilops tauschii</i>] ^b	21	2.3
M0XBC0	86% identical to 'predicted: protein CHROMATIN REMODELING 4' [<i>Brachypodium distachyon</i>] ^b	14	2.5
F2DXK0	Nucleosome assembly ^a ; 91% identical to 'histone H1 WH1B.1' [<i>Triticum aestivum</i>] ^b	21	3.9
Primary metabolism			
F2EIG7	Citrate synthase ^a	21	2.5
F2DZ70	Aminomethyltransferase ^a ; 93% identical to 'predicted: aminomethyltransferase, mitochondrial' [<i>Brachypodium distachyon</i>] ^b	21	2.0
F2E7B8	Sulfite reductase (ferredoxin) activity ^a ; 98% identical to 'sulfite reductase' [<i>Triticum durum</i>] ^b	14	1.5
F2D8X4	Glutamine synthetase ^a ; 99% identical to 'plastid glutamine synthetase 2' [<i>Triticum aestivum</i>] ^b	21	2.2
Hormone metabolism and signaling			
Q0PD05	ABA 8'-hydroxylase ^a ; 100% identical to 'ABA 8'-hydroxylase 1' [<i>Hordeum vulgare</i> subsp. <i>vulgare</i>] ^b	21	1.8
F2E5Y1	89% identical to 'serine/threonine-protein kinase CTR1' [<i>Aegilops tauschii</i>] ^b	21	1.8
F2CZK2	87% identical to 'auxin efflux carrier family protein-like' [<i>Oryza sativa japonica</i>] ^b	21	2.1
Other functions			
F2DBM9	Endoplasmic reticulum ^a ; 91% identical to 'predicted: B-cell receptor-associated protein 29-like' [<i>Brachypodium distachyon</i>] ^b	21	4.2
M0XNJ9	Endoplasmic reticulum ^a ; 85% sequence identity with 'B-cell receptor-associated protein 31-like' [<i>Setaria italica</i>] ^b	21	2.7

dpa, days past anthesis

^aID, name and gene ontology characterization retrieved from UniProt database (<http://www.uniprot.org>) (The UniProt Consortium, 2015).

^bNCBI protein sequence homology search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (NCBI Resource Coordinators, 2015).

^cMEROPS protease database (<http://merops.sanger.ac.uk>) (Rawlings et al., 2014).

^dPhyre2 protein fold recognition server (<http://www.sbg.bio.ic.ac.uk/phyre2>) (Kelley et al., 2015).

^eRice kinase Phylogenomics database (<http://ricephylogenomics.ucdavis.edu/kinase/>) (Jung et al., 2010).

^fCarbohydrate-active enZymes database (<http://www.cazy.org>) (Lombard et al., 2014).

Table 3

Proteins which are present at significantly higher levels in line '10_11' vs. variety 'Karl' at 14 or 21 dpa: comparison with transcript data (from Jukanti et al., 2008)

Protein ID	Affymetrix contig/probeset ^a	Name/gene ontology/homology ^b	10_11/Karl ratio			
			Transcript		Protein	
			14 dpa	21 dpa	14 dpa	21 dpa
O64938	Contig1637_s_at	β-1,3-glucanase 2; glycoside hydrolase (GH) family 17		3.9	2.4	
M0YUE3	Contig1637_at	Glucan endo-1,3-β-glucosidase GII; glycoside hydrolase (GH) family 17	1.8	2.4	2.3	
F2DLS4	Contig4970_at	99% identical to 'β-D-glucan exohydrolase isoenzyme ExoI' (<i>H. vulgare</i>)		3.4		9.7
F2CYK3	Contig3635_s_at Contig3636_at	87% identical to 'somatic embryogenesis receptor kinase 1' (SERK1) [<i>T. urartu</i>]		1.7 2.0	4.0	
F2DKJ2	Contig20799_at	94% identical to 'tyrosine-sulfated glycopeptide receptor 1' [<i>A. tauschii</i>]	0.57			2.4
M0W8C6	Contig1402_at	85% identical to 'GDSL esterase/lipase' [<i>A. tauschii</i>]		1.6		3.3
Q0PD05	Contig11708_at	ABA 8'-hydroxylase		5.4		1.8
F2CT33	Contig9938_at	87% identical to 'predicted: 50S ribosomal protein L4, chloroplastic' [<i>B. distachyon</i>]	2.1	0.54	1.7	
F2CTV4	Contig9121_at	87% identical to '30S ribosomal protein S1, chloroplastic', [<i>T. urartu</i>]		0.51		2.1
F2DFK1	Contig12029_s_at	Serine-type endopeptidase; S08.A28		3.6	3.5	
F2DXK0	Contig1778_s_at Contig1776_s_at	91% identical to 'histone H1 WH1B.1' [<i>T. aestivum</i>]	1.6	2.4 1.5		3.9
F2E7B8	Contig4569_at Contig4570_s_at	98% identical to 'sulfite reductase' [<i>T. durum</i>]	2.5 1.5		1.5	

^aOnly those proteins encoded by genes with a corresponding probeset on the Affymetrix Barley1 GeneChip (Close et al., 2004), and for which transcript levels are significantly different between variety 'Karl' and line '10_11' (Jukanti et al., 2008) are shown.

^bFrom Table 2.

Fig. 1. Representative 2D gels showing total soluble proteins from barley flag leaves. Late-senescent variety 'Karl' (A, C) and early-senescent line '10_11' (B, D) flag leaf proteomes at 14 (A, B) and 21 days past anthesis (dpa) (C, D) are shown. Exactly 332 and 323 spots were common in all samples imaged with CyDye-based labeling in 14- and 21-dpa samples, respectively. Protein spots with significantly higher abundance in line '10_11' as compared to 'Karl' (based on three replicates) at either 14 or 21 dpa are identified on the gels. Rubisco large (R_L) and small subunits (R_S) are highlighted in panels A and B.

Fig. 2. Shotgun proteomic analysis of late-senescent barley variety 'Karl' and early-senescent line '10_11' flag leaves. Protein abundances, based on ion intensities of three peptides across three replicates, were compared at 14 (A) and 21 days past anthesis (B). Proteins that are not significantly different in abundance are shown with small grey symbols; proteins that are significantly (p value <0.05 ; difference ≥ 1.5 -fold) more or less abundant in line '10_11' as compared to 'Karl' are shown with large symbols.

Fig. 3. Protein domains in identified proteins. Proteins identified in 'Karl' and '10_11' flag leaf samples at 14 (filled columns) and 21 days past anthesis (open columns) are shown.

Fig. 4. Analysis of identified proteins by gene ontology/biological process. Proteins identified in 'Karl' and '10_11' flag leaf samples at 14 (filled columns) and 21 days past anthesis (open columns) are shown.

Fig. 5. Protein domains in quantifiable proteins at 14 (A) and 21 days past anthesis (B). Proteins without significant differences in abundance between variety 'Karl' and line '10_11' (black), proteins which are significantly less abundant (p value <0.05 ; ≥ 1.5 -fold) in line '10_11' (dark grey), and proteins which are significantly more abundant (p value <0.05 ; ≥ 1.5 -fold) in line '10_11' (light grey) are shown. Data for higher-abundance proteins include those identified by 2D-DIGE.

Fig. 6. Analysis of quantifiable proteins by gene ontology/biological process at 14 (A) and 21 days past anthesis (B). Proteins without significant differences in abundance between variety 'Karl' and line '10_11' (black), proteins which are significantly less abundant (p value <0.05 ; ≥ 1.5 -fold) in line '10_11' (dark grey), and proteins which are significantly more abundant (p value <0.05 ; ≥ 1.5 -fold) in line '10_11' (light grey) are shown. Data for higher-abundance proteins include those identified by 2D-DIGE.

Fig. 7. Analysis of quantifiable proteins by gene ontology/cellular metabolic process at 14 (A) and 21 days past anthesis (B). Proteins without significant differences in abundance between variety 'Karl' and line '10_11' (black), proteins which are significantly less abundant (p value < 0.05 ; ≥ 1.5 -fold) in line '10_11' (dark grey), and proteins which are significantly more abundant (p value < 0.05 ; ≥ 1.5 -fold) in line '10_11' (light grey) are shown. Data for higher-abundance proteins include those identified by 2D-DIGE.