COMPARISON OF EARLY- AND LATE-SENESCENCE NEAR-ISOGENIC BARLEY GERMPLASM: PROTEOMICS AND BIOCHEMISTRY SHED NEW LIGHT ON AN OLD PROBLEM

by

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DEDICATION

Be it known that my grandfather, Walter Mason and mother, Mrs. Judith Ann Mason during the course of my study, through personal sacrifice, selflessness, and without material reward, bestowed the support and understanding without which my degree would not have been possible. This statement is hereby conferred to honor and recognize the confidence, devotion, guidance and assistance freely given in helping me obtain this important milestone in my life.

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ABSTRACT

Before their death, plant tissues undergo the essential process of senescence. Senescence is characterized by a coordinated recovery of nutrients and their retranslocation to surviving structures, such as seeds of annual plants. In monocarpic crops (e.g., maize, wheat, and barley), timing and efficiency of senescence can impact yield and grain quality. However, our understanding of senescence regulation and nutrient remobilization is limited, and protein- and metabolite-level analyses of the process are scarce, particularly in crops. To improve understanding of physiology in barley (*Hordeum vulgare* L.) leaf senescence, a systems-level comparison of near-isogenic germplasm, late-senescing/low-grain protein content variety 'Karl' and an early-senescing/high-grain protein content line ('10_11'), was performed. Protein levels in flag leaves (topmost leaves) of 'Karl' and '10_11' were compared at 14 and 21 days past anthesis (dpa) using both two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) and shotgun proteomic approaches. Conspicuously, proteins with roles in plant pathogen defense were present at higher levels in '10_11' as compared to 'Karl'. These included membrane receptors, glucanases, pathogenesis-related and disease resistance proteins. Proteins involved in protein degradation and organic acid/amino acid metabolism were upregulated in line '10_11' as compared to 'Karl', expectedly in early-senescing leaves involved in nitrogen remobilization. Metabolite levels were compared in the same plant material as protein levels except that analyses were also performed at anthesis (0 dpa), using mass spectrometry-based non-targeted metabolic profiling techniques. Metabolites with higher abundance in early-senescing line '10_11' included gibberellin catabolites, Yang cycle intermediates and intermediates of jasmonic acid biosynthesis. These differences were mostly observed at 0 dpa, indicating an early shift in phytohormone metabolism that may be important for senescence regulation and plant disease resistance between 'Karl' and '10_11' during the senescence phase, as jasmonic acid and ethylene have roles in plant pathogen defense. Overall, proteomic and metabolomic analyses performed here shed new light on the regulation of the senescence process, on the importance of plant defense against pathogens during senescence, and possibly on crosstalk between senescence regulation and pathogen defense. Proteins and metabolites identified in this study may become targets for ongoing efforts at improving crop yield, quality and environmental stress resistance.

INTRODUCTION TO DISSERTATION CHAPTER

Overview

Senescence is an integral part of normal development and fitness of all plants. During this process, essential nutrients are mobilized from vegetative tissues and transported to surviving structures (e.g., grains for annuals and roots for perennials) (Humbeck 2013; Zhou and Gan 2010; Lim, Kim, and Nam 2007). The process of senescence allows for plants to utilize growth-limiting nutrients (e.g., trace elements, nitrogen, and phosphorus) efficiently by recycling tissues that are no longer needed and transforming them into soluble metabolites that are transported to and stored in developing grains (Kim, Lim, and Nam 2007). Processes that influence the onset and progression of senescence in major crops (e.g., wheat, rice, and barley) can have a substantial impact on yield and quality (Suzuki et al. 2014c; Saez, Canton, and Forde 2012; Distelfeld, Avni, and Fischer 2014; He and Dijkstra 2014). In annual/monocarpic species, the timing of senescence is governed by developmental age (Jing et al. 2005; Ghanem et al. 2012), but it can also be influenced by various external and internal factors. Specifically, endogenous cues (i.e., age and hormones) and environmental stresses (e.g., nutrition deficiencies, extreme temperatures, drought, and light-deprivation) are intimately linked to the rate and molecular nature of senescence (Zhang and Zhou 2013). Developing an understanding of this process at the molecular level can help highlight the processes which plants utilize to ensure that growth-limiting nutrients are efficiently utilized and protected.

Leaf Senescence

Senescence typically starts in older tissues and progresses towards younger plant parts. In cereals, the leaf tip senesces first, followed by progression towards the base of the leaf. Leaf mesophyll cells contain light-harvesting complex proteins (thylakoid membrane proteins) and Rubisco (soluble protein of the chloroplast stroma) that serve as a rich source of nitrogen that is remobilized during senescence (Fischer 2007, Fischer 2012). Processes that influence senescence of leaf tissue can therefore directly influence the quality of resulting seeds or grains. In leaves, senescence occurs in three overlapping stages: initiation/induction, execution/nutrient remobilization and the terminal phase that results in cell death (Cai et al. 2015; Davies and Gan 2012; Miloud and Ali 2012).

The initiation phase of senescence leads to changes in the gene expression profile. Genes that are induced during senescence are defined as 'senescence-associated genes' (*SAGs*) (Zentgraf et al. 2004) and are of particular interest, assuming their expression indicates a role in senescence regulation. Senescence is initiated by *SAGs* encoding signaling and regulatory proteins including receptor protein kinases, mitogen-activated protein kinases, transcription factors and epigenetic factors (e.g., DNA methyltransferases) (Zhang and Zhou 2013; Breeze et al. 2008). Consequently, these genes and proteins influence downstream events that occur throughout senescence and may also influence quality trait characteristics. These aspects of their functions make them useful candidates for future research in crop improvement.

Execution of senescence causes dismantling of the chloroplasts, cytoskeleton, endoplasmic reticulum (ER) and ribosomes of leaves. A multitude of reactions transform carbohydrates, lipids, proteins and RNAs into soluble components during this phase. Several enzymes are involved in chlorophyll catabolism during senescence including pheophytinase, pheophorbide a oxygenase and red chlorophyll catabolite reductase (Hörtensteiner 2006; Christ et al. 2013; Matile et al. 1996). Degradation of proteins in leaf tissues is catalyzed by an array of specific and non-specific peptidases. These enzymes include cysteine, serine and aspartic peptidases (Li et al. 2012). Membrane lipid associated enzymes including lipoxygenase, acyl-CoA oxidases, long-chain acyl-CoA synthetases (LACS) and ketoacyl-CoA thiolases are involved in membrane conversation and degradation during senescence (Troncoso-Ponce et al. 2013; Edwards, Humphry, and Sanchez-Tamburrino 2012; Watanabe et al. 2013; Thompson et al. 1987; Cao et al. 2014). Cellulose, hemicelluloses and pectins that compose the cell wall are hydrolyzed by β-glucosidases, xylosidases and glucanases during the execution of senescence (Wingler et al. 1998; Minic and Jouanin 2006; Mohapatra et al. 2010; Bollhoner, Prestele, and Tuominen 2012; van Doorn 2008). Additionally, nucleases are important for the degradation of RNA and DNA (Gan and Amasino 1997; Kim, Lim, and Nam 2007; Taylor et al. 1993; Pérez-Amador et al. 2000; Lers et al. 1998). The catabolic processes that occur during senescence rely on the function of proteins that degrade macromolecules in vegetative tissues. Identifying and characterizing abundant enzymes in senescing leaves can provide information about metabolic pathways that are important for senescence execution.

During the execution of senescence soluble metabolites are transferred from senescing tissues to reproductive or storage organs. The composition of soluble metabolites available for transport to grains is dependent on transformation reactions of cellular components. Accumulated metabolites that have been observed in senescing leaves include purines, polar amino acids, polyamines (Werner and Witte 2011; Galili and Höfgen 2002), glutathione, homoglutathione (Miloud and Ali 2012), nucleic acids (Gregersen 2011; Veneklaas et al. 2012; Werner and Witte 2011); and other nutrient-containing compounds (Stephan and Scholz 1993). The reactions and pathways associated with the accumulation of certain metabolites during senescence (e.g., Yang Cycle intermediates and S-adenosylmethionine) can be used as a platform for crop improvement.

The timing of senescence exhibited by commercially important crops impacts overall yield and grain quality for several reasons: (1) onset of senescence reduces the accumulation of biomass by terminating photosynthetic activity, (2) up to 90% of the nutrients in grains are remobilized from vegetative plant parts during senescence, and (3) pre-mature senescence caused by environmental stress can decrease yield and nutrition (Leakey and Lau 2012; Locato, Cimini, and Gara 2013; Engelhard 1989; Zhao et al. 2007). Overlapping features between senescence and stress responses can be seen at the transcript, protein and metabolite levels. Modulations in senescence can have an influence on important traits such as higher nutrition, tolerance to stress, tolerance to disease, and higher yield (Rollins et al. 2013; Mittler 2006; Allu et al. 2014; Munné-Bosch and Alegre 2004; Stefanov et al. 2011). These results underline the complexity of the regulatory network controlling leaf senescence (Breeze et al. 2011; He et al. 2001). Despite the importance to plant nutrient metabolism and consequently to agriculture, the essential features that play a major role in the regulation and execution of senescence remain to be fully defined in economically important crops (Mrízová et al. 2014b). Overlapping features that are shared between senescence and stress response also remain poorly defined (Quirino et al. 2000b; Gan 2014). Investigation of important programs in senescence provides avenues for crop improvement by highlighting processes that influence stress tolerance and nutritional value in crops.

Regulatory mechanisms underlying leaf senescence have made it possible to devise strategies for manipulating senescence for increasing crop yield and improving quality of horticultural crops (Gan 2014). Changes observed in gene expression, modulation of hormones, (e.g., ethylene, jasmonic acid, salicylic acid, abscisic acid, and cytokinins), production of reactive oxygen species (ROS), signaling via carbon and nitrogen metabolic flux, expression of stress and pathogenesis-related (PR) proteins and macromolecular hydrolysis (Schippers et al. 2007) each influence the progression of senescence. Overlapping mechanisms that regulate the efficiency of senescence, plant-pathogen interactions, and stress responses may provide useful avenues for crop improvement by influencing resistance to herbicides, insects, and pathogens, tolerance to abiotic stress conditions, and nutritional value (Dunwell 2014).

Hormone Modulation

Senescence can be influenced by phytohormones such as ethylene (Graham et al. 2012; Grbic and Bleecker 1995), jasmonic acid (JA) (He et al. 2002; Parthier 1990), abscisic acid (ABA) (Liu, Jia, et al. 2013), and salicylic acid (SA) (Manacorda et al. 2013; Morris et al. 2000). These hormones are also involved in regulating responses to various abiotic and biotic stresses (Lim, Kim, and Nam 2007; Gottwald et al. 2012; Vallélian-Bindschedler, Métraux, and Schweizer 1998). The exact role played in senescence remains elusive for many hormones (Hou, Wu, and Gan 2013; Mueller-Roeber and Balazadeh 2013; Ellis et al. 2005; Zwack and Rashotte 2013; Kappers et al. 1998; Wang et al. 2007; Hung and Kao 2003; Niu and Guo 2012). Research suggests that plant hormones play a complex role that intersects senescence and stress responses in plants (Allu et al. 2014). Defining the roles hormones (and their pathway intermediates) play during senescence may also offer insight about responses to environmental stresses.

Reactive Oxygen Species

Reactive oxygen species (ROS; e.g., superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen) play a critical role in the natural course of senescence (Arora, Sairam, and Srivastava 2002). They are one of the earliest responses generated in plant cells undergoing senescence and abiotic stress (Khanna-Chopra 2012). High levels of observed ROS in senescent leaves are caused by various mechanisms (Cui et al. 2013). Leaking of ROS by electron transport chains (ETC) in chloroplasts and mitochondria (Hideg et al. 2006) occurs during senescence (Vacca et al. 2004; Thompson, Legge, and Barber 1987). Damage generated from the production of ROS increases over time because detoxification systems are declining (Schippers et al. 2007; Khanna-Chopra 2012). The deconstruction of the photosynthetic machinery that occurs during senescence results in the impairment of photosynthetic electron transport systems. Lastly, a loss of cellular integrity observed during senescence, resulting from the action of membrane-attacking oxidases that produce ROS (Thompson et al. 1987) can also increase oxidative stress in senescent tissues. Similar membrane peroxidation damage is also associated with aging and environmental stresses (Bhattacharjee 2014).

Biotic Stress

Plants are sessile organism, making it difficult to avoid the broad range of environmental stresses that commonly occur (Zhang and Zhou 2013; Suzuki et al. 2014b). Biotic stress in the form of pathogen infection can often cause pre-mature senescence of surrounding tissues (Kim, Lim, and Nam 2007) resulting in substantial production losses (Arshad et al. 2014; Marçais and Bréda 2006). The immune system of plants largely relies on recognition of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) and secreted virulence effectors by pattern-recognition receptors (PRRs) and resistance (R) proteins, respectively (Bernoux, Ellis, and Dodds 2011; Zipfel 2008). PRR-type proteins are mainly located on the cell surface whereas R-type receptors are intracellular (Li, Lu, and Shan 2014; Distelfeld, Avni, and Fischer 2014; Van Loon and Van Strien 1999; Dardick, Schwessinger, and Ronald 2012). Important domains commonly found in PRRs include leucine-rich repeat (LRR-RLK) and lysine motif subdomains (LysM) combined with kinase domains (Zipfel 2014). R proteins commonly consist of LRR domains found in combination with nucleotide-binding site (NBS), coiled-coil (CC) and ARC (‘apoptosis R gene products and CED-4’) subdomains (Dangl 1992; Abramovitch, Anderson, and Martin 2006; Chisholm et al. 2006). Activated PRR and R proteins elicit numerous downstream responses that include intracellular signaling, transcriptional reprogramming, and induction of ‘pathogenesis-related’ (PR) proteins (Borrell et al. 2014; Maeda et al. 2005). Recognized families of PR proteins include β-1,3-glucanases (PR-2), chitinases of type I-VII (PR-2,3,8,11), proteinase inhibitors (PR-6), peroxidases (PR-9), ribonuclease-like proteins (PR-10), lipid-transfer proteins (PR-14) and several other PR proteins encompassing a wide range of functions such as cell wall rigidification, nutrient removal (Van Loon and Pieterse 2006; Broekaert et al. 1995), signal transduction, and anti-microbial activity in order to resist pathogen invasion (Christiansen and Gregersen 2014; Van Loon and Pieterse 2006).

Abiotic Stress

Abiotic stresses (e.g. drought, salinity, heat, cold, low nutrient availability, high light intensity, ozone) can have a substantial impact on growth and yield under field conditions (Wang, Vinocur, and Altman 2003; Chaves and Oliveira 2004; Agarwal and Grover 2006; Nakashima and Yamaguchi-Shinozaki 2006; Hirel et al. 2007; Bailey-Serres and Voesenek 2008). In recent years, drought alone caused up to $50 billion in damage to worldwide agricultural production (http://www.ncdc.noaa.gov/billions/events) (Wang, Vinocur, and Altman 2003). Wide ranges of metabolic and signaling changes have been reported in the response of plants to different stresses (Atkinson, Lilley, and Urwin 2013; Koussevitzky et al. 2008; Prasch and Sonnewald 2013; Rasmussen et al. 2013; Rizhsky et al. 2004). There are three major categories of stress-related genes: (i) those that are involved in signaling cascades and transcriptional control; (ii) those that function directly in the protection of membranes and proteins such as (Fe- or Mn)-superoxide dismutases (SOD) (Alscher, Erturk, and Heath 2002), catalases, late-embryogenesis abundant (LEA) proteins (Xu et al. 1996), heat shock proteins (Al-Whaibi

2011); and (iii) those that are involved in H_2O and ion uptake (aquaporins and K^+/Na^+ ion transporters) (Wang, Vinocur, and Altman 2003; Zhu 2001). Other stress-related proteins that have also been characterized in plant response to stress include stress-related carbohydrate-binding proteins (lectins) (Fouquaert and Van Damme 2012), C-repeat/dehydration-responsive element-binding (CBF/DREB) proteins (Ma et al. 2014), and glycine-rich RNA-binding proteins (GR-RBPs) (Staiger et al. ; Zhang, Zhao, et al. 2014; Staiger 2001; Staiger and Green 2011). Plants have mechanisms tailored to combat specific stresses, but the overlap between such responses is not fully understood. The ability of plants to respond to individual or combined stresses is important for plant fitness and crop productivity (Suzuki et al. 2014b; Göbl et al. 2014). The mechanisms that occur in senescence and stress response programs are essential to the fitness of plants as they are intimately linked with efforts made by plants to protect and reutilize nutrients (Guiboileau et al. 2010).

Complexities underlying the processes controlling leaf senescence should not be understated (Penfold and Buchanan-Wollaston 2014; Breeze et al. 2008; Buchanan-Wollaston et al. 2005). Advances in systems biology due to high-throughput techniques have made it possible to uncover processes controlling plant productivity at a systems level (Fukushima and Kusano 2014; Kusano and Fukushima 2013; Fukushima et al. 2009). Widely used platforms that have been used in systems biology include transcriptomics, which measures mRNA transcript levels; proteomics, which quantifies protein abundance; and metabolomics, which determines abundance of small cellular metabolites (Yizhak et al. 2010; Zhang, Li, and Nie 2010). While transcriptomics and proteomics provide important insights into the hierarchical regulation of metabolism, metabolomics shed light on the actual enzyme activity through metabolic regulation and mass action effects (Yizhak et al. 2010). No single platform analysis can fully unravel the complexities of senescence in leaves. Therefore, in order to acquire a complete picture of senescence processes in agriculturally important species, integration of multiple layers of information systems is required (Zhang, Li, and Nie 2010).

Senescence and Barley (*Hordeum vulgare* L.)

Barley (*Hordeum vulgare* L.) is a small-grain cereal (closely related to wheat, *Triticum aestivum* L.) and is among the oldest cultivated crop species and presently is the fourth most important cereal crop in the world (Mrízová et al. 2014b). The robust nature of barley is reflected by its ability to be grown on all habitable continents. Common barley pathogens include powdery mildew, leaf rust, stem rust, scab (fungal diseases), leaf/kernel blight (bacterial) and several viral diseases (Meng, Moscou, and Wise 2009; Chang et al. 2013; Risk et al. 2013). Industries that rely on the production of barley include those that produce malt liquors (beer and hard alcohol), animal feed, and human food (Mrízová et al. 2014b). Modulating senescence behavior in a versatile species such as barley benefits commercial production in two ways. (1) Delaying senescence onset and extending the photosynthetic period in malting grains can increase grain starch content. (2) Accelerating the onset of senescence increases nitrogen content in grains used for animal feed. Increases in value of production, advancements in creating stable transgenic lines by biolistic transformation, and the rising number of patents indicate that barley is a promising candidate for future crop development worldwide (Sanford 1988;

Mrízová et al. 2014b). Establishing genes, transcripts, proteins, and metabolites that are important during senescence and stress in barley can inform breeding practices (Zhou et al. 2011; Zhao et al. 2013; Edgerton 2009).

A system-wide comparison of well-defined germplasm can link genetic information to physiological outcomes (Amiour et al. 2012). Integrating systems biology platforms (e.g., genomics, transcriptomics, proteomics, and metabolomics) can help define the regulatory, signaling, and execution processes that occur during senescence by determining the macromolecules that are present in senescing tissues. Established genomic and transcriptomic data sets were integrated with proteomic and metabolomic data in order to achieve this aim. This allowed for all levels of biological molecules (i.e., DNA, mRNAs, proteins, and metabolites) that are important for senescence in barley flag leaves to be characterized.

Examples of transcriptional analysis implemented on barley can be found from various environmental/genetic combinations (McGrann et al. 2014; Cao et al. 2014; Hackenberg et al. 2014). Of particular interest are the extensively characterized barley variety 'Karl' and a line which is near-isogenic to it, '10_11' (Heidlebaugh et al. 2008; Jukanti et al. 2008a; Jukanti and Fischer 2008). Importantly, 'Karl' exhibits later senescence and lower grain protein content (GPC) compared to line '10_11'(Heidlebaugh et al. 2008; Jukanti et al. 2008a; Jukanti and Fischer 2008). Previous studies established '10_11's ability to remobilize increased amounts of N into its grains while experiencing an accelerated senescence period in comparison to 'Karl' (Jukanti and Fischer 2008). Transcriptomic data revealed highly regulated transcripts that belong to families that are associated with signal perception, transduction and/or regulation, including several leucine-rich repeat (LRR) transmembrane protein kinases and a glycine-rich RNA-binding protein (Jukanti et al. 2008a). The previous physiological and transcriptomic analyses (Heidlebaugh et al. 2008; Jukanti et al. 2008a; Jukanti and Fischer 2008) provide a solid foundation, which can be expanded through proteomic and metabolomic studies. In order to advance this comparison further, the work presented in this thesis is aimed at defining the next levels of biological molecules by characterizing senescence-associated proteins and metabolites.

The proteomes and metabolomes of late-senescence/low-GPC variety ‘Karl’ and fast-senescing/high-GPC line ‘10_11’ were analyzed using three independent techniques. The proteome was compared by two-dimensional difference in-gel electrophoresis (2D-DIGE) and label-free quantitative mass-spectrometry-based proteomics (‘shotgun proteomics’) as described in Chapter Two. Metabolic profiling was achieved *via* mass spectrometric non-targeted metabolic profiling techniques. Performing 2D-DIGE techniques can give reliable/accurate quantification of the most abundant proteins (Aebersold, Rist, and Gygi 2000; Lilley and Friedman 2004; May et al. 2012). Shotgun proteomics allow for increased numbers of identifications with reliable quantification by means of precursor intensities/peak areas of both highly and less abundant proteins (Weisser et al. 2013; Neilson et al. 2011). Applying both techniques increases the dynamic range of protein identifications, offering a view of both the heavily abundant and less abundant proteins expressed in the proteome. With the addition of metabolite information, the macromolecular hierarchy is completed and allows for global metabolic pathway mapping analysis. Proteins and metabolites exhibiting statistically distinct behaviors between flag leaves of ‘10_11’ and ‘Karl’ throughout senescence were quantified and characterized. These data provide a framework to establish the molecular differences between the two barley lines, ultimately providing insight into senescence regulation and nutrient metabolism pathways important for the senescence processes.

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CHAPTER TWO

PROTEOMIC COMPARISON OF NEAR-ISOGENIC BARLEY (*HORDEUM VULGARE* L.) GERMPLASM DIFFERING IN THE ALLELIC STATE OF A MAJOR SENESCENCE QTL IDENTIFIES NUMEROUS PROTEINS INVOLVED IN PLANT PATHOGEN DEFENSE

Contribution of Authors and Co-Authors

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Manuscript Information Page

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Abbreviations

2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; LC, liquid chromatography; MS; mass spectrometry; MS, tandem mass spectrometry; PRR, pattern recognition receptor.

Abstract

Before plants die they undergo the essential process of senescence, a coordinated recovery and translocation of mobile nutrients from vegetative tissues (e.g., leaves) to surviving tissues (e.g., reproducing seeds). In the case of important monocarpic cereals (such as maize, wheat, rice, and barley), the timing and efficiency of senescence has a major impact on crop yield as it is intimately linked to nutrient metabolism. To further investigate this process, a proteomic comparison of near-isogenic barley germplasm, late-senescence/low-grain protein (GPC) parental variety 'Karl' and a near-isogenic early-senescence/high-GPC line, '10_11', was performed by label-free quantitative mass spectrometry-based (shotgun) and two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) proteomic techniques. Analyzing the differences between senescent flag leaf proteomes of line '10_11' and variety 'Karl' at 14 and 21 days after anthesis,, a total of 17 proteins (7 at 14 dpa and 10 at 21 dpa) were found to be significantly (and >1.5-fold) upregulated in line ‘10_11’ as compared to variety ‘Karl’ by 2D-DIGE, while 49 additional proteins (9 at 14 dpa and 49 at 21 dpa) were identified as significantly (again >1.5-fold) upregulated in line ‘10_11’ by the shotgun approach, indicating that the two techniques are complementary. Conspicuously, a number of proteins with putative roles in plant-pathogen interactions were upregulated in line ‘10_11’. These included membrane receptors, a cyclic nucleotide-gated ion channel, glucanases, pathogenesis-related and disease resistance proteins. Additionally, enzymes and proteins involved in protein degradation (including a family S8 serine protease and a tripeptidyl protease) and organic/amino acid metabolism (citrate synthase, glutamine synthetase) were upregulated, as may be expected in early-senescing germplasm involved in nitrogen remobilization. These data shed new light on senescence regulation, on the importance of plant pathogen defense during senescence, and possibly on crosstalk between senescence regulation and pathogen defense.

Introduction

Senescence is the last life phase of plant tissues, organs and, in the case of monocarpic species, entire plants. Senescence can be divided into an initiation/signaling, an execution, and a terminal phase (Guo and Gan 2014; Fischer 2012). During senescence initiation, receptor protein kinase signaling, reactive oxygen species generation and transcription factor regulation all occur (Fischer 2012). Whole-plant senescence in temperate cereals progresses from the bottom to the top of the plant, while leaf senescence starts with the oldest part (the tip of the blade) and continues towards the base (Feller, Anders, and Mae 2008). Plants recover nutrients accumulated in leaves during vegetative growth. Macromolecules including proteins and nucleic acids are degraded and metabolized, resulting in amino acids that are retranslocated to storage tissues and organs such as the endosperm of developing seeds (Distelfeld, Avni, and Fischer 2014). Execution of senescence therefore relies on the activities of proteases, lipases, nucleases, and membrane transport proteins. Senescence timing (both onset and rate of the process) can have a substantial impact on yield as it limits the duration of photosynthesis (Geldner 2014; Furuta et al. 2014; Ledford 2010; Michaels 2009; Claesson et al. 2012; Distelfeld, Avni, and Fischer 2014).

Leaf senescence is thought to have a significant role in plant adaptation because nutrients, often limiting for growth, can be retained by plants instead of being lost to the environment (Gornicki et al. 2014). During nutrient remobilization plants may become more susceptible to opportunistic pathogens, thus risking nutrient loss (Quirino et al. 2000b). Conversely, pathogen infection can lead to pre-mature senescence. Given this connection, it is not surprising that defense- and pathogenesis-related (PR) protein transcripts accumulate in healthy senescing leaf tissues (Slusarenko 1996; Guo and Gan 2005; Buchanan-Wollaston 1997). Plants perceive conserved molecular features (microbe or pathogen-associated molecular patterns; MAMPS/PAMPS), present in a wide range of microbes including pathogens (Boller and Felix 2009), through ‘Pattern Recognition Receptors’ (PRRs) that are present at the cell surface (Dardick and Ronald 2006). PRRs localized at the cell surface are represented by leucine-rich repeat (LRR) and other receptor(-like) protein kinases, while the main classes of intracellular immune receptors are represented by proteins consisting of LRRs commonly found in combination with nucleotide-binding site (NBS), coiled-coil (CC) and ARC subdomains (apoptosis R gene products and CED-4) (Sueldo et al. 2014; Palacio et al. 2014; Mosher and Kemmerling 2013; Tabata et al. 2014). Proteins triggered within the immune system elicit intracellular signaling, transcriptional reprogramming, and the synthesis of conserved PR proteins and defense metabolites (Borrell et al. 2014; Maeda et al. 2005; Buchner and Hawkesford 2014; Baird et al. 2014). Recognized families of PR proteins include β-1,3-glucanases (PR-2), type I-VII chitinases (PR-2,3,8,11), proteinase-inhibitors (PR-6), peroxidases (PR-9), ribonuclease-like proteins (PR-10), lipid-transfer proteins (PR-14), and several others (Mrízová et al. 2014a). PR proteins perform a wide range of functions including cell wall rigidification, nutrient removal and storage (Van Loon and Pieterse 2006; Lor et al. 2014; Brooks et al. 2014), signal transduction, and anti-microbial activity in order to resist pathogen invasion (Van Loon and Pieterse 2006; Reid and Ross 2011; Martí and Webb 2014). An example is the ability of plants to modulate both physical and chemical barriers to defend against pathogens by using proteins involved in cuticle (Parent and Tardieu 2014) and cell wall modification (long-chain fatty acid ligase and PR 2, β-1,3-glucanase, respectively). During biotic stresses, plant cells exhibit rapid synthesis and accumulation of reactive oxygen species (ROS) that can be controlled by the function of alcohol dehydrogenases, peroxidases and catalases (Betti et al. 2014; Suzuki et al. 2014a). The observation of these proteins both during senescence and in response to pathogens indicates crosstalk between the molecular mechanisms governing these processes (Yoshida 2003; Quirino et al. 2000b).

Defining the impact that proteins involved in senescence regulation and pathogen defense have on grain yield and quality may offer new opportunities for crop improvement and pathogen management.

Barley (*Hordeum vulgare* L.), one of the world's oldest domesticated crops, is a member of the Poaceae family that includes additional agriculturally important species such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.). The fact that barley has a smaller and simpler genome ($2n = 2x = 14$) than durum and bread wheats, that a whole-genome shotgun assembly and an integrated physical map have recently become available (Mayer et al. 2012), as well as recent advances in the efficiency of creating stable transgenic lines (Bellincampi, Cervone, and Lionetti 2014) make barley a useful model system for the study of all temperate cereal crops.

We have previously characterized barley germplasm varying in senescence timing, namely the late-senescing variety 'Karl' and a near-isogenic early-senescing line (termed '10_11'), using biochemical, molecular and transcriptomic methods (Heidlebaugh et al. 2008; Jukanti and Fischer 2008; Jukanti et al. 2008a). This germplasm varies in the allelic state of a chromosome six locus, which encompasses the *Hv*NAM-1 transcription factor (Distelfeld et al. 2008). Our experiments have identified numerous genes that are upregulated in flag leaves of line '10_11' as compared to 'Karl' at 14 and particularly at 21 days past anthesis (dpa) (Jukanti et al. 2008a). Strong (>10-fold) upregulation was found for genes that may be involved in the control of senescence. Proteins encoded by these highly regulated transcripts belong to families that are associated with signal perception, transduction and/or regulation, including a glycine-rich

RNA-binding protein, leucine-rich receptor-like protein kinases, polyubiquitin and a WRKY transcription factor (Jukanti et al. 2008a).

To establish whether differences between ‘10_11’ and ‘Karl’ are also found at the protein level, comparisons of transcriptomic (Lawson et al. 2014) and proteomic data are necessary.(Canovas et al. 2004; Ghazalpour et al. 2011). Plant productivity parameters for both model and crop species (Jackson et al. 2011) can be defined by integrating plant genome and transcriptome annotations with proteomic and metabolomic studies (Fukushima and Kusano 2014). For the present study, separation and quantification of ‘10_11’ and ‘Karl’ proteins was achieved by two-dimensional difference in-gel electrophoresis (2D-DIGE) and label-free quantitative mass spectrometry based proteomics (shotgun proteomics), both of which comprise a final liquid chromatographic (LC) mass spectrometric analysis (LC-MS). The 2D-DIGE method provides reliable and accurate quantification of the most abundant proteins (Liu, Li, et al. 2013; Zelazny and Vert 2014; Pierik and Testerink 2014), 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 (Anderson and Catona 2014). In this paper, we present an extensive quantitative proteomic analysis that implements a combination of orthogonal proteomic techniques to characterize and compare the proteomes of early-senescing barley variety ‘10_11’ and late-senescing line ‘Karl’ (Figure 2.1). Applying both techniques increases the dynamic range of protein identifications, offering a view of both more and less abundant proteins.

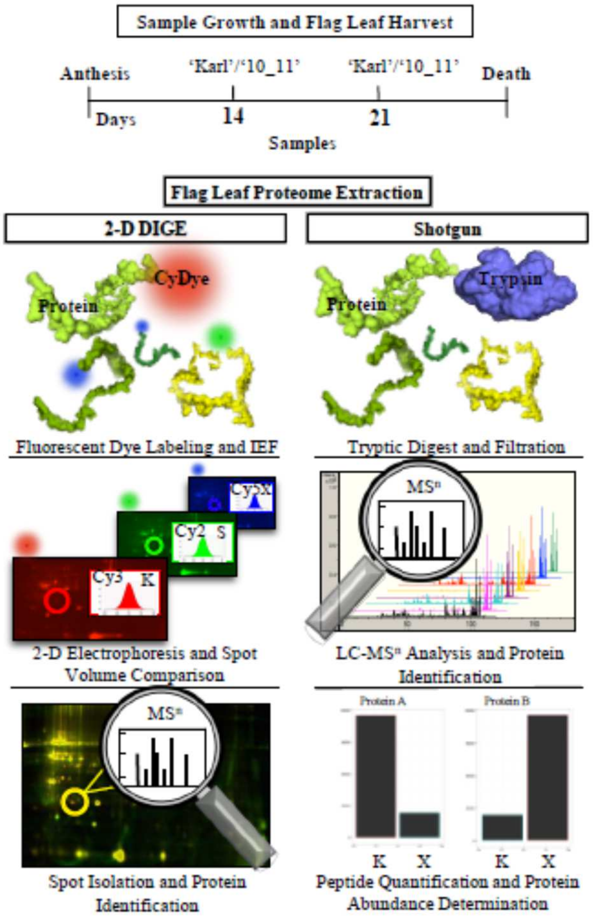

Figure 2.1 Proteomic Experimental Pipeline. Barley flag leaves harvested at 14 and 21 days past anthesis were subjected to one of two simultaneous proteomic analyses: (1) 2-D DIGE (CyDye, gel comparisons, in-gel digest, LC-MS^n data collection, and identification by database query) or (2) shotgun proteomic techniques (tryptic digest, LC-MS data collection, identification by database query, and protein abundance calculations) (CyDye; GE Healthcare).

Materials and Methods

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). Line '10_11' is characterized by earlier senescence and higher grain protein as compared to variety 'Karl', due to allelic variation at a chromosome 6H locus controlling these traits (Jukanti et al. 2008a). Seeds were planted on February 1st, 2013 in a small randomized block design containing four blocks each of line '10_11' and variety 'Karl' (eight blocks total) in a glasshouse of the Montana State University Plant Growth Center. A 22 °: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. Samples harvested at 14 and 21days past anthesis were flash-frozen in liquid N_2 and stored at -80 °C until protein extraction. For each harvest time point, four to six samples consisting of flag leaves from 8 to 10 plant shoots each were collected.

Sample Preparation and Quantification for 2D-DIGE Analysis

Frozen flag leaves were ground for 1 minute in liquid N_2 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) insoluble polyvinylpolypyrrolidone (PVPP) and 0.1% (v/v) β-mercaptoethanol). In order to block proteolysis 20 μL of protease inhibitor cocktail (Sigma, St. Louis, MO, USA, product P8849, containing AEBSF, phosphoramidon, pepstatin A, bestatin and E-64) was added to each sample. Extracts were vortexed for 2 min, centrifuged (5 min, 21 000 g, 4 °C), and resulting supernatants were transferred into separate tubes. Soluble proteins in the supernatants were concentrated by precipitation with a 5-fold volume of cold acetone and resuspended for 1 hour 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 from each sample by centrifugation at 24,700 g for 20 minutes followed by careful removal of supernatant and storage at -80 °C. The concentration of protein samples was determined with the Bradford Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Samples were diluted to concentrations of 2 mg/mL with suspension buffer and were used as stocks for experiments. To check protein sample quality, 10 μg (5 μL) of each sample were removed, diluted 1:2 with Laemmli sample buffer (Bio-Rad Laboratories), then analyzed by 1D SDS-PAGE electrophoresis.

2D-DIGE

The protocol was as previously described with minor changes noted (Maaty et al. 2012). Minimal fluorescent labeling of lysine side chains (1 per 100) was achieved with N-hydroxysuccinimide ester cyanine dyes (CyDyes; Cy2, Cy3, Cy5; GE Healthcare Bio-Sciences Corp., Piscataway, NY, USA) according to the manufacturer's protocol. Briefly, 50 □g protein from each biological replicate was reacted with 400 pmol of CyDye (either Cy3 or Cy5; dissolved in 99.8% DMF; Sigma-Aldrich Co. LLC, St. Louis MO, USA). For the internal standard, equal volumes of 2 mg/mL protein sample stocks were taken from each extraction, pooled, and then labeled with Cy2. Reactions were quenched by the addition of 1 □L of a 10 mM L-lysine solution (Sigma) and left on ice for 10 minutes. Cy2-, Cy3-, and Cy5-labeled samples were combined appropriately (e.g. 50 □g 'Karl' Cy3, 50 □g '10_11' Cy5, and 50 □g internal standard Cy2) and diluted with IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 2.5% IPG, GE Healthcare).

Isoelectric focusing (IEF) used pre-casted IPG strips (pH 3-11 NL [nonlinear], 24 cm length; GE Healthcare). Each of the three fluorescent-dye labeled samples were combined and brought to a volume of 450 □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). Experiments for DIGE and for protein spot analysis required the addition of 150 □g and 400 □g of protein to each IPG strip, respectively. IEF was carried out with an IPGPhor II (GE Healthcare) at 20 °C, with a maximum of 50 mA/strip. After isoelectric focusing separation, the strips were equilibrated twice for 15 minutes with equilibration buffer (50 mM Tris/HCl, pH 8.8, 6 M Urea, 30% glycerol, 20% SDS and

0.007% bromophenol blue) where the first equilibration step included the reducing agent DTT (65 mM), and the second, 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, using constant current.

Gels were scanned using a Typhoon Trio Imager according to the manufacturer's suggested protocol (GE Healthcare) at 100 μm resolution. The Cy3 gel images were scanned at an excitation wavelength of 532 nm with a long pass emission wavelength of 570 nm, Cy5 gel images were scanned at an excitation wavelength of 633 with a long pass emission wavelength of 670 nm, while the Cy2 gel images were scanned at an excitation wavelength of 488 nm with 520 nm emission filter. Images were subjected to an automated difference in-gel analysis using Progenesis SameSpots software version 3.0.2 (Nonlinear Dynamics Ltd.). Within the software, individual gel spots were co-detected as DIGE image pairs, which were linked to the corresponding in-gel Cy2 standard ultimately allowing for between-gel comparisons and overall statistical analysis. Gels used for protein identification were stained with Coomassie GelCode Blue Safe (Thermo Scientific Inc., Rockford, IL, USA), destained in water, then stored at 4 °C in 10% acetic acid for visible spot excision.

Protein Spot Identification and Data Analysis

Protein spots of interest were excised from the gels, washed in 100 mM NH_3HCO_3 (200 μL), in-gel reduced and S-alkylated, followed by digestion with porcine trypsin (Promega Corp., Madison WI, USA) overnight at 37 °C. The reduction and acetylation of cysteine residues was carried out by the addition of 10 mM DTT in 100 mM NH_3HCO_3 at 60 °C for 1 hr preceded by the addition of 40 mM iodoacetamide (IAA) in 100 mM NH_3HCO_3 for 30 minutes at room temperature (RT) in the dark, respectively. A solution containing 1 pmol of porcine trypsin in 100 mM NH_3HCO_3 (Promega) was added to the gel pieces until submerged, then in-gel protein digestion was carried out overnight at 37 °C. The tryptic peptides were extracted from the gel pieces with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile (ACN) using three times 200 □L by centrifugation (24,652 g, 5 min) and removal of supernatant into a single pooled sample. Peptides were dried under a speed vacuum, suspended with 20 □L 0.07% TFA in water and transferred to sample tubes for mass analysis. 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). Samples were trapped and desalted on the Zorbax 300SB-C18 Agilent HPLC-Chip enrichment column (40 nl volume) in 5% acetonitrile and 0.1% formic acid delivered by an auxiliary CapLC pump at 4 □l/min. The peptides were then eluted and loaded onto the analytical capillary column (43 mm x 75 µm ID, also packed with 5 um Zorbax 300SB-C18 particles) connected in-line to the mass spectrometer with a flow of 600 nl/min. Peptides experienced a 5 to 90% acetonitrile gradient over 16 min. Data-dependent acquisition of collision-induced dissociation tandem mass spectrometry (MS) was utilized with the following parameters: mass-to-charge-ratio (*m/z)* range of 200 to 2,200 at 24,300 *m/z*-s. Raw data files were then exported for data analysis.

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 (Aanen 2014) by using the module FileConverter, a script that converts each data file into molecular graphic format (mgf) producing one feature map per sample. Peptide identification was initiated first by generating a custom protein sequence file (.FASTA) that contains known proteins identifiable in barley plants (retrieved 2014; http://www.uniprot.org; containing 52,289 protein entries). Using the graphical user interface for proteomics identification search engines software (SearchGUI; (Zeng et al. 2014) these sequences were subjected to an *in silico* tryptic digest and resulting peptide masses were outputted as a target library. In order to discern the false discovery rate (FDR) of peptide identifications, a set of reversed-decoy sequences were also appended to the target library. The final target-decoy library was queried against data files using the following search parameters: up to two missed cleavages allowed, precursor charges +2; +3; +4, precursor ion mass tolerance 20 parts per million (ppm), fragmentation mass tolerance of 0.5 Da, and three variable post translational modifications (PTMs) (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 (Xu et al. 2014) and OMSSA (Gray et al. 2014) 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 (Endo et al. 2014; Zeng et al. 2014). Searched data was 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 (Zeng et al. 2014; Endo et al. 2014).

Sample Preparation and Quantification for Shotgun Proteomics

Shotgun protein samples were acquired as described above for 2D-DIGE sample preparation. Stock samples of 2 mg/ml (determined by Bradford analysis) were used to obtain 50 mg 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 digest was performed by the initial addition of 50 mM NH_4HCO_3 and Trypsin Gold (Promega Corp., Madison WI, USA) in a 50:1 ratio (soluble protein / trypsin) proceeded 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) by centrifugation (24,652 g) prior to mass spectroscopic analysis of peptides. Overall, three biological replicates from each of the two lines (‘10_11’ and ‘Karl’) and time point (14 and 21 days past anthesis) were harvested and treated for MS experiments, totaling 12 samples.

LC-MS for Shotgun Mass Spectrometry Samples

LC-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 in-line to the mass spectrometer with a flow of 600 nl/min. Peptides experienced a 5 to 90% acetonitrile gradient over 120 min while delivered to the online mass spectrometer (Bruker Daltonics). Data-dependent acquisition of collision induced dissociation tandem mass spectrometry was performed in Auto(MS^n) mode with 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.

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 determination rate, and exported by a minimum coverage. Separately, this output was matched to a dataset that was made-up of normalized/aligned mass lists to obtain precursor ion intensities that correspond to abundance of each peptide/protein in a run. As described previously, a theoretical target library is necessary for quality management of experimental data. The acquired experimental MS data files outputted directly from the mass spectrometer were converted using OpenMS FileConverter scripts (Aanen 2014) into two separate file formats: (1) molecular graphic file format, (2) MZxml for quantification in later analysis. Analysis was performed on two distinct sets of biological replicates depending on time point. Each set was composed of 6 samples; three from variety 'Karl', and three from line '10_11'. Database searching and peptide identification was performed as described for 2D-DIGE analysis. Final protein identifications that met software requirements were consolidated into three non-distinct groups depending on the number of unique peptide representatives contributing to their assignment. In each case where a protein was characterized with two or more peptides, the status of 'identified' was given. The various members of this group possessing additional unique peptides (three or more) were preliminarily designated 'quantifiable'. By exporting the potentially 'quantifiable' group members and their precursor ion information (m/z and retention time; RT; in minutes) a new mass library was created from assigned experimental data values (experimental mass library). Quantifiable proteins with a fold-change of 1.5 or more and a p-value <0.05 were additionally categorized as having 'significantly different abundance'.

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 (Nishii et al. 2014). Analyses were performed simultaneously on each set of separate biological replicates. All features within each sample were detected in centroid mode ignoring features below an intensity of 1,500. The remaining mass lists were then assigned RT in seconds with the following settings: minimum intensity 2,500; timespan of (0.1 min); m/z tolerance of 20 ppm. Resulting chromatograms were normalized by RT (0.01 m/z unit tolerance; 20% relative RT tolerance; minimum intensity 5,000, then subsequently aligned to one another (0.01 m/z unit tolerance; 20% RT tolerance;

weighted equally; same charge state required), and finally gap-filled (50% intensity tolerance; 0.01 *m/z* unit tolerance; 20% RT tolerance). The remaining mass list contained features aligned to one another that could further be assigned by using the previously generated experimental mass library (search criteria considered: 0.01 *m/z* unit tolerance; 20% RT tolerance). The resulting assignments were filtered to include only scenarios in which three or more unique peptide precursor ions were present in every sample. A well-known issue of label-free shotgun proteomics is the sequence dependence of peptide ionization efficiency (Craig et al. 2009). To limit false-negatives and false-positives resulting from this phenomenon, peptides (>3 found) with the closest ion intensity were chosen for subsequent quantification. Proteins represented by each set of three ions chosen were confidently determined to be 'quantifiable'.

Quantification, Statistics, and Protein Function Analysis

Protein abundances were determined for each sample type ('10_11' or 'Karl') and time point (14 dpa or 21 dpa) by averaging intact tryptic peptide ion intensities from three biological replicates of each sample. The resulting average intensity values were combined according to protein (sum of three '10_11' precursor ion intensities and sum of three 'Karl' intensities) giving the abundance values of each protein for both sample types. 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 (Shao et al. 2014), XLStats

(Blee et al. 2014) (version 2013.5.01; Addinsoft 1995-2013) and R (de Jong et al. 2014). Graphics were also generated using this group of software with addition of the PyMol Molecular Graphics System (Zhang, Han, et al. 2014).

In order to be consistent with protein function assignment, each amino acid sequence, and accession numbers of each protein of interest, were queried against several databases including the UNIPROT database (http://www.uniprot.org), conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al. 2013), NCBI protein sequence homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (NCBI Resource Coordinators, 2015), rice kinase database (http://rice.plantbiology.msu.edu) (Dardick et al. 2007), and MEROPS protease database (http://merops.sanger.ac.uk) (Rawlings et al. 2014). All available database gene ontology and functional information was combined and summarized for analysis of each protein function (Supplementary Table 2.3, Appendix A).

Results

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 (Heidlebaugh et al. 2008; Jukanti and Fischer 2008; Jukanti et al. 2008a). 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 days past anthesis (dpa), using both 2D-DIGE and shotgun approaches (Figure 2.1). The proteomic data from 2D gels and shotgun type analyses are complementary. In the combined data set, ~9,000 proteins were identified (Supplementary Table 2.1, Appendix A), approximately one third of which were quantified from senescing ‘Karl’ and ‘10_11’ leaves. Among these, our analysis focused on proteins significantly upregulated in line ‘10_11’, as these may be important for the regulation or execution of senescence-associated processes.

2D-DIGE Comparison of Barley Line ‘10_11’ and Variety ‘Karl Flag Leaves

Differences in protein abundances between ‘10_11’ and ‘Karl’ flag leaves at 14 and 21 dpa were first investigated by 2D-DIGE proteomic techniques. Image analysis showed that 332 (14 days) and 323 (21 days) protein spots were present in both ‘10_11’ and ‘Karl’ (Figure 2.2). The most visible difference between time points was the decrease in Rubisco abundance (highlighted in blue and designated with ‘R’ in the 14-day gels; Figure 2.2 A,B and 21-day gels; Figure 2.2 C,D). Differences between ‘10_11’ and ‘Karl’ increased slightly from 14 to 21 days. At 14 days, seven protein spots were more abundant (≥ 1.5-fold, $p \leq 0.05$) in early-senescing line ‘10_11’ samples, whereas ten were more abundant at 21 days (Table 2.1).

Proteins showing larger abundance in ‘10_11’ as compared to ‘Karl’ at 14 dpa included a pentatricopeptide repeat-containing protein potentially involved in mitochondrial RNA metabolism (UniProt ID: F2EGM4), a DNA polymerase □□(F2DD24), a mitochondrial import receptor subunit (M0WZV1), a protein functioning in chromatin remodeling and DNA repair (M0XBC0) and, importantly, a rine peptidase which could be involved in nitrogen remobilization from

ves (F2DFK1) (Table 2.1).

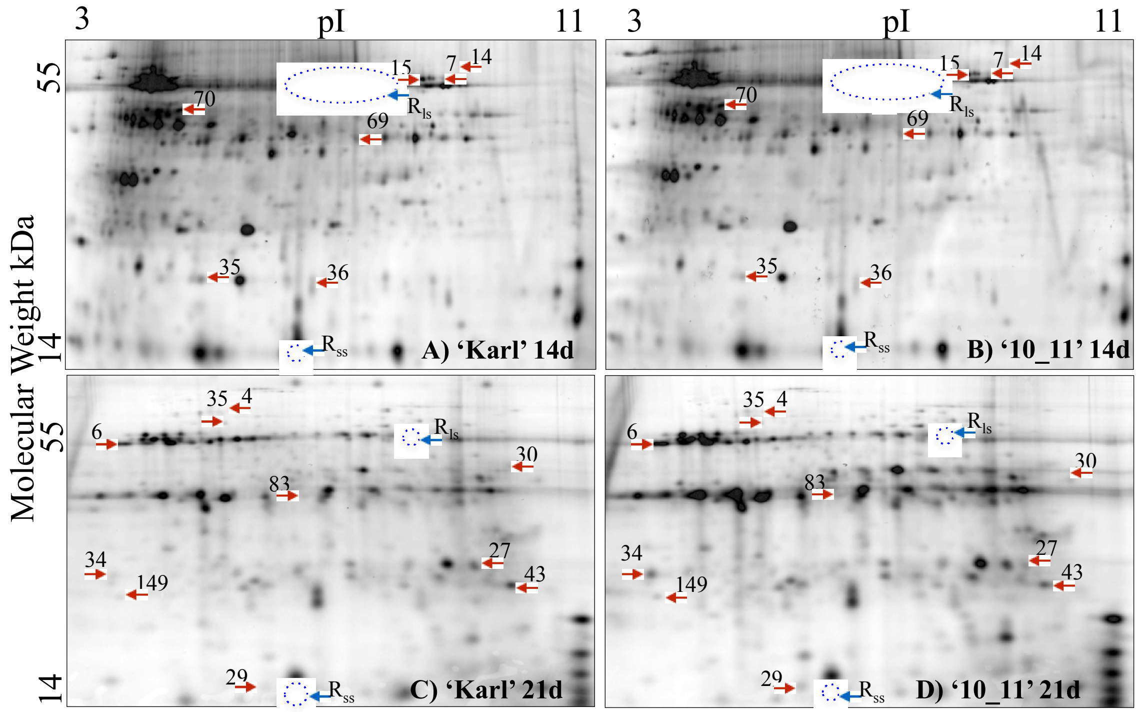

Figure 2.2 Representative 2D-DIGE Gels Showing Total Soluble Proteins From Barley Flag Leaves. Late-senescing variety 'Karl' (A,C) and early-senescing line '10_11' (B,D) flag leaf proteomes were compared at 14 and 21 days past anthesis (dpa). Exactly 332 and 323 spots were common in all samples imaged with CyDye-based labeling in 14- and 21-dpa samples, respectively. A total of 17 spots (proteins) were identified to have significantly higher relative abundances (1.5 ≤ fold change, 0.05 ≥ p-value) in '10_11' compared to 'Karl'. These proteins are designated with numbers and red arrows in the gel images (numbers correspond to Table 2.1). Rubisco large and small subunits are denoted as 'R_{ls}' and 'R_{ss}' and highlighted with blue arrows and circles.

Table 2.1 Proteins which are significantly and >1.5-fold upregulated in ‘10_11’ *vs.* ‘Karl’ at 14 and 21 dpa, as determined by 2D-DIGE. Corresponding spot numbers are shown in Fig. 2.2.

No.	**ID[1]**	**Name**	**Gene ontology (GO)[1]**	**Functions[2,3,4]**	**p-value**	**Ratio X/K**
14 days						
7	F2EGM4	Predicted protein	No gene ontology information	83% identical to ‘predicted: pentatricopeptide repeat-containing protein, mitochondrial-like’ [*Brachypodium distachyon*]; 100% query cover. Contains two pentatricopeptide repeat (PPR) domains.	0.032	4.8
14	F2DD24	Predicted protein	DNA binding; DNA repair; DNA-dependent DNA replication; DNA-directed DNA polymerase activity.	91% identical to ‘DNA polymerase lambda’ [*Triticum urartu*]; 100% query cover. Contains nucleotidyltransferase (NT) domain of family X DNA polymerases, which fill in short gaps during DNA repair. A member of the Rel-Spo domain superfamily. The *Arabidopsis thaliana* Rel-Spo proteins, *At*RSH1, -2, and -3 appear to regulate a rapid (p)ppGpp-mediated response to pathogens and other stresses.	0.006	3.6
15	F2DFK1	Predicted protein	Negative regulation of catalytic activity; proteolysis; serine-type endopeptidase activity.	72% identical to ‘subtilisin-like protease’ [*Aegilops tauschii*]; 100% query cover. Contains (serine) peptidase S8 family (subtilase) domain. MEROPS[4] classification S08.A28, ‘senescence-associated subtilisin protease’.	0.047	3.5

Table 2.1 Continued

35	M0WZV1	Uncharacterized protein	Mitochondrial outer membrane translocase complex; protein import into mitochondrial outer membrane	98% identical to 'putative mitochondrial import receptor subunit TOM20' [*Aegilops tauschii*]; 31% query cover. Contains TOM20 (translocase of outer membrane 20 kDa subunit) domain.	0.009	2.5
36	M0XBC0	Uncharacterized protein	ATP binding; DNA binding; helicase activity; metabolic process; nucleus; zinc ion binding	86% identical to 'predicted: protein CHROMATIN REMODELING 4' [*Brachypodium distachyon*], 100% query cover. 88% identical to 'CHD3-type chromatin remodeling factor PICKLE' [*Aegilops tauschii*], 94% query cover. Contains 'probable chromatin-remodeling complex ATPase chain, provisional'; 'superfamily II DNA/RNA helicases, SNF2 family domain'.	0.043	2.5
69	F2CT33	Predicted protein	Chloroplast stroma; chloroplast thylakoid membrane; cytosolic ribosome; nucleus; plastid ribosome; poly(U) RNA binding; structural constituent of ribosome; translation	87% identical to 'predicted: 50S ribosomal protein L4, chloroplastic [*Brachypodium distachyon*]; 87% query cover. Contains 50S ribosomal protein L4 domain.	0.025	1.7

Table 2.1 Continued

70	F2DCS3	Kinesin-like protein	ATP binding; kinesin complex; metabolic process; microtubule; microtubule motor activity; microtubule-based movement	83% identical to ‘predicted: kinesin-related protein 11 [*Brachypodium distachyon*]; 100% query cover. 78% identical to ‘centromeric protein E, putative, expressed’ [*Oryza sativa*]; 100% query cover. Contains kinesin motor domain, CENP-E/KIP2-like subgroup, involved in chromosome movement and/or spindle elongation during mitosis.	0.033	1.7
21 days						
34	F2EE16	Predicted protein	ATP binding; ATP catabolic process; ATPase activity	79% identical to ‘elongation factor 3’ [*Cryptococcus neoformans*], 100% query cover. Contains ‘ATP-binding cassette domain of elongation factor 3, subfamily f’; also contains ‘ATPase components of ABC transporters with duplicated ATPase domains’, ‘putative ABC transporter ATP-binding domain, provisional’.	0.031	2.3
4	F2D8X4	Glutamine synthetase	ATP binding; glutamate-ammonia ligase activity; glutamine biosynthetic process	99% identical to ‘plastid glutamine synthetase 2’ [*Triticum aestivum*], 98% query cover. Contains glutamine synthetase catalytic domain.	0.015	2.2
6	F2DZ70	Aminomethyltransferase	Aminomethyltransferase activity; transaminase activity; glycine catabolic process	93% identical to ‘predicted: aminomethyltransferase, mitochondrial’ [*Brachypodium distachyon*], 100% query cover. Contains glycine cleavage system T protein (also known as folate-binding domain).	0.008	2.0

Table 2.1 Continued

83	M0VXI9	Uncharacterized protein	Exonuclease activity; mediator complex; nucleic acid binding; nucleic acid phosphodiester bond hydrolysis	89% identical to 'predicted: small RNA degrading nuclease 1-like' [*Brachypodium distachyon*], 100% query cover. 87% identical to 'small RNA degrading nuclease 3' [*Triticum urartu*], 97% query cover. Contains 3'-5'-exonuclease domain of RNA exonucleases 1, 3 and similar eukaryotic proteins.	0.005	1.9
35	F2DIB4	Predicted protein	RNA binding; RNA processing; cell wall; cytosol; nucleoplasm; nucleotide binding; ribonucleoprotein complex	95% identical to 'La protein-like protein' [*Aegilops tauschii*], 100% query cover. Contains La motif of plant proteins similar to the La autoantigen and two RNA recognition motifs (RRMs). Appears to function as an RNA-binding protein.	<0.001	1.6
29	F2CTE6	Predicted protein	Ubiquitin ligase complex; ubiquitin-protein ligase activity	86% identical to 'U-box domain-containing protein 44-like [*Brachypodium distachyon*], 99% query cover. Contains modified RING finger domain; probably involvement in E2-dependent ubiquitination.	0.033	1.6
27	M0VN45	Uncharacterized protein	ATP binding	86% identical to 'luminal-binding protein 5' [*Aegilops tauschii*], 99% query cover. Contains nucleotide-binding domain of the 70-kDa heat shock protein family, heat shock protein 70 domain.	0.010	1.6

Table 2.1 Continued

30	M0YJW4	Uncharacterized protein	Endoplasmic reticulum; heme binding; iron ion binding; monooxygenase activity; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	95% identical to 'cytochrome P450 704C1' [*Aegilops tauschii*], 99% query cover. Contains fatty acid omega-hydroxylase (provisional) domain and cytochrome P450 domain.	0.030	1.6
149	F2DQ10	Predicted protein	Regulation of protein catabolic process; regulation of catalytic activity; enzyme regulatory activity; proteasome complex.	94% identical to 'predicted: 26S proteasome non-ATPase regulatory subunit 2 homolog A-like [*Brachypodium distachyon*], 100% query cover. Contains proteasome/cyclosome repeat.	0.049	1.5
43	P22244	Protein synthesis inhibitor I	Cytoplasm; defense response to fungus; killing of cells of other organism; metabolic process; negative regulation of translation; rRNA N-glycosylase activity	100% identical to 'protein synthesis inhibitor I/ribosome-inactivating protein I/rRNA N-glycosidase chain A' [*Hordeum vulgare*], 100% query cover. Contains RIP (ribosome inactivating protein) domain.	0.038	1.5

X, early-senescing barley line '10_11'; and K, late-senescing barley variety 'Karl'.

[1] Gene ontology characterization / ID (retrieved from UniProt database; http://www.uniprot.org).

[2] Conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

[3] NCBI protein sequence homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

[4] MEROPS protease database (http://merops.sanger.ac.uk/index.shtml); Rawlings *et al*. (2014), Nucleic Acids Res. 42: D503-D509.

Among proteins with higher abundances in '10_11' at 21 dpa were an ATP-binding protein (F2EE16, possibly the ATPase component of an ABC transporter), a glutamine synthetase (F2D8X4) and an aminomethyltransferase (F2DZ70; involved in glycine cleavage in mitochondria), an RNA degrading nuclease (M0VXI9), two components of the ubiquitin-proteasome system (F2CTE6, F2DQ10), a cytochrome P450 protein possibly involved in fatty acid hydroxylation (M0YJW4) and a ribosome-inactivating protein/protein synthesis inhibitor (P22244) (Table 2.1). Overall, 2-D DIGE data suggest enhanced proteolysis and N metabolism, possible upregulation of mitochondrial functions, and upregulation of components of the ubiquitin-proteasome system in line '10_11' as compared to 'Karl'. As for proteins identified through shotgun proteomics (see below), some of the upregulated proteins may have functions in plant pathogen defense, including the pentatricopeptide repeat-containing protein and proteins involved in DNA repair and chromatin remodeling, as homologous proteins from other species have been implicated in plant defense responses (Park et al. 2014; Song and Bent 2014).

Shotgun Proteomic Comparison of Barley Line'10_11'and Variety 'Karl' Flag Leaves

To increase the number of identified and quantified proteins, a shotgun proteomic analysis was applied to the system. Proteins with two or more peptides contributing to their characterization were considered as 'identified' (Supplementary Table 2.1, Appendix A) Proteins for which three or more peptides were identified met the criterion of 'quantifiable' (Schulze and Usadel 2010) (Supplementary Table 2.2, Appendix A).

Proteins with a fold-change of 1.5 or more and a p-value <0.05 were additionally categorized as 'significantly regulated'. After combining data from all samples, a total of 9,258 proteins were identified and 3,015 (1176 + 1839; 32.6%) were classified as quantifiable (Table 2.2). Considering the different data sets by time point, 14-day samples generated 1,176 quantifiable proteins out of 5,055 identifications, while the 21-day samples generated 1,839 quantifiable proteins out of 5,338. Overlap in identified proteins between 14- and 21-day sample identifications was 12.3%. Proteome coverage was determined from the percentage of identified proteins based on the total number of predicted barley proteins (52,289 entries, retrieved from http://www.uniprot.org). The calculated coverage for 14-day samples was 9.7% and for 21-day samples 10.2%.

A majority of significantly regulated proteins were lower in abundance in the early-senescence line '10_11' compared to the late-senescence variety ('Karl'). In total, 65 proteins had significantly different abundances between 'Karl' and '10_11' flag leaves at 14 days ($p < 0.05$; >1.5fold) (Table 2.2, Figure 2.3A). Most quantified proteins did not show a significant difference in abundance between samples (grey circles in Figure 2.3); proteins that changed significantly are colored by abundance level (red = higher abundance in '10_11; blue = lower abundance in '10_11') (Figure 2.3). The number of proteins that were more abundant in '10_11' was 9, while 56 were less abundant in '10_11' as compared to 'Karl' (Table 2.2). The 21-day samples had a substantial increase in significant differences between '10_11' and 'Karl'. This was consistent with the previous transcriptomic analysis (Jukanti et al. 2008a). A total of 347 proteins had different abundances between '10_11' and 'Karl' at the later time point, with 49 having higher abundance and 298 having lower abundance in ‘10_11’ as compared to ‘Karl’ (Table 2.2, Figure 2.3B). These results suggest that ‘10_11’ senesces faster, again confirming previous physiological and transcriptomic analysis of the system (Jukanti and Fischer 2008; Heidlebaugh et al. 2008; Jukanti et al. 2008a). However, it may be argued that those proteins which are more abundant in earlier-senescing flag leaves are enriched for functions necessary for senescence regulation and nutrient remobilization, and are therefore of particular interest.

Table 2.2 Label-free Shotgun Data Summary

	Sample time point (dpa)	
	14	21
Protein group category	Number of protein groups	
Identified		
≥ 2 peptides	5055	5338
≥ 3 peptides	1176	1839
Significantly regulated total		
≥ 3 peptides $p \leq 0.05$	65	347
Significantly regulated up		
≥ 1.5-fold $p \leq 0.05$ ‘10_11’ *vs.* ’Karl’	9	49
Significantly regulated down		
≥ 1.5-fold $p \leq 0.05$ ‘10_11’ *vs.* ’Karl’	56	298

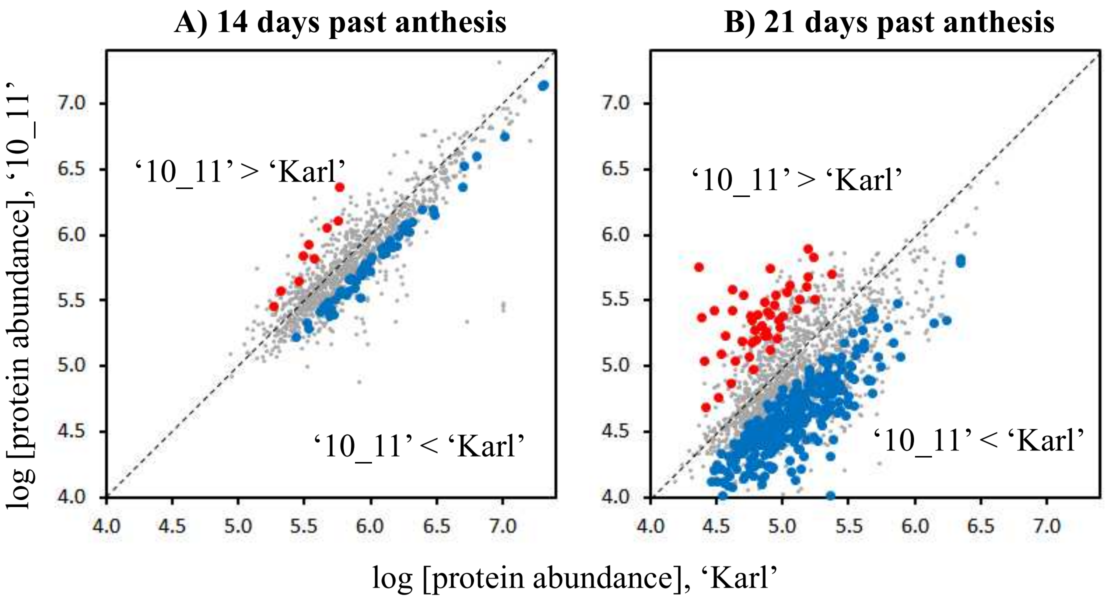

Figure 2.3 Differential Proteomic Analysis of ‘10_11’ And ’Karl’ by Shotgun Proteomics. A) Normalized protein abundances in 14 dpa samples and B) 21 dpa samples. Proteins that did not show significant differences in abundance are shown in grey, and the remaining groups ($p \leq 0.05$) were colored by abundance level (red designates significant identifications with higher abundance in ’10_11’ and blue represents lower abundance in ‘10_11’).

Analysis of Protein Domains Present in Proteins Which are Differentially Regulated Between Barley Line ‘10_11’ and Variety ‘Karl’ Flag Leaves

Protein sequence serves as a rich source of functional information. Protein domains are the main structural components of globular proteins and provide a foundation for function and pathway analysis (Shao and Hegde 2014). Using the NCBI BLAST module (http://blast.ncbi.nlm.nih.gov/Blast.cgi), all proteins with differential abundances between ‘10_11’ and ‘Karl’ at 14 and 21 days (fold change >1.5, $p < 0.05$) were searched to identify conserved domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Figure 2.4, Supplementary Table

2.3, Appendix A). Several of the domains present in proteins which were of higher abundance in line ‘10_11’ at 21 days indicate functions which were also identified as upregulated in our previous transcriptomic analysis (Jukanti et al. 2008a). Of particular interest were a protein kinase domain (PKc, cl09925), a leucine-rich repeat domain (LRR-RI; cl19743), an ATPase domain (AAA, cl18944), and an RNA recognition motif (RRM_SF; cl17169) (Figure 2.4C). Proteins with these domains often have important roles in signal perception, signal transduction and RNA processing. As for entire proteins, a lower number of domains were regulated at day 14, with 19 domains recognized as upregulated in ‘10_11’ as compared to ‘Karl’. Among these were the C2 protein kinase (cl14603), family 17 glycosyl hydrolase (cl21706), HCP-like (cl19102), nitrite/sulfite reductase ferredoxin-like (cl08392), SCP-like extracellular (cl00133), leucine-rich repeat N-terminal (cl08472), plant peroxidase-like (cl00196) and peptidase C19 domains (cl02553) (Figure 2.4A). Upregulation of these domains suggests a number of functions including again, signal perception and transduction.

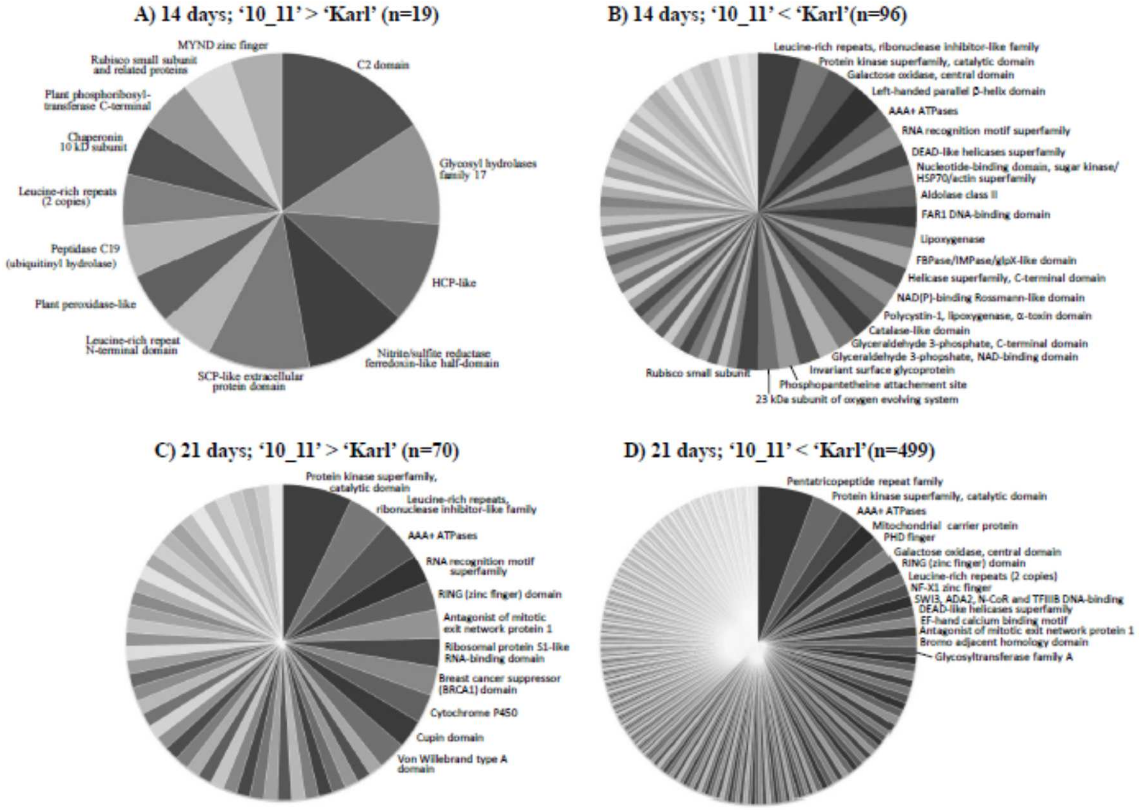

Figure 2.4 Frequency Of Conserved Domains Of Low And High Abundance Proteins Within Barley Line '10_11' Compared to Variety 'Karl' Flag Leaves. Proteins which are up- (A,C) or downregulated (B,D) in line '10_11' as compared to 'Karl' flag leaves at 14 (A,B) and 21 dpa (C,D).

Among the numerous (499) protein domains present in proteins that were lower in abundance at 21 days in '10_11' flag leaves, the most frequently represented domains were pentatricopeptide repeat (cl03252), protein kinase (cl09925), AAA ATPase (cl18944), mitochondrial carrier (cl02813), PHD finger (cl17040), Kelch_3/galactose oxidase (cl02701 and RING (zinc finger) domains (cl17238) (Figure 2.4D). At 14 days, 96 different domains were present in the lower abundance proteins. The most frequently observed set at this time point included leucine-rich repeat (LRR_RI; cl19743), protein kinase (PKc-like; cl21453), Kelch_3/galactose oxidase (cl02701), LbetaH (cl00160), AAA ATPase (cl18944) and RNA Recognition Motif (RRM_SF; cl17169) domains (Figure 2.4B). Overall, these data indicate that numerous signaling, regulatory and metabolic pathways are differentially regulated between 'Karl' and '10_11' flag leaves at 14 dpa, and to an even larger extent at 21 dpa. However, domain analysis does not provide a detailed picture of the most important functions controlling the early-senescence phenotype of line '10_11'.

Functions Associated With Proteins That Are Upregulated In Line '10_11' As Compared To Variety 'Karl'

To obtain additional information on the function of proteins with higher abundances in '10_11', each protein sequence that was significantly and >1.5-fold upregulated in line '10_11' as compared to variety 'Karl' at either 14 or 21 dpa was analyzed using BLAST searches against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Coordinators 2015), information from the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al. 2015), from gene ontology characterization (retrieved from UniProt database; http://www.uniprot.org) (Consortium 2015) and, when appropriate, the rice kinase database (http://phylomics.ucdavis.edu/kinase) (Dardick et al. 2007; Jung et al. 2010) or the MEROPS peptidase database (http://merops.sanger.ac.uk) (Rawlings et al. 2014) (Table 2.3).

Several of the proteins which are upregulated in line '10_11' flag leaves at 14 days have putative or likely functions in plant-pathogen interaction and defense. These include F2CYK3 (which may act as a co-receptor for microbe/pathogen-associated molecular pattern recognition, similar to BRASSINOSTEROID INSENSITIVE 1 ASSOCIATED RECEPTOR KINASE 1 [BAK1]) (Liebrand, van den Burg, and Joosten 2014), two enzymes with glucanase activity (O64938, M0YUE3; β-1,3-glucanases have been classified as family 2 pathogenesis-related proteins) (Van Loon and Pieterse 2006), two family 1 pathogenesis-related proteins (F2DMI6, Q05968), and a putative peroxidase (F2DVY5).

Proteins which are upregulated in line '10_11' at 21 days comprise a more varied group, but putative or likely roles in plant-pathogen interaction are again conspicuous. Obvious candidates for such a function include, again, a glucanase (F2DLS4), two proteins with NB-ARC domains (van der Biezen and Jones 1998; Takken and Tameling 2009) and gene ontology annotations indicating a role in defense response (F2DAV7; M0Z6M8), an NBS-LRR protein (Takken and Goverse 2012; Takken and Tameling 2009) (A9UKM2), and a leucine-rich repeat receptor-like protein kinase which could be involved in microbe/pathogen-associated molecular pattern recognition (F2DLM6).

Additional proteins which may be involved, or also involved, in plant-pathogen interaction include the most highly upregulated of all proteins, F2DS71, with similarity to quinone reductases. A role for such enzymes in the interaction between wheat and powdery mildew has previously been suggested (Greenshields et al. 2005). Several upregulated proteins have functions in lipid metabolism/signaling and possibly in modification of the epidermal cuticle layer, and thus in preventing pathogen entry (F2E0G6, F2E985, F2DEY4, M0W8C6, M0XVD2) (Tang, Simonich, and Innes 2007; Kiba et al. 2014; Gottwald et al. 2012; Lopez-Marques et al. 2014; Ariizumi et al. 2004).

Further proteins with putative roles in pathogen defense are functionally diverse. They include M0W4D3, a protein homologous to At4g22120, which codes for a calcium-permeable cation channel. Related genes have been shown to be upregulated in response to both abiotic and biotic stress (Hou et al. 2014) and could be involved in signaling. Numerous *Arabidopsis* ABC transporter C family members / multidrug resistance-related proteins (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 (Wanke and Kolukisaoglu 2010; 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 into 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, cyclic nucleotide gated ion channels (CNGCs, homologous to F2EHG6), particularly *Arabidopsis* CNCG2 and CNCG4, have been implicated in plant pathogen defense (Ma and Berkowitz 2010; Ma and Berkowitz 2007), and CNCG2 is also involved in senescence regulation (Ma et al. 2010). Protein M0UVB4 contains an SCP-like extracellular domain; proteins with this domain include plant pathogenesis-related protein 1 (PR-1), which accumulates after infection with pathogens (Van Loon and Pieterse 2006). A role for an ABA 8'-hydroxylase in abscisic acid catabolism, leading to upregulation of defense compound synthesis has been described (Mialoundama et al. 2009), and GO annotation for this enzyme also suggests a role in defense against fungal pathogens (Table 2.3). Finally, upregulation of a protein with homology to BREAST CANCER SUSCEPTIBILITY 1 is conspicuous. It has been demonstrated that DNA damage responses are both upregulated and important for plant immune responses (Song and Bent 2014; Yan et al. 2013). The upregulation of both a DNA polymerase □ (F2DD24) and a protein involved in chromatin remodeling (M0XBC0) discovered through 2D-DIGE (Table 2.1) should be reiterated in this context.

The largest group of proteins upregulated in line ‘10_11’, besides those with (potential) functions in plant-pathogen interactions, consists of those involved in protein synthesis and protein degradation. Upregulation of such proteins was observed at both 14 and 21 days (Tables 2.1 and 2.3). They include three ribosomal proteins (F2CT33, F2CRD7, F2CTV4), chaperonins (M0VN45, F2DB07) and a (putative) prolyl-tRNA synthetase (F2CTM7). Functions involved in protein degradation include elements of the ubiquitin-proteasome pathway (F2DQ10, a proteasome regulatory subunit and two ubiquitin carboxyl-terminal hydrolases, F2DPJ3 and MOWZ82), a subtilisin-like 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 for senescence-associated nitrogen remobilization (Feller, Anders, and Mae 2008; Distelfeld, Avni, and Fischer 2014) as well as for the regulation of the senescence process (Fischer 2012).

Additional functional groups that were upregulated in line ‘10_11’, but with only one or two representatives each, include two putative B cell receptor-associated proteins (BAPs; F2DBM9, M0XNJ9) (Table 2.3). 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 (Heath-Engel, Wang, and Shore 2012). Intriguingly, BAP31 has also been demonstrated to be involved in the initiation of apoptotic processes (Namba et al. 2013), and both leaf senescence and the hypersensitive response lead to plant cell death, albeit there are important (time and scale) differences between these processes (Thomas et al. 2003). It should also be briefly mentioned that a homolog of *Arabidopsis* CTR1 (F2E5Y1; involved in ethylene signaling), a putative auxin efflux carrier (F2CZK2) and a citrate synthase were identified as upregulated in line ‘10_11’. Taken together, these results indicated that the differences in hormone signaling, hormone transport and organic acid/amino acid metabolism observed between line ‘10_11’ and variety ‘Karl’ flag leaves may be important to the differences in senescence behaviors.

Table 2.3 Proteins Which are Significantly and >1.5-fold Upregulated in ‘10_11’ *vs.* ‘Karl’ Flag Leaves at 14 and 21 dpa, as Determined by Shotgun Proteomics

ID[1]	**Name**	**Gene ontology (GO)**[1]	**Functions**[2,3,4,5]	**p-value**	**Ratio X/K**
			14 days		
F2CYK3	Predicted protein	No gene ontology information	87% identical to ‘somatic embryogenesis receptor kinase 1’ (SERK1) [*Triticum urartu*]; 84% query coverage. 65% identical to ‘predicted: somatic embryogenesis receptor kinase 1-like isoform 1’ [*Brachypodium distachyon*]; 99% query coverage. 71% identical (156 amino acid overlap) to Os04g38480, ‘BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 PRECURSOR’, putative’.	0.001	4.0
F2DB07	Predicted protein	Cytoplasm; protein folding	96% identical to ‘10 kDa chaperonin’ [*Aegilops tauschii*]; 100% query cover. Domain search also indicates 10 kDa chaperonin.	0.048	2.4
O64938	Beta-1,3-glucanase 2	Carbohydrate metabolic process; hydrolase activity, hydrolyzing O-glycosyl compounds	100% identical to ‘β-1,3-glucanase 2’ [*Hordeum vulgare*]; 100% query cover.	0.036	2.4
M0YUE3	Uncharacterized protein	Carbohydrate metabolic process; hydrolase activity, hydrolyzing O-glycosyl compounds	Glucan endo-1,3-β-glucosidase GII [*Hordeum vulgare*]; member of the glycoside hydrolase family 17.	0.013	2.3

Table 2.3 Continued

F2DMI6	Predicted protein (Uncharacterized protein)	Extracellular region	100% identical to ‘PR-1a pathogenesis related protein (Hv-1a)’ [*Hordeum vulgare* subsp. *vulgare*]; 100% query cover. 96% identical to ‘pathogenesis-related protein 1.1’ [*Triticum aestivum*]; 100% query cover.	0.008	2.2
F2DVY5	Predicted protein (Uncharacterized protein)	Heme binding; metal ion binding; peroxidase activity; response to oxidative stress	70% identical to 'peroxidase 52 isoform 1' [*Zea mays*]; 100% query cover. Contains plant peroxidase superfamily domain (secretory peroxidase, specific).	0.049	1.8
Q05968	Pathogenesis-related protein 1	Defense response; extracellular region; response to biotic stimulus	100% identical to 'pathogenesis-related protein 1, precursor [*Hordeum vulgare*]'; 100% query cover. Contains SCP PR-1 like superfamily domain.	0.042	1.8
F2DPJ3	Predicted protein (Uncharacterized protein)	Metal ion binding; ubiquitin-dependent protein catabolic process	88% identical to ‘ubiquitin carboxyl-terminal hydrolase 19’ [*Triticum urartu*]; 93% query cover; Contains zf-MV and peptidase_C19 superfamily conserved domains.	0.045	1.5
F2E7B8	Predicted protein	4 iron, 4 sulfur cluster-binding; heme binding; sulfite reductase (ferredoxin) activity	98% identical to ‘sulfite reductase’ [*Triticum durum*]; 100% query cover. 4 iron, 4 sulfur cluster-binding; heme binding; sulfite reductase (ferredoxin) activity; ferredoxin or sulfite reductase hemoprotein-like protein. Contains both nitrite and sulfite reductase 4Fe-4S domain and nitrite/sulfite reductase ferredoxin-like half domains.	0.047	1.5

Table 2.3 Continued

F2DS71	Predicted protein	Oxidoreductase activity; zinc ion binding	100% identical to 'predicted protein' [*Hordeum vulgare*]; 100% query cover. 50% identical to 'NADPH:quinone reductase' [*Sphingobacterium sp*. H1ai]; 93% query cover. Also ~50% identical to quinone reductases from various additional bacteria. Contains AL_MDR (arginate lyase and other MDR family members) domain; members of this group contain an arginate lyase, quinone reductases and proteins related to the zinc-dependent dehydrogenases/reductases.	0.002	23.9
F2DLS4	Predicted protein	Carbohydrate metabolic process; hydrolase activity, hydrolyzing O-glycosyl compounds	99% identical to 'β-D-glucan exohydrolase isoenzyme ExoI' (first 25 AA missing in best match; GenBank: AAD23382.1).	0.048	9.7
F2E0G6	Predicted protein	Catalytic activity; membrane	95% identical to 'long-chain fatty acid-CoA ligase 4 [*Aegilops tauschii*]; 100% query cover. 95% identical to long chain acyl-CoA synthetase 9 [*Triticum urartu*]; 97% query cover.	<0.001	9.3
M0VYQ7	Uncharacterized protein	No gene ontology information	Predicted, uncharacterized protein. No conserved domains.	0.027	8.8
F2DAV7	Predicted protein	ADP binding; defense response	45% identical to 'putative disease resistance protein RGA3' [*Aegilops tauschii*]; 99% query cover. Contains NB-ARC domain, a domain shared by plant resistance gene products and regulators of cell death in animals.	0.002	6.7

Table 2.3 Continued

M0YRC6	Uncharacterized protein (Fragment)	Chloroplast; cytosolic ribosome; tripeptidyl-peptidase activity; vacuolar membrane	93% identical to 'tripeptidyl-peptidase 2' [*Aegilops tauschii*]; 98% query cover. Contains TPPII (tripeptidyl peptidase II) domain. TPPII is a crucial component of the proteolytic cascade acting downstream of the 26S proteasome.	0.047	6.7
M0W4D3	Uncharacterized protein	Golgi apparatus; plasma membrane; plasmodesma	97% identical to 'uncharacterized membrane protein C2G11.09 [*Triticum urartu*], 100% query cover. 97% identical to ‘putative membrane protein [*Aegilops tauschii*], 100% query cover. 68% identical to ‘early-responsive to dehydration stress protein isoform 2’ [*Theobroma cacao*]; 99% query cover. Contains domain of unknown function DUF221. 48% identical to the only characterized DUF221 protein, At4g22120, a stress-inducible calcium-permeable cation channel.	0.004	6.4
M0XH58	Uncharacterized protein	Nutrient reservoir activity	‘Embryo globulin’ [*Hordeum vulgare* subsp. *vulgare*]; 100% identical.	0.041	4.9

Table 2.3 Continued

F2CTM7	Predicted protein	ATP binding; chloroplast stroma; embryo sac development; mitochondrion; ovule development; proline-tRNA ligase activity; prolyl-tRNA amino acylation; regulation of photosynthesis; seed development	88% identical to ‘putative prolyl-tRNA synthetase’ [*Oryza sativa* Japonica Group]; 100% query cover.	0.002	4.7
F2E985	Predicted protein (Uncharacterized protein)	Vegetative to reproductive phase transition of meristem; protein desumoylation; hydrogen peroxide biosynthetic process	75% identical to 'CRAL/TRIO domain-containing protein’ [*Zea mays*], 98% query cover. Contains CRAL/TRIO / SEC14 lipid-binding domain.	0.042	4.3
M0WB67	Uncharacterized protein (Fragment)	No gene ontology information	62% identical to 'PREDICTED: proline-rich protein 4-like' [*Setaria italica*]; 88% query cover.	0.002	4.3
F2DBM9	Predicted protein	Endoplasmic reticulum; integral component of membrane; intracellular protein transport	91% identical to 'predicted: B-cell receptor-associated protein 29-like [*Brachypodium distachyon*], 100% query cover. Contains ‘B-cell receptor-associated protein 31-like’ domain.	0.007	4.2
F2DXK0	Predicted protein	DNA binding; nucleosome; nucleosome assembly; nucleus	91% identical to ‘histone H1 WH1B.1’ [*Triticum aestivum*]; 47% query cover. Belongs to Histone H1/H5 family.	0.042	3.9

Table 2.3 Continued

M0Z6M8	Uncharacterized protein	ADP binding; defense response	75% identical to ‘disease resistance protein RPM1’ [*Aegilops tauschii*]; 100% query cover. Domain search indicates presence of NB-ARC domain.	0.046	3.9
F2DEY4	Predicted protein	No gene ontology information.	No conserved domains. 66% identical to ‘no exine formation 1 isoform 1’ [*Theobroma cacao*]; 93% query cover. 58% identical to ‘no exine-formation-1’ [*Arabidopsis thaliana*]; 100% query cover.	0.001	3.8
M0UJZ3	Uncharacterized protein	No gene ontology information	76% identical to ‘predicted: flocculation protein FLO11-like isoform X1’ [*Oryza brachyantha*]; ‘predicted: flocculation protein FLO11-like isoform X2’ [*Oryza brachyantha*]. Contains domain of unknown function DUF936.	0.007	3.8
M0ZER7	Uncharacterized protein	No gene ontology information	88% identical to ‘serine/threonine-protein kinase SMG1’ [*Aegilops tauschii*]; 100% query cover.	0.038	3.7
M0WMI0	Uncharacterized protein	No gene ontology information	No functional information; no conserved domains.	0.004	3.6
A9UKM2	NBS-LRR resistance-like protein	ADP binding; defense response	100% identical to 'NBS-LRR resistance-like protein' [*Hordeum vulgare*]; 100% query cover. 88% identical to 'disease resistance protein RPM1' [*Triticum urartu*], 100% query cover.	0.001	3.4

Table 2.3 Continued

M0VRP3	Uncharacterized protein	ATP binding; ATPase activity, coupled to transmembrane movement of substances; integral component of membrane	92% identical to ‘ABC transporter C family member 10’ [*Aegilops tauschii*], 99% query cover.	0.010	3.4
M0W8C6	Uncharacterized protein	Hydrolase activity, acting on ester bonds; lipid metabolic process	85% identical to ‘GDSL esterase/lipase’ [*Aegilops tauschii*]; 94% query cover. Domain search also indicates esterase/lipase function.	0.008	3.3
F2DDX0	Predicted protein	No gene ontology information	35% identical to ‘carboxylesterase’ [*Flavobacterium* sp. F52]; 92% query cover. Contains domain of unknown function (DUF) 1254.	0.001	3.1
M0UK31	Uncharacterized protein	No gene ontology information	71% identical to ‘predicted: kinectin-like’ [*Brachypodium distachyon*]; 70% query cover. 51% identical to 'lisH domain-containing protein C1711.05-like' [*Oryza brachyantha*], 92% query cover.	0.003	3.1
F2CRD7	Predicted protein	Ribosome; structural constituent of ribosome; translation	98% identical to ‘predicted: 60S ribosomal protein L4-like isoform 1’ [*Acyrthosiphon pisum*]; 100% query cover. Contains a 60S ribosomal protein L4 C-terminal domain.	0.003	3.0
M0XBQ5	Uncharacterized protein	Heme binding; iron ion binding; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	88% identical (99% query coverage) to ‘tyrosine N-monooxygenase/hydroxylase’ [*Aegilops tauschii*, *Triticum urartu*]. Contains tryptophan N-hydroxylase (PLN02971) domain. Belongs to the cytochrome P450 family.	0.001	3.0

Table 2.3 Continued

F2DDU0	Predicted protein	No gene ontology information	48% identical to ‘copine family protein’ [*Tetrahymena thermophila*]; 76% query cover. Domain search: VWA Copine.	0.033	2.9
M0YBE3	Uncharacterized protein (Fragment)	No gene ontology information	58% identical to ‘serine/threonine-protein kinase SMG1’ [*Aegilops tauschii*]; 94% query cover. 60% identical to ‘long-chain-fatty-acid-CoA ligase 1’ [*Aegilops tauschii*]; 83% query cover.	0.009	2.9
M0XNJ9	Uncharacterized protein	Endoplasmic reticulum; integral component of membrane; intracellular protein transport	85% sequence identity with ‘B-cell receptor-associated protein 31-like’ [*Setaria italica*], 97% query cover.	0.006	2.7
M0WZ82	Uncharacterized protein (Fragment)	Ubiquitin-dependent protein catabolic process	91% identical to ‘inactive ubiquitin carboxyl-terminal hydrolase 54’ [*Triticum urartu*]; 98% query cover. Contains domain of unknown function (DUF) 629. Also contains a domain corresponding to a subfamily of peptidase C19.	0.019	2.6
F2DLM6	Predicted protein	ATP binding; protein kinase activity	80% identical to ‘predicted: probable LRR receptor-like serine/threonine protein kinase At1g14390-like’ [*Brachypodium distachyon*]; 99% query cover. Domains search indicates presence of LRRs and protein kinase domain.	0.040	2.5
F2EIG7	Citrate synthase	Cellular carbohydrate metabolic process; transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer; tricarboxylic acid cycle	Citrate synthase	0.031	2.5

Table 2.3 Continued

F2DKJ2	Predicted protein	ATP binding; protein serine/threonine kinase activity	36% identical to ‘inter-alpha-trypsin inhibitor heavy chain H3 precursor, putative’ [*Ricinus communis*], 98% query cover. Contains vWA_C3HC4_type [cd01466], VWA C3HC4-type: Von Willebrand factor type A (vWA) domain.	0.016	2.4
F2EHG6	Predicted protein	No gene ontology information	93% identical to ‘predicted: cyclic nucleotide-gated ion channel 4-like’ [*Brachypodium distachyon*]; 99% query cover. 92% identical to ‘predicted: cyclic nucleotide-gated ion channel 4-like [*Setaria italica*]; 99% query cover. 91% identical to ‘cyclic nucleotide-gated ion channel 2’ [*Zea mays*]; 99% query cover.	0.015	2.4
M0VCY2	Uncharacterized protein	No gene ontology information	Function unknown; no conserved domains found.	0.044	2.4
M0WWN5	Uncharacterized protein	Cytosol; small-subunit processome	83% identical to 'predicted: nucleolar protein 14-like' [*Brachypodium distachyon*]; 99% query cover. Contains a Nop14 superfamily domain.	0.030	2.4
F2E0F7	Predicted protein	ATP binding; protein serine/threonine kinase activity	100% identical to ‘predicted protein’ [*Hordeum vulgare*]; 100% query cover. Contains kinase domain and numerous LRRs (C-terminally to kinase domain).	0.018	2.3

Table 2.3 Continued

F2EFZ8	Predicted protein	DNA binding; DNA recombination; DNA repair; nucleus; response to gamma radiation; ubiquitin-protein transferase activity; zinc ion binding	86% identical to 'BREAST CANCER SUSCEPTIBILITY 1-like protein' [*Aegilops tauschii*]; 100% query cover. Contains a zinc finger and BRCT domain.	0.044	2.3
M0UVB4	Uncharacterized protein	ATP binding; plasmodesma; zinc ion binding	96% identical to ‘Sacsin’ [*Triticum urartu*] SCP: SCP-like extracellular protein domain, found in eukaryotes and prokaryotes. This family includes plant pathogenesis-related protein 1 (PR-1), which accumulates after infections with pathogens, and may act as an anti-fungal agent or be involved in cell wall loosening. SCP has also been proposed to be a Ca^{++} chelating serine protease.	0.034	2.2
F2CTV4	Predicted protein (Fragment)	RNA binding	87% identical to ‘30S ribosomal protein S1', chloroplastic, [*Triticum urartu*]; 73 % query cover. Contains two ribosomal protein S1 (RPS1) domains and an S1 RNA-binding domain.	0.024	2.1
F2CZK2	Predicted protein	Integral component of membrane; transmembrane transport	99% identical to ‘uncharacterized transporter C5D6.04’ [*Triticum urartu*]; 99% query cover. 87% identical to ‘auxin efflux carrier family protein-like’ [*Oryza sativa japonica* group]; 99% query cover.	0.039	2.1
F2DXZ6	Predicted protein	Membrane; plant-type cell wall; plasmodesma; vacuole	α/β hydrolase family; this family contains α/β hydrolase enzymes of diverse specificity.	0.036	2.1

Table 2.3 Continued

M0XVD2	Uncharacterized protein	ATP binding; cation-transporting ATPase activity; integral component of membrane; magnesium ion binding; phospholipid-translocating ATPase activity	95% identical to 'phospholipid-transporting ATPase 1' [*Aegilops tauschii*]; 100% query cover. Contains E1-E2 ATPase, Hydro1, and COG4087 superfamily domains.	0.031	2.1
F2DVR4	Predicted protein	Nucleic acid binding; nucleotide binding	Contains two RRM1_NUCls domains. 88% identical to ‘protein gar2’ [*Aegilops tauschii*].	0.019	1.8
F2E5Y1	Predicted protein	ATP binding; cytosol; protein serine/threonine kinase activity	‘Serine/threonine-protein kinase CTR1’ [*Aegilops tauschii*]; 100% query cover. Involved in ethylene signaling.	0.040	1.8
F2EKD0	Predicted protein (Uncharacterized protein)	No gene ontology information	67% identical to ‘predicted: F-box/LRR-repeat protein 14-like’ [*Setaria italica*]; 97% query cover. Contains two AMN1 domains (antagonist of mitotic exit network protein 1).	0.036	1.8
M0Z797	Uncharacterized protein	Chloroplast thylakoid; oxidoreductase activity, acting on iron-sulfur proteins as donors; photosynthetic electron transport in photosystem I	91% identical to ‘predicted: PGR5 (proton gradient regulation5)-like protein 1A, chloroplastic-like’ [*Brachypodium distachyon*]; 100% query cover.	0.043	1.8

Table 2.3 Continued

Q0PD05	ABA 8'-hydroxylase	(+)-abscisic acid 8'-hydroxylase activity; abscisic acid metabolic process; defense response to fungus; heme binding; iron ion binding; release of seed from dormancy; response to red or far red light; response to water deprivation	HvCYP707A1. 100% identical to 'ABA 8'-hydroxylase 1' [*Hordeum vulgare* subsp. *vulgare*]; 100% query cover. Contains P450 superfamily domain.	0.008	1.8
F2CWU3	Predicted protein	No gene ontology information	96% identical to 'predicted: nuclear distribution protein nudE homolog 1' [*Acyrthosiphon pisum*]; 100% query cover. Contains a DUF342 and a NUDE_C super-family domain.	0.022	1.6
F2D818	Predicted protein	Nucleic acid binding; protein methyltransferase activity	85% identical to 'predicted: protein methyltransferase hemK homolog' [*Brachypodium distachyon*]; 100% query cover. Domain search indicates 'S-adenosylmethionine-dependent methyltransferases (SAM or AdoMet-MTase), class I'.	0.044	1.6

X, early-senescing barley line '10_11'; and K, late-senescing barley variety 'Karl'.

[1] Gene ontology characterization / ID (retrieved from UniProt database; http://www.uniprot.org) (UniProt Consortium, 2015).

[2] Conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer *et al.*, 2015).

[3] NCBI protein sequence homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (NCBI Resource Coordinators, 2015).

[4] Rice kinase database (http://rice.plantbiology.msu.edu) (Dardick *et al.*, 2007; Jung *et al.*, 2010).

[5] MEROPS protease database (http://merops.sanger.ac.uk) (Rawlings *et al.*, 2014).

Discussion

Senescence is a carefully controlled process of molecular degradation and nutrient reallocation (Lim, Kim, and Nam 2007) that is shown to have substantial impact on the yield of cereal crops. This study focused on the identification and quantification of global changes in the proteome of barley (*Hordeum vulgare* L.) flag leaves during senescence. This strategy was distinct because both2D-DIGE and shotgun proteomic techniques were employed. According to the UniProt protein database (http://www.uniprot.org), the barley proteome comprises 52,289 putative proteins that are encoded by the 30,000 confirmed barley genes (~70% of genes are regulated by alternative splicing) (Mayer et al. 2012). In total, 9,258 proteins were identified in this study and one third of them were quantifiable, corresponding to 17.7% of the barley proteome. Considering that only late-mature and senescing barley flag leaves were investigated, these experiments report on a substantial percentage of the proteome.

Functions of highly abundant enzymes and regulatory proteins identified in the fast-senescing/high-GPC barley line ’10_11’ point to key processes that occur during senescence signaling and execution. Distinctions between ‘10_11’ protein abundance compared to ‘Karl’ were determined from results collected by both 2D-DIGE and shotgun experiments. Specifically, several trends exhibited in highly abundant proteins in ‘10_11’ were functionally associated with N-metabolism and, interestingly, plant defense response programs. Distinctions in abundance between ‘10_11’ and ‘Karl’ were observed in proteins that share common features with those important for perception, signal transduction, and defense reactions which plants initiate when challenged by pathogen attack. Proteins involved in the perception/signaling of the plant defense system (LRR-RLKs and NBS-LRRs) including several previously characterized as barley pathogen induced proteins were present at higher levels (1.5 – 24-fold) in fast-senescing/high-GPC barley line ‘10_11’ (Table 2.3). These proteins are known to elicit downstream responses within the plant that decrease the magnitude of pathogen damage. For example, the loss of cell wall integrity induced by pathogens during infection is sensed by membrane receptors within plants and a variety of defense responses are elicited (Tierney, Wiechert, and Pluymers 1988). During senescence, the decomposition of the cell wall is an inevitable outcome, resulting in oligosaccharide breakdown products. Oligosaccharide hydrolase (β-glucosidase) proteins were substantially more abundant in senescing flag leaves of early-senescing/high-GPC line ‘10_11’ compared to late-senescing/low-GPC line ‘Karl’. Plant membrane receptors can perceive oligosaccharide signatures that are present on pathogens themselves, or released from the plant cell wall due to pathogen attack by hydrolytic enzymes (Wang et al. 2010; Afzal, Wood, and Lightfoot 2008; Tör, Lotze, and Holton 2009). A characterized β-glucosidase enzyme found in *A. thaliana* was also found to be upregulated during senescence (Bessire et al. 2011). β-glucosidase activity is utilized by plants during senescence to loosen cell walls to accelerate the breakdown of tissues or alternatively as a signaling mechanism to start hypersensitive response (rapid response that can result in the death of cells; HR) (Lee et al. 2007; Sasidharan, Voesenek, and Pierik 2011; Nuhse 2012). Additional proteins that serve protection-associated roles of higher abundance at both time points analyzed in high-GPC line ‘10_11’ flag leaves were various enzymes that are involved in long-chain fatty acid synthesis metabolic processes. These proteins have been found to be essential to plant defense against threats caused by bacterial and fungal pathogens, and insect invasions (Dietrich, Ploss, and Heil 2004; Dechorgnat et al. 2012) because of their function in cuticle (waxy layer on leaf surface) synthesis. These findings indicate active long-chain fatty acid (LC-FA) processes by LC-FA enzymes during senescence in '10_11', possibly suggesting their role in protecting tissues and nutrients during senescence. The observation of highly abundant defense-related HR domains/proteins (ROS-associated) in the early-senescing/high-GPC line '10_11' during the senescence period might further provide evidence they serve a protective role during senescence..

At both 14 and 21 dpa, a number of proteins with possible roles in pathogen defense were found to be upregulated in flag leaves of line '10_11'. Such proteins may allow fast-senescing germplasm to defend nitrogen (and other nutrients) from pathogens during the remobilization process, resulting in higher grain protein concentration. The specific contribution of these proteins to accelerating senescence, increasing grain nitrogen, and enhancing resistance to pathogens remains to be determined. Profiling differences between '10_11' and 'Karl' during inoculation with known barley pathogens could help clarify these points.

The overlap between leaf senescence and the defense/pathogenesis response is clearly demonstrated by proteomic comparisons of early-senescing/high-GPC line '10_11' and late-senescing/low-GPC variety 'Karl' flag leaves at the protein level. The results indicate that similarities between defense processes and senescence transcend into the protein level in barley. Overlap between proteins important for senescence and defense mechanisms may be due to the senescence program incorporating features of the pathogen-defense responses to protect leaves against opportunistic pathogens (Coleman 1986; Obregón et al. 2001; Quirino et al. 2000b; Gan 2014). Alternatively, the role of PR proteins during senescence may also serve as a pathogen-resistant form of soluble nitrogen storage macromolecules (Gan and Amasino 1997; Wisniewski, Bassett, and Arora 2004; Pommerrenig et al. 2011). In *Alfalfa* several vegetative storage proteins were shown to not exclusively serve as nitrogen reserves during specific phases of legume development, but also play an important adaptive role in plant protection against abiotic (low temperature) and biotic (pathogen attack) stresses (Dordas 2008). Identification of similarities between defense and senescence programs may provide insight into proteins/pathways that influence quality characteristics (Geldner 2014; Furuta et al. 2014) (Distelfeld, Avni, and Fischer 2014; He and Dijkstra 2014; Shen, Hsu, and Chen 2007; Catford and Jansson 2014; Pausas and Keeley 2014). Modulating the proteins that serve important roles in both senescence and defense can facilitate cereal improvement in two ways, (1) increasing grain nutrition (affected by N-associated enzymes and total % N) and strengthening resistance to biotic stresses (Distelfeld, Avni, and Fischer 2014; He and Dijkstra 2014; Shen, Hsu, and Chen 2007; Catford and Jansson 2014; Pausas and Keeley 2014; Panchen et al. 2014).

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CHAPTER THREE

METABOLOMIC COMPARISON OF POST-ANTHESIS BARLEY FLAG LEAVES FROM NEAR-ISOGENIC GERMPLASM DIFFERING IN ITS SENESCENCE BEHAVIOR

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Abstract

Senescence is the last stage of leaf development and it is characterized by a series of coordinated reactions plants utilize in order to remove nutrients from vegetative tissues and transfer them into reproductive tissues (e.g., from leaves to grains). In addition to developmental age, other factors such as plant hormones, pathogen infection, and environmental stresses can influence senescence onset, progression, and duration. Differences in the behavior of senescence (e.g., early- or late-onset) directly impacts yield and grain quality of commercially produced cereals including wheat, barley, and rice. Mechanisms that influence senescence and nutrient remobilization in barley (*Hordeum vulgare* L.) were investigated using mass spectrometry-based metabolic profiling and pathway mapping. Flag leaf metabolite extractions from two near-isogenic barley lines that exhibit distinctly different senescence behaviors were compared at anthesis (flowering) and throughout senescence. Metabolic profiling of line '10_11' (early-senescing) and 'Karl' (late-senescing) flag leaves revealed differences in their global metabolism over time, highlighted features that were upregulated in '10_11' as compared to 'Karl', and provided targets for molecular feature identification techniques. The metabolites found at high levels in '10_11' were associated with pathways involved in cellular oxidation state management, gibberellin degradation, jasmonic acid synthesis, and the Yang cycle (ethylene synthesis), implicating changes in phytohormone and oxidative stress levels in the regulation of barley flag leaf senescence.

Introduction

Leaf senescence is an age-dependent program that is coordinated by plants in order to recycle valuable nutrients for use in subsequent generations (Bleecker 1998, Quirino, Noh et al. 2000, Gepstein 2004, Lim, Kim et al. 2007, Zhang and Zhou 2013). Vegetative tissues serve as a primary source of essential nutrients that are mobilized during senescence (Yuan and Chen 2009). The biochemical reactions that occur during senescence are associated with hydrolysis of carbohydrates (Wingler, von Schaewen et al. 1998, Minic and Jouanin 2006), chlorophylls (Matile, Hörtensteiner et al. 1996, Hörtensteiner 2006), lipids (Thompson, Froese et al. 1998, Troncoso-Ponce, Cao et al. 2013), proteins (Brouquisse, Masclaux et al. 2001, Goud and Kachole 2011), and RNA (Gan and Amasino 1997) by the actions of enzymes that include glucosidases (Veyres, Danon et al. 2008), pheophorbide a oxygenase (Hörtensteiner 2006), lipoxygenases, lipases (Thompson, Froese et al. 1998, Jia, Tao et al. 2013), endo- and exopeptidases (Granell, Cercós et al. 2004, Parrott, Martin et al. 2010, Roberts, Caputo et al. 2012), and RNases (Taylor, Bariola et al. 1993, Lers, Khalchitski et al. 1998). The transformation of cellular components into transportable metabolites results in nitrogen in the form of purines, polar amino acids, and polyamines (Galili and Höfgen 2002, Werner and Witte 2011); sulfur in glutathione and homoglutathione (Miloud and Ali 2012); phosphate from nucleic acids (Gregersen 2011, Werner and Witte 2011, Veneklaas, Lambers et al. 2012); and other mineral nutrients. The availability of, and capacity to relocate, these compounds during senescence directly impacts the speed, timing and percentage of nutrient accumulation in developing grains (Pearson and Rengel 1994, Garnett and

Graham 2005, Fischer 2007). For example, the percentage of nitrogen remobilized during senescence affects seed/grain quality, specifically the grain protein content (GPC) (Fageria and Baligar 2005, Yuan and Chen 2009, Fageria, Baligar et al. 2010). Manipulating senescence behavior by genetic modification can improve the quality of resulting grains, increase yield, enhance resistance to pathogen attack, strengthen tolerance to herbicides, and enhance the ability to withstand abiotic/biotic stresses in crops (Dunwell 2000, Chandler and Dunwell 2008, Dunwell 2014). Developing an understanding of metabolic features that influence senescence is essential for the improvement of crop traits.

Barley (*Hordeum vulgare* L.) is cultivated worldwide and its crop value continues to increase (USDA 2014). In addition, and perhaps more significantly, the number of patents issued for engineered forms of barley has also increased (Dunwell 2014, Mrízová, Holasková et al. 2014). Particularly useful for understanding senescence in barley is an extensively characterized near-isogenic barley germplasm program that differ in senescence behavior (Heidlebaugh, Trethewey et al. 2008, Jukanti and Fischer 2008, Lacerenza, Parrott et al. 2010). Specifically, line '10_11' has an earlier onset and faster progression of senescence, and increased GPC when compared to parental line 'Karl' (Jukanti and Fischer 2008, Lacerenza, Parrott et al. 2010). Defining differences that are observed in the metabolism of '10_11' and 'Karl' flag leaves during senescence can help to determine regulatory and execution pathways that influence nutrient remobilization .

Systems biology platforms including genomics, transcriptomics, proteomics, and metabolomics are now powerful tools in crop development efforts (Zhang, Chen et al.

2006, Fukushima and Kusano 2014, Gong, Yang et al. 2014, Simons, Saha et al. 2014). The majority of studies aimed at dissecting senescence have used transcriptional and, to a lesser extent, proteomic methods. The application of metabolomics to the study of senescence is limited (Guo 2013). To address this, mass-spectrometry based non-targeted metabolic profiling techniques were used to compare '10_11' and 'Karl' during senescence (Figure 3.1).

Differences in metabolite abundances between '10_11' and 'Karl' were observed throughout senescence. The identities of changing features that were associated with barley metabolism highlighted active pathways that may be important for senescence in each barley line. Global trending of features indicated shifts in the overall metabolism of '10_11' and 'Karl' as senescence progressed. Mapping of metabolites to pathways revealed that several putatively identified compounds that were more abundant in '10_11' as compared to 'Karl' are intermediates of several hormone-associated pathways including gibberellin catabolism, Yang cycle (ethylene biosynthesis) and jasmonic acid biosynthesis. The results may point to the importance of hormone regulation during senescence. Moreover, barley breeders can utilize information obtained in the performed experiments to inform targeted breeding practices aimed at enhancing senescence and nutrient remobilization in crops.

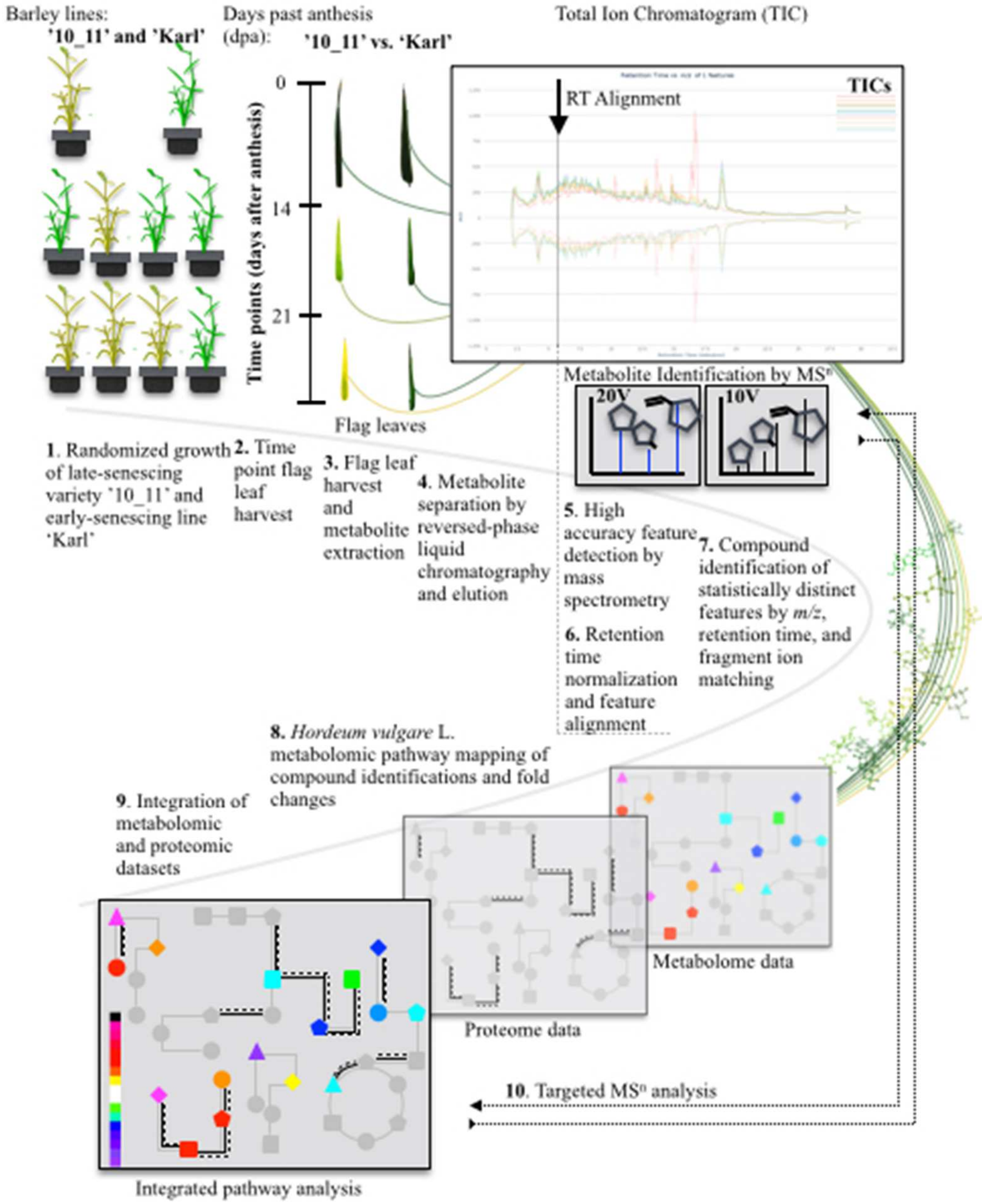

Figure 3.1 Metabolomic Comparison of Barley Near-isogenic Line '10_11' and Variety 'Karl' Flag Leaves by LC-MS. Metabolite extracts from flag leaves of '10_11' and 'Karl' were compared by LC-MS. Features of interest were selected for identification through targeted MS analysis, integrated with proteomic data, and mapped into barley metabolic pathway networks (BarleyCyc), available from the Plant Metabolic Network (PMN) website (http://pmn.plantcyc.org/BARLEY/class-tree?object=Pathways) on (www.plantcyc.org).

Materials and Methods

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). Variety 'Karl' is characterized by delayed senescence and lower grain protein content as compared to line '10_11', due to allelic variation at a chromosome 6H locus controlling these traits (Mickelson et al. 2003; Jukanti et al. 2008a). Seeds were planted on 1 February 2013 in a small randomized block design containing four blocks each of line '10_11' and variety 'Karl' (eight blocks total) in a glasshouse of the Montana State University Plant Growth Center. The growth light cycle and temperature parameters were replicated from the previous transcriptional analysis (Jukanti et al. 2008b). 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. Samples were harvested at three different time points: (1) upon the onset of anthesis (0 days past anthesis; dpa), (2) 14 days (14 dpa), and (3) 21 days past anthesis (21 dpa). Excised leaves were immediately flash-frozen in liquid N_2 and stored at -80°C. For each harvest time point, three biological replicates were collected, each consisting of randomly selected flag leaves (from ten different plant shoots).

Metabolite Extraction

Frozen flag leaves were ground for 1 minute in liquid N_2 with a mortar and pestle. The procedure for metabolite extraction was adapted from Roessner et al. (Roessner et al. 2006). Leaf powder (approximately 150 mg per sample) was immersed in 100% methanol (MeOH) at 70°C for 15 minutes. Samples were vortexed for 1 min and then centrifuged (25,000 *g*, 10 minutes, 4°C) to remove cellular debris from the soluble fraction. Proteins were separated from the metabolites by an acetone precipitation (two and a half parts acetone to one part MeOH from extraction) at -80°C overnight, followed by centrifugation (25,000 *g*) at 4°C for 10 minutes. The resulting supernatant fraction was dried in a speed vacuum (low heat setting), and stored at -80°C. Prior to analyses by LC-MS, samples were resuspended in 40 □L of 50% HPLC grade water / 50% MeOH.

Agilent LC-MS 6538 Mass Spectrometry

Barley metabolite analysis was conducted using an Agilent 1290 ultra-performance liquid chromatography (UPLC) interface (Agilent Technologies, Santa Clara, CA, USA) fitted to an Agilent 6538 Accurate-Mass quadrupole time-of-flight mass spectrometer. Volumes of 5 □L from each metabolite sample were injected in a randomized order. Metabolites were separated by reversed phase (RP) chromatography on a Kinetex 1.7 □m C18 150 x 2.1 mm column (Phenomenex, Torrance, CA) kept at 50°C with a flow rate of 600 □L min $^{-1}$. Using a method adapted from Hamerly *et al.* (Hamerly et al. 2014), the elution profile implemented started with a two minute step of 98% solvent A (0.1% formic acid in H_2O; waste) with 2% solvent B (0.1% formic acid in acetonitrile) followed by a 2% to 95% solvent B gradient over 24 min, a continued 95% solvent B for two min, and then a return to 2% solvent B over two min.

Mass detection was performed in positive mode, with a cone voltage of 3,500 V and a fragmentor voltage of 120 V. Drying gas temperature was 350°C (flow of 12 L min $^{-1}$) and the nebulizer was set at ~5.2 bar. Data was acquired with the following parameters: m/z range of 50-1,000 at 25,200 mass-to-charge-ratio (*m/z)*-s. Mass analyzer resolution was 18,000 and post calibration tests had mass accuracy of approximately one ppm. For MS^n acquisition, both standard compounds and ions of interest within a tolerance window of 1.7 *m/z* units were fragmented at 10 and 20 V.

Data Analysis

The data analysis and processing workflow used was adapted from Hamerly et al. (2014). Raw data files obtained from the Agilent 6538 instrument in centroid mode were converted to MZxml format from raw format using Masshunter Qualitative software (Agilent, Santa Clara, CA). Output from Masshunter software was then imported into XCMS online (Tautenhahn et al. 2012). Data files from each triplicate analysis were loaded into separate datasets that were cross-compared. Biological replicates for each barley line ('10_11' and 'Karl') were analyzed at each time point (0, 14, and 21 days past anthesis). Paired analysis between barley lines at each time point was performed using the 'meta-analysis' option of XCMS online (https://xcmsonline.scripps.edu). Features within each sample were detected using an intensity cut off of 5,000 counts and an elution time tolerance of 0.1 min (Zhu et al. 2013). The mass features that remained were assigned retention times (RT) with the following parameters: minimum intensity 10,000, time span of 0.25 minutes, and *m/z* tolerance of 10 ppm. The resulting features were then cross-compared between samples, manipulated by various statistical analyses (see below), and features considered to be significantly different (fold change; $FC \geq 3.0$ and p-value ≤ 0.01) were then considered for additional identification and characterization. Comparisons were then performed on the three resulting paired outputs. Statistical analyses included one-way ANOVA, principal component analysis; PCA, k-means clustering and Welch's t-test performed using the XLSTAT software package (Addinsoft 2013) and XCMS online (https://xcmsonline.scripps.edu). The ratio comparing '10_11' relative abundance *vs*. 'Karl' relative abundance ('10_11'/'Karl') was used to determine fold-change differences of features. Global trend normalization was achieved by $\log_2$ transformation and normalization of each average relative intensity value by the total sum of relative intensities for each feature (i.e., average intensity / Σ[average intensity at 0, 14, and 21dpa]) so that the sum of the normalized values was equivalent to unity.

Initial metabolite identifications were achieved by alignment of features by retention time (RT) and *m/z* to a small in-house library of common biological molecules analyzed using equivalent LC-MS method settings. Anticipated biologically relevant compound identifications were assigned based on high-mass accuracy alignment (<30ppm *m/z* tolerance) relative to a custom database specific to *Hordeum vulgare* L. (Supplementary Table 2.2, Appendix A). The database was exported and reformatted from 'BarleyCyc', available from the Plant Metabolic Network (PMN) website (http://pmn.plantcyc.org/BARLEY/class-tree?object=Pathways) on www.plantcyc.org). Putative compound features were selected for MS fragment analysis that was performed on one of two samples; either a pooled sample of '10_11' (equivalent mixture of nine samples) or a pooled ‘Karl’ sample (equivalent mixture of nine samples) depending on which sample had higher calculated relative abundances. In order to identify compounds of interest from annotated pathways in the BarleyCyc database (Plant Metabolic Network (PMN), [http://pmn.plantcyc.org/organism-summary?object=BARLEY], on www.plantcyc.org), *in silico* fragmentation analysis was implemented. Chemical structures of candidate compounds were generated using the HighChem structure editor (http://www.highchem.com/structureeditor/index.html). The resulting structures were used as theoretical parent ions that were fragmented *in silico* in order to obtain MS substructures and their corresponding *m/z* values. Features with matching experimental and theoretical *m/z* parameters were assigned to candidate compounds and putatively identified. The putative metabolites that exhibited significantly different levels (fold change (FC) $\geq$ 3.0 and p-value $\leq$ 0.01) between ’10-11’ and ‘Karl’ were mapped to known *Hordeum vulgare* L. metabolic pathways retrieved from the BarleyCyc metabolic pathway database (Zhang et al. 2005). Putative identifications were described by 'super-pathway' (cross-species annotation and cases where metabolic pathway is not fully known) and 'sub-pathway' (indicates linear sub-pathway of the enzymatic reaction associated with candidate metabolite) terms (Morgat et al. 2012). Metabolic pathway mapping was performed using Pathway Tools v17.5 software (SRI International) (Karp 2000). Metabolites were combined with previously obtained proteomic datasets (*in preparation;* Mason et al. 2015, Thesis Chapter Two) and compared for common trends at 14 and 21 dpa.

Results

Metabolic Profiles of Barley Line ‘10_11’ and Variety ‘Karl’ Flag Leaves

Extracts of metabolites from flag leaves were analyzed using reversed-phase LC-MS. Samples from early-senescing line ‘10_11’ and near-isogenic parent variety ‘Karl’ at 0, 14, and 21 days past anthesis (dpa). Features detected by MS were aligned by mass-to-charge-ratio (*m/z)* and retention time (RT), then averaged across biological replicates (Supplementary Figure 3.1, Appendix A) to obtain a relative abundance value for comparison. In the 18 samples that were analyzed, 5,131 shared features were detected (intensity > 5,000 counts) in at least one biological replicate of both '10_11' and 'Karl'. There were 2,901, 1,801, and 2,455 of those features found in common between '10_11' and 'Karl' at 0, 14, and 21 dpa (Figure 3.2 and Supplementary Table 3.1, Appendix A). The number of significantly different features (>3.0-fold; p value <0.01) were initially found by comparing every biological replicate of '10_11' and 'Karl' against one another (18 samples total) by an one-way ANOVA statistical analysis. There were 187 (0 dpa), 36 (14 dpa) and 35 (21 dpa) found to be significantly regulated between '10_11' and 'Karl' samples. These results indicated that significant differences found between the metabolic profiles of '10_11' and 'Karl' decreased as senescence progressed.

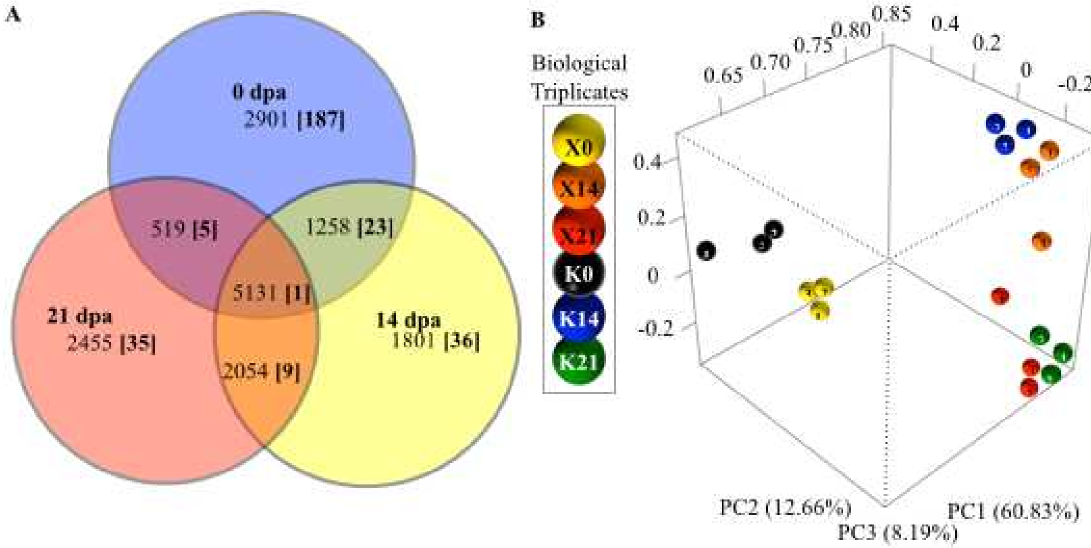

Figure 3.2 Statistical Analysis of Metabolomic Profiles of Barley Line ‘10_11’ and Variety ‘Karl’ Flag Leaves. A) Venn diagram indicating the number of unique and shared MS molecular features found in each biological replicate comparison (min intensity of 5000). The number of features with significant differences in abundance (>3.0-fold; $p<0.01$) are shown bracketed. The diagram was generated using XCMS online (https://xcmsonline.scripps.edu).B) Principal component analysis (PCA) of all features above significance cut-off.

Differences in the biological replicates of '10_11' and 'Karl' at each time point were then assessed by principal component analysis (PCA, Figure 3.2B). Sample groups clustered well at 0 dpa and revealed the most separation between '10_11' and ‘Karl’ populations. As the plants progressed through senescence their metabolic profiles were less distinct from one another and at 21dpa, the sample groups were the least differentiated.

To highlight any significantly changing features between '10_11' and 'Karl' at each time point independently, relative abundances of each feature detected in biological replicates were compared by one-way ANOVA and designated with a corresponding p-value (Figure 3.3 A-C). These results revealed significant fold-changes between '10_11' and 'Karl' at each time point. There were distinctly more features that exhibited significantly higher abundances in '10_11' (168) compared to 'Karl' (20) at 0 dpa than observed at the other time points (Figure 3.4 A-C). These results indicated that prior to senescence, during anthesis, ‘10_11’ and ‘Karl’ had the greatest difference in intracellular metabolite composition. At 14 and 21 dpa, the number of significantly regulated features that had higher levels in '10_11' *vs.* 'Karl' ('10_11'; 23, 22 and 'Karl'; 12, 13) were similar. The disparity observed between '10_11' and 'Karl' at 0 dpa compared to later stages in senescence may indicate regulatory differences at the metabolic level that occur before senescence and during anthesis. Distinct features that were found in ‘10_11’ may also have potential as biomarkers to establish N-remobilization and senescence behaviors in barley lines.

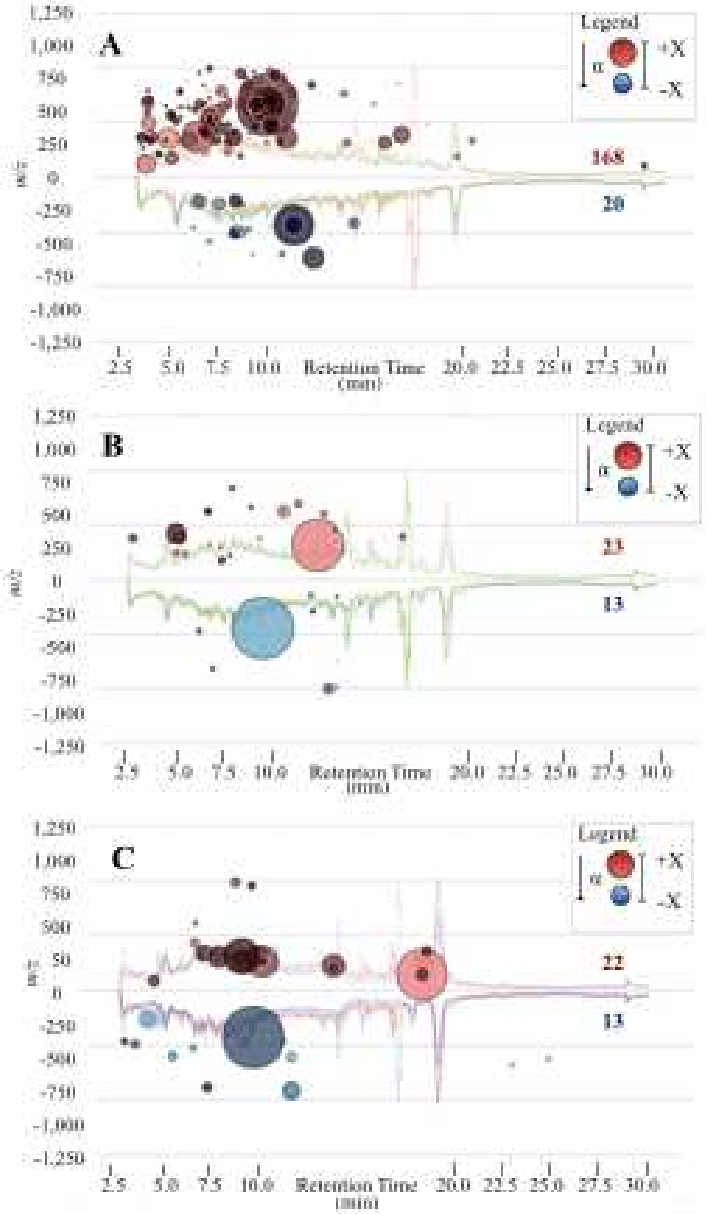

Figure 3.3 Significantly Regulated Features Found Between Barley Line ‘10_11’ and Variety ‘Karl’ Flag Leaves. Cloud-plots from features that were filtered by significance (fold change ≥ 3.0 and a p-value ≤ 0.01) are shown as colored circles indicating the degree and direction of the fold difference (red hues; higher abundance in '10_11'; +X and blue hues; lower abundance in '10_11'; -X). Molecular feature retention times are displayed on the x-axis with the corresponding mass-to-charge ratios (*m/z)* on the y-axis. Comparisons of biological replicates of ’10_11’ *vs*. ’Karl’ at different time points (0, 14 and 21 dpa) are shown in panels A-C, respectively).

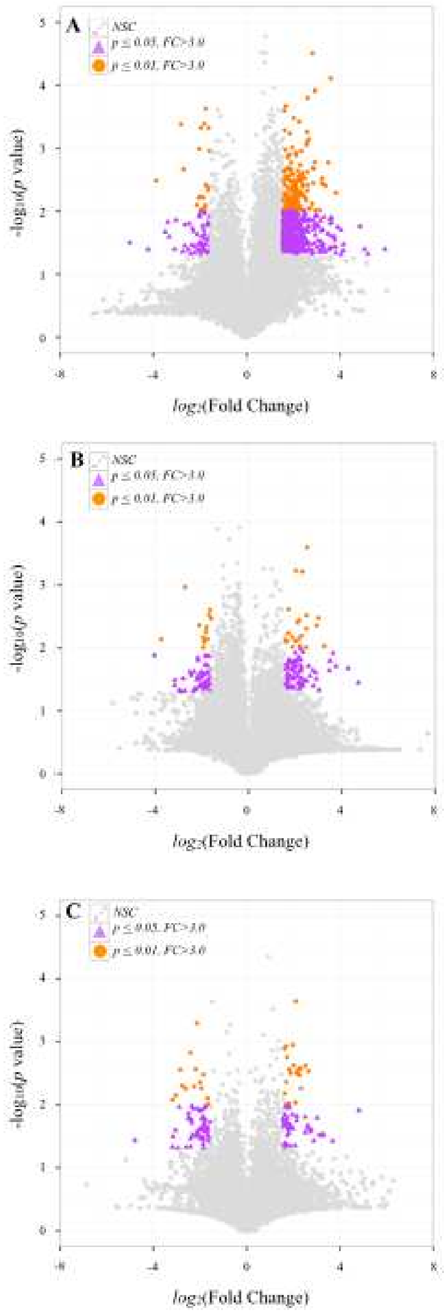

Figure 3.4 Volcano Plots of Detected Features. For each time point comparison; A) 0 dpa, B) 14 dpa, and C) 21 dpa, points represent log$_2$(Fold Change) x -log$_{10}$(p-value) of each feature. The shape of the point indicates the level of significance and the color indicates the group of features that are significant ($p \leq 0.05$, FC ≥ 3.0) and those that are highly significant ($p \leq 0.01$, FC ≥ 3.0). Fold change values were calculated via relative feature abundances of '10_11' compared to 'Karl'.

Metabolites With Significantly Different Abundances in ‘10_11’ and ‘Karl’

To identify the molecular features of interest, a combination of targeted techniques were implemented. Specifically, four strategies were applied: 1) *m/z* and RT matching to a list of standard N-containing compounds; 2) high mass accuracy comparisons of features with a library of known barley metabolites; 3) MS fragmentation analysis was performed using a series of theoretical fragment masses and substructures generated by *in silico* dissociation of hypothetical compound structures; 4) mapping of features to known metabolic reactions and integrating with proteomic data.

In total, there were 19 authentic standards identified by *m/z* and RT (Table 3.1 and Supplementary Figure 3.1, Appendix A). Candidate assignments were made for 405 compounds that were detected within 30 ppm of expected [M+H] molecular weight (Supplementary Table 3.1, Appendix A). These values were queried against a barley mass list (Supplementary Table 3.2, Appendix A). Features that exhibited differential abundance between ‘10_11’ and ‘Karl’ determined by statistical analyses were compared at each time point (Table 3.2). Putative identifications were made for amino acids (3), cofactors (3), amines (2), pyrimidines (2), and others (13, Supplementary Figure 3.3, Appendix A). Candidate identifications were related to barley metabolism by their associated to known reactions (e.g., precursors, products, reactants, and reaction intermediates) that were retrieved from the BarleyCyc, database (http://pmn.plantcyc.org/BARLEY/class-tree?object=Pathways). There were 21 sub-pathways and 10 super-metabolic pathways represented by the group of significantly regulated metabolites. The degree of difference between ‘10_11’and ‘Karl’ was determined by the corresponding ratio of measured relative abundances.

Table 3.1 Identified Standard Metabolites.

Descriptions	**Feature *m/z***			**Retention Time (sec)**		**Feature Significance**			**Fold Change**		
ID	*[M+H]*	*mzmed*	*m/z error*	*Stnd rt*	*rtmed*	*p value 0*	*p value 14*	*p value 21*	*FC 0*	*FC 14*	*FC 21*
beta-Phenylethyl-amine	122.0955	124.62			168.27	0.432			1.79		
L-Isoleucine	132.1007	124.62			150.94	0.623	0.425		1.18	1.89	
Ala-Leu	203.1369	124.62			228.54		0.851	0.347		-1.06	1.47
Cytidine-3',5'-monophosphateCytidine-2',3'-monophosphate	306.0519	127.5			155.91		0.328	0.331		-1.62	-1.52
L-phenylalanine	166.0843	127.56	-0.0028	165.08	130.84	0.339	0.305	0.743	2.67	-2.67	-1.48
xanthosine	285.083	134.58	-0.0043	284.08	179.04	0.545			1.25		
thymine	127.0502	136.56	-0.0024	126.04	153.63	0.004			3.56		
xanthine	153.0407	136.56	-0.0018	152.03	129.96			0.081			-24.32
Thymidine	243.0954	136.56			152.82	0.68			1.41		
adenosine	268.101	144.54	-0.0013	267.1	206.97		0.617			1.3	
L-tryptophan	205.0972	209.28	-0.0018	204.09	230.53	0.02	0.088	0.179	1.79	-1.5	-2.69
L-proline	116.0706	231.12	-0.0012	115.06	312.54	0.109			1.62		
L-Pyroglutamic Acid		239.16			274.88		0.286			1.61	
(-)-sativan	287.1278	239.2	-0.0028	286.12	242.72	0.902	0.462		1.01	-1.2	
Amygdalin	458.1593	316.86			364.03		0.074			1.85	
Sorbic Acid	113.0588	423.48			428.27	0.004	0.056	0.182	1.19	1.49	-1.27

Table 3.1 Continued

2-Hydroxy Benzoic Acid4-Hydroxy Benzoic Acid	139.0375	432.42	481.49	0.395	0.264	0.974	1.43	1.03	-1
Triethyl Phosphate	183.0765	478.26	480.22	0.157			3.11		
Sebacic Acid	203.1252	553.98	561.65	0.249	0.001	0.063	1.63	1.41	-1.5

FC- fold change, 1- identified by MS/MS; MT- matches metlin. X denotes if the feature was detected in > three of six of samples in the comparison. Determination of pathway was retrieved from PlantCyC Hordeum Vulgare L. database (http://pmn.plantcyc.org/BARLEY/substring-search?type=NIL&object=). rt- retention time (seconds), S-sulfur, Stnd-standard.

Abundant metabolites that were found upregulated in ‘10_11’ compared to ‘Karl’ correspond to common plant super-pathway classes (Benton et al. 2014). This group of candidates represented super-pathways that include amino acid biosynthesis (AAB), cofactor, prosthetic group, and electron carriers (CPGEC), nucleoside and nucleotide degradation (NND). There were metabolites found at high levels in ‘10_11’ that contain various percentages of N, namely phosphoribosyl-ATP (17.91% N; 6.0-fold) thymine (22.21%), S-adenosyl-L-methionine (23.85%), and amino acids such as L-isoleucine (10.68%) and L-arogenate (6.89%). Three sulfur (S) containing candidate metabolites had 3.5-fold higher relative abundance in ‘10_11’ flag leaves at different time points (at 0-dpa, S-adenosyl-L-methioninamine; SAM, and at 21-dpa S-methyl-5-thio-D-ribose; MTR and 2-keto-4-methyl-thiobutyrate; KTMB). These three S containing metabolites correspond to intermediates produced by the methionine-salvage pathway or Yang cycle in plants (Figure 3.5) (Miyazaki and Yang 1987). The Yang Cycle is essential for salvaging S from methionine during ethylene biosynthesis and in leaf tissue specifically, it is suggested to play a primary role in recycling of 5-methylthioadenosine (MTA) during polyamine and nicotianamine/phytosiderophore biosynthesis (Miyazaki and Yang 1987; Pommerrenig et al. 2011). The reactions that were associated with upregulated metabolites in '10_11' are known to produce compounds that may be involved in pathogen responses in plants, (Walters 2000, 2003; Gill and Tuteja 2010; Gupta et al. 2013; Pommerrenig et al. 2011) as well as in senescence. The difference in levels of metabolite intermediates measured in ‘10_11’ compared to ‘Karl’ suggests the possibility that the activity of the Yang cycle is increased in ‘10_11’ during senescence.

Table 3.2 Candidate Metabolites Exhibiting Differential Behaviors Between ’10_11’ and ‘Karl’

Name	**m/z error**	**[M+H]**	**RT**	**Ratio 0**	**Ratio 14**	**Ratio 21**	**p 0**	**p 14**	**p 21**	**Hordeum vulgare Subpathway**	**Up/downstream enzyme**
S-adenosyl-L-methioninamine	0.0052	358.1776	358.18	3.45			0.015			spermidine biosyntehsis I	met-adenosyltransferase EC: 2.5.1.6
glycine betaine		118.0852	118.09		-3.2	24.13		0.25	0.139	methionine degradation; Amines and Polyamines Biosynthesis	
phosphoribosyl-ATP	0.0078	708.2926	708.29	6	-1.92	-1.25	0.001	0.168	0.603	histidine biosynthesis	
2-keto-4-methyl-thiobutyrate (KMTB)	0.0044	149.0261	149.03	-1.21	1.5	3.51	0.719	0.349	0.01	cysteine and methionine metabolism; glucosinolate biosynthesis; secondayr metabolite synthesis, Yang Cycle	met-adenosyltransferase EC: 2.5.1.6
L-isoleucine		133.148	132.1	3.85			0.046			Isoleucine Biosynthesis	
L-arogenate	0.0034	228.0861	228.09			3.11			0.041	phenylalanine biosynthesis	
GA8-catabolite	0.0009	363.1427	363.14	-1.81	3.17		0.157	0.033		gibberellin degradation	13(S)-lipoxygenase EC: 1.13.11.12
GA44-catabolite		347.2153	347.18	4.62	2.68		0.007	0.327		gibberellin degradation	13(S)-lipoxygenase EC: 1.13.11.12
Ga51-catabolite		331.1342	331.15	2.49			0.059			gibberellin degradation	13(S)-lipoxygenase EC: 1.13.11.12
thymine	0.0024	127.0502	127.05	3.56			0.004			thymine degredation	
S-methyl-5-thio-D-ribose (MTR)	0.0006	181.0529	181.05			3.48			0.027	S-methyl-5' -thioadenosine degradation I	met-adenosyltransferase EC: 2.5.1.6
p-coumaroyltyramine		284.1616	284.13		-2.07	-2.46		0.15	0.378	hydroxycinnamic acid tyramine amides biosynthesis	
curcumin diglucoside	0.0005	693.0747	693.07	3.49			0.013			curcumin glucoside biosynthesis	

Table 3.2 Continued

jasmonoyl-CoA	0.0224	951.2401	951.24	4.48	-1.28	1.02	0.024	0.382	0.97	jasmonic acid biosynthesis	long chain fatty acid ligase EC: 6.2.1.3
"5,10-methylenetetrahydrofolate"	0.0078	458.1772	458.18	3.31	-1.47	-1.41	0.033	0.161	0.479	folate transformations II; pyrimidine deoxyribonucleosides salvage	
"cyanidin-3,5-diglucoside"	-0.0119	611.2134	611.21	1.5	1.71	3.6	0.576	0.223	0.017	chlorophyllilide a biosynthesis	
protoporphyrin IX	-0.0019	563.2642	563.26	9.09	-2.5	-18.81	0.039	0.05	0.15	heme biosynthesis from glutamate	
Nomega-hydroxy-L-arginine	-0.0145	192.1217	192.12			-3.88			0.012		
3-deoxy-D-manno-octulosonate 8-phosphate	-0.0162	319.0408	319.04		-4.71	9.48		0.045	0.238	CMP-KDO biosynthesis II	
(+)-sesamin		449.1048	355.12	1.09	-1.25	-1.12	0.232	0.027	0.597	sesamin biosynthesis	
16-oxo-palmitate	-0.0184	271.2262	271.23	-3.3	1.01	4.48	0.029	0.991	0.219	cutin, suberine and was biosynthesis	
benzylbenzoate	-0.0007	213.091	213.09	1.41	-2.74	-4.26	0.712	0.275	0.027	volitile benzenoid biosynthesis I	
dUMP	-0.0094	309.0471		-3.49			0.005			gentiodelphin biosynthesis	

FC- fold change, 1- identified by MS/MS; MT- matches metlin. X denotes if the feature was detected in > three of six of samples in the comparison. Determination of pathway was achieved with PlantCyC Hordeum Vulgare L. database, rt- retention time (sec), SM- secondary metabolite biosynthesis. Ratio calculated '10_11' vs 'Karl' where ratio >0 and -('Karl vs '10_11') when ratio <0.

Metabolites that exhibited low levels in ‘10_11’ compared to ‘Karl’ were associated with a dissimilar group of super-metabolic pathways, including secondary metabolite (SMB), carbohydrate, cell structure, and nucleotide/nucleoside (NNB) biosynthesis. The low level candidate metabolites in ‘10_11’ were 16-oxo-palmitate (-3.3-fold; 0 dpa), benzyl benzoate (-4.3-fold; 21 dpa), and 3-deoxy-D-manno-octulosonate 8-phosphate (-4.7-fold, 14dpa). Other low abundant candidate compounds include dUMP (-3.5-fold, 0 dpa) and N-omega-hydroxy-L-arginine (-3.9-fold; 21 dpa).

Integration of Metabolomic and Proteomic Datasets *via* Pathway Analysis

Highly abundant metabolites that were associated to upregulated enzymes (*in preparation;* Mason et al. 2015, Thesis Chapter Two) were integrated using pathway analysis. Enzyme-metabolite interactions that correspond to metabolic pathways in barley were found upregulated including, met-adenosyltransferase in the Yang cycle (Waduwara-Jayabahu et al. 2012), 13(*S*)-lipoxygenase responsible for catalyzing the first step of α-linoleate biosynthesis (Feussner and Wasternack 2002), and long-chain fatty acid (LCFA) ligase involved in jasmonic acid biosynthesis (Pauwels and Goossens 2011) (Figure 3.5). Overlapping results found at the proteomic and metabolomic levels were used to provide a possible explanation for the accumulation of putatively identified metabolites in ’10_11’.

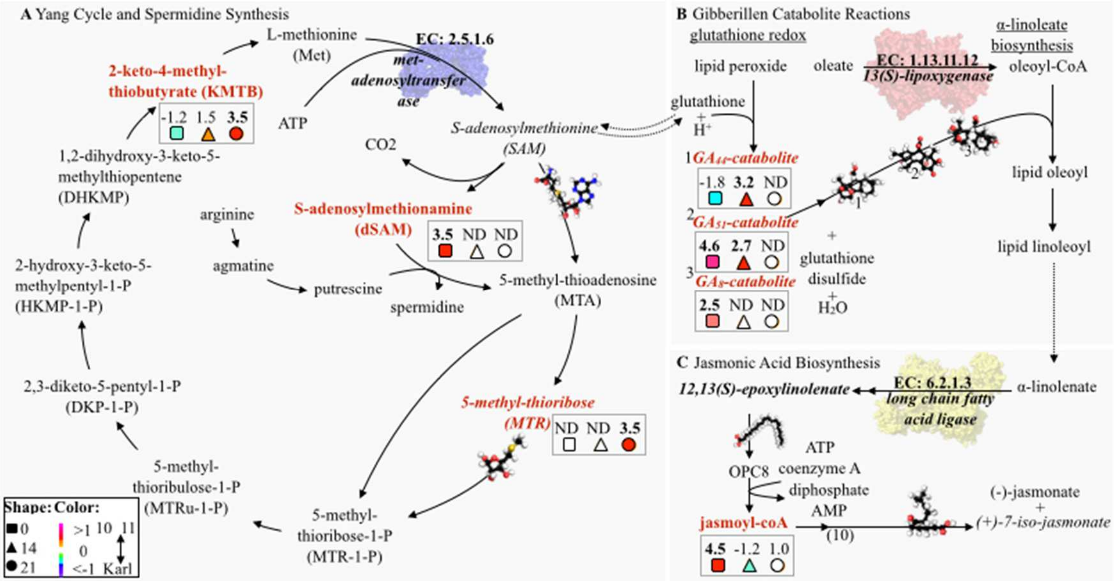

Figure 3.5 Pathway Analysis of Upregulated Metabolites and Proteins. Metabolite and protein metabolic pathway mapping of the Yang cycle, glutathione reduction-oxidation, α-linoleate biosynthesis, and jasmonic acid biosynthesis pathways in *Hordeum vulgare*. Candidate compound identifications that were exhibiting significant fold changes (FC) are displayed in red bold lettering; –fold change values are designated with a unique shape depicting each time point, and a color dependent on degree of fold-change value. Molecular structures and italics indicate cases where MS data was collected to supplement feature identifications (Supplementary Figure 3.3, Appendix A) [FC ratios were calculated as '10_11' *vs.* 'Karl'; color scale- 0 < more in '10_11', 0 > less in '10_11']. Not detected -ND; GA- gibberellins, CoA - Co-enzyme A.

Temporal Analysis of '10_11' and 'Karl' During Senescence

Separate time-course analyses were performed to monitor any differences in the global metabolism between '10_11' and 'Karl' throughout the progression of senescence (Supplementary Figure 3.4, Appendix A). Relative intensities were normalized for each feature across corresponding time points in order to generate slope values for comparison. The resulting slopes established the degree and direction of each molecular feature trend overtime. Features that were only found at a single time point were omitted from this analysis. A total of 8,961 trends were calculated for ‘10_11’ and ‘Karl’ (Figure 3.6 A,B). conservation of a distinct pattern was observed between the two barley lines. Each feature analyzed in both ‘10_11’ and ‘Karl’ was found to follow one of four general trends. There were subtle differences found between ‘10_11’ and ‘Karl’ in the overall degree and number of features exhibiting each general trend. Specifically, there were a larger group of negative sloping trends (decreasing relative abundance) observed in '10_11' compared to 'Karl' and a smaller group of positive sloping trends that had positive sloping trends (increasing relative abundance). These results revealed a faster decrease feature intensities detected in '10_11' compared to 'Karl' and subsequently, that '10_11' remobilized metabolites faster than 'Karl'. Additionally, these results also provided a possible explanation for the ability of '10_11' to experience accelerated senescence. Overall, feature trends indicated that on a global scale, metabolic fingerprints were shared between ‘10_11’ and ‘Karl’, with exception of subtle differences. In consequence, the established differences in N-metabolism and senescence onset and timing between ‘10_11’ and ‘Karl’ may occur on a more intimate scale (i.e., individual metabolites or pathways).

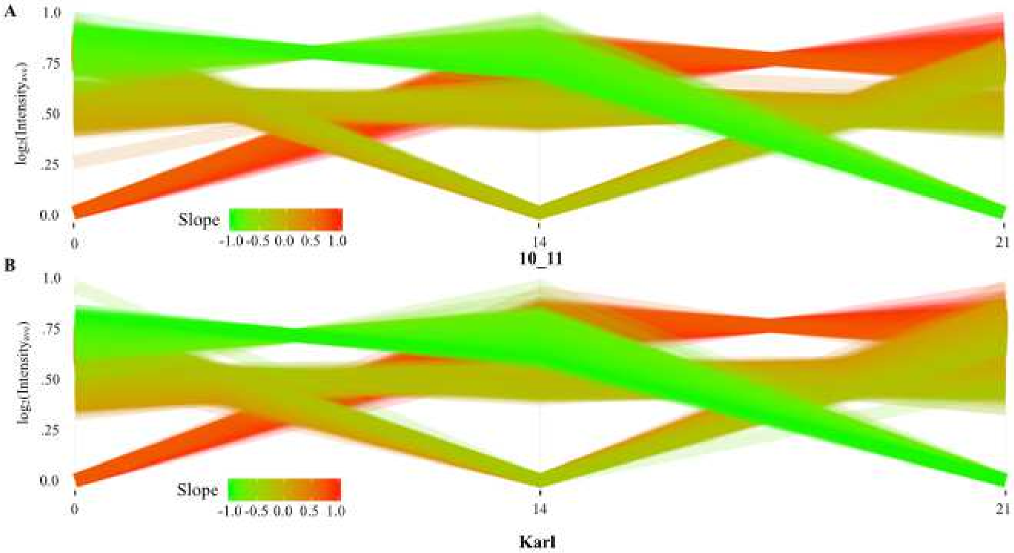

Figure 3.6 Global Feature Trends. Slope trends were calculated for features that were detected at two or three time points. Trends from A)'10_11' and B) 'Karl' are plotted with time points (0, 14, or 21 dpa) designated on the x-axis and the corresponding normalized intensity values on the y-axis. The slope of each line is indicated by its color (decreasing slopes; yellow-green and increasing slopes; yellow-red).

Discussion

During senescence in plants, cellular components that make up photosynthetic tissues (leaves and shoots) are transformed by enzymatic reactions to produce soluble metabolites that are exported to surviving structures (Thomas 2013; Humbeck 2013). Defining the physiological features that influence senescence in small-grain cereals such as barley is particularly important because of its intimate connection to nutrient metabolism (50-90% of grain protein in cereals stems from remobilization from vegetative/photosynthetic tissues) (Gregersen, Holm, and Krupinska 2008; Wu et al. 2012). To examine this process in barley, the flag leaf metabolomes of barley germplasm differing in senescence behavior were compared at different times during senescence (0, 14 and 21 dpa). Standardized non-targeted LC-MS metabolic profiling, followed by statistical and metabolic pathway analysis, were used to probe the differences in the chemical composition of flag leaves between '10_11' and 'Karl'.

A comparison of each time point focusing on statistically significant differences revealed that the largest differences between ‘10_11’ and ‘Karl’ occurred before initiation of senescence and at anthesis; 0 dpa. This was based on the number of features that differed at the first time point (Figure 3.2, Fold change ≥ 3.0; p-value ≤ 0.01) compared to the others. Increasing similarity during the later stages of senescence suggests that molecular mechanisms are conserved, despite phenotypic differences in the ability to employ the massive scale breakdown and remobilization of nutrients. The convergence exhibited between ‘10_11’ and ‘Karl’ in this study corroborated the conclusions from a large study that compared 27 senescence-promoting conditions in *Arabidopsis* (e.g., nutrient deprivation, drought, salinity, age) and found that gene expression profiles differed significantly at early stages, but shared considerable similarities at late stages of developmental leaf senescence (Guo and Gan 2012). Together, these results suggest that the processes occurring prior to visible senescence have a large impact on the execution of the process.

Characterizing molecular features that have significantly higher levels in flag leaves of '10_11' compared to 'Karl' at anthesis and during senescence can provide the opportunity for targeted identification and classification of metabolites that play a role in senescence processes in ‘10_11’. The lack of biologically relevant standards with available MS data, expensive or commercially unavailable authentic standards (e.g., hormone catabolites), and ambiguous MS patterns make it difficult to resolve the molecular identities of many molecular features of interest (Benton et al. 2014; Creek et al. 2014; Zhou et al. 2014). However, putative identifications were made for several features by matching experimental *m/z* (within 30 ppm) with a list of candidate compounds retrieved from the BarleyCyc metabolic pathway database (Zhang et al. 2005). Using the resulting assignments, metabolic pathway analysis was performed and integrated with proteomic data. The observation of up- or downstream enzymes in combination with the observation of accumulated metabolites associated with a specific metabolic pathway can provide additional evidence that the pathway is active during a particular developmental phase. Furthermore, the observation of high levels of enzymes and corresponding metabolite intermediates specifically in senescent tissues can highlight important pathways active during this last phase of plant development (Benton et al. 2014; Mochida and Shinozaki 2011; Leonelli et al. 2013).

Several putative identifications of compounds from the Yang cycle were more abundant in '10_11' than in 'Karl' at each time point analyzed, along with other overlapping pathways, namely α-linoleate biosynthesis, glutathione reduction/oxidation, gibberellin (GA) catabolism, and jasmonic acid (JA) biosynthesis (Figure 3.5) (Dubousset et al. 2009; Pommerrenig et al. 2011). It has been established that the Yang cycle in plants is essential for sulfur recycling, detoxification of 5'-methylthioadenosine (MTA) produced during ethylene biosynthesis, and nicotianamine/polyamine biosynthesis (Burstenbinder et al. 2007; Pommerrenig et al. 2011; Waduwara-Jayabahu et al. 2012). *S*-adenosylmethionine (SAM) is transformed from methionine in the first reaction of the Yang cycle (Pommerrenig et al. 2011; Roje 2006; Sauter et al. 2013). At this point in barley metabolism, SAM can participate as a precursor for ethylene biosynthesis, be converted into MTA (third reaction) and continue through the Yang cycle, or be transformed into S-adenosylmethioamine (dSAM) to serve as a precursor for polyamine biosynthesis. Ethylene, is an important phytohormone that orchestrates many aspects of plant growth and development including defense against pathogens and senescence (Theologis 1992; Glick et al. 1997; Bleecker and Kende 2000; Pandey et al. 2000). A product of the Yang cycle; nicotianamine, is a non-protein amino acid that protects plants from the formation of reactive oxygen species (ROS) by chelating to free metal ions (Waduwara-Jayabahu et al. 2012; Weber et al. 2004; Gratão et al. 2005). Finally, polyamines (putrescine, spermine and spermidine) are small, transportable aliphatic amines that are involved in mitigating the adverse effects of abiotic stress in plants by their antioxidant properties (Pommerrenig et al. 2011; Wi, Kim, and Park 2006; Walters 2000, 2003; Bouchereau et al. 1999; Del Duca, Serafini-Fracassini, and Cai 2014; Pandey et al. 2000). The size, N composition and soluble nature of polyamines are optimal characteristics for metabolites that can be utilized for nutrient transport during senescence. Moreover, the nutrient rich (N and S) products of the Yang cycle may provide a means for '10_11' to transport increased nutrients into its developing grains during senescence.

The identification of significantly changing hormone metabolites between '10_11' and 'Karl' include those involved in ethylene, jasmonic acid synthesis, and GA degradation. This observation indicates that there is an importance of hormone signaling throughout senescence in '10_11'. There were three GA catabolites that were significantly more abundant in ‘10_11’ compared to ‘Karl’ (GA_{44}, GA_{51}, and GA_{25}), suggesting increased degradation of GA in '10_11'. The ability of '10_11' to degrade GA during senescence may serve as an important regulatory mechanism, as gibberellins have previously been associated with senescence-delaying effects (Fischer 2012). The oxidative state of cells can be influenced by GA metabolism as revealed by the regulation of transcripts that encode for ROS-scavenging enzymes by GA reactions (Achard et al. 2008; Tognetti, MÜHlenbock, and Van Breusegem 2012). GA catabolites are aliphatic alcohols that can be consumed by glutathione oxidation/reduction (redox) reactions (Fletcher and Osborne 1965; Goldthwaite and Laetsch 1968). Reduction of oxidized glutathione is essential for maintaining the homeostasis of cells experiencing oxidative stress (Foyer and Shigeoka 2011). The production of ROS can be increased through aging, photosynthesis, stress responses, and senescence in plants (Buchanan and Balmer 2005; Gechev et al. 2006; Mhamdi et al. 2010; Sedigheh et al. ; Zimmermann and Zentgraf). Glutathione redox and the Yang cycle reactions are both modulated by sulfur containing compounds; this may serve as an interconnection between the two pathways (BarleyCyc metabolic pathway database, (Zhang et al. 2005). α-linoleate biosynthesis consumes GA catabolites and also provides the precursor (α-linoleate) for JA biosynthesis (BarleyCyc metabolic pathway database, (Zhang et al. 2005). Other precursors to the hormone JA were more abundant in '10_11' compared to 'Karl' in the earlier time points that were analyzed (0 and 14 dpa). JA is a senescence-promoting hormone that is associated with plant stress (biotic) responses (Fischer 2012; Guo and

Gan 2012). Together these results putatively define metabolic pathways found upregulated in ‘10_11’ flag leaves during senescence and highlight possible mechanisms associated with hormone metabolism and cellular redox that influence its senescence behavior.

Global feature intensity trends between ‘10_11’ and ‘Karl’ revealed by temporal analysis showed a distinct pattern that was shared by both lines (Figure 3.6). Convergence shown by the global metabolic profiles of ‘10_11’ and ‘Karl’ throughout senescence suggests that the two largely share common metabolic pathways. The surprising similarity of these results indicated that the difference in the senescence behavior of ‘10_11’ and ‘Karl’ is influenced by more intricate mechanisms. Therefore, the appearance of subtle differences in ‘10_11’ compared to ‘Karl’ may influence its early onset phenotype, while largely the same metabolic processes are employed in ‘10_11’ and ‘Karl’.

These results provide evidence of the importance of the Yang cycle and several other pathways for senescence in '10_11' flag leaves. Underlying trends in function were revealed by the upregulated pathways in '10_11' including, management of cell redox homeostasis, N and hormone metabolism. In order to establish the true function of the Yang cycle and other pathways that were identified by this study in senescence and nutrient metabolism, further validation of metabolite identifications using authentic standards is necessary. Overall, information gathered by this analysis provide a context for the biochemical differences that are exhibited by ‘10_11’ and ‘Karl’ during senescence and highlights potential pathways that can be used for future improvement of barley or other related species.

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CONCLUSION TO DISSERTATION

Age- and environmental stress-induced senescence are intimately linked to nutrient metabolism in plants and can often limit yield and nutrition in commercially produced crops (Munné-Bosch and Alegre 2004; Wingler and Roitsch 2008). An understanding of the molecular processes that plants undergo during senescence is essential for crop improvement efforts (Gregersen 2011; Edwards, Humphry, and Sanchez-Tamburrino 2012; Thomas et al. 2009). Regulatory and execution factors that are responsible for nutrient recycling from senescing plant organs may offer ways of manipulating senescence for agricultural applications (Alegre 2004; Powell et al. 2012; Distelfeld, Avni, and Fischer 2014; Munné-Bosch and Alegre 2013). Onset and progression of these processes depend on numerous interactions of proteins with various substrates (nucleic acids, proteins, carbohydrates, etc.) (Quirino et al. 2000a; Guo and Gan 2005; Noodén, Guiamét, and John 1997; Zhou and Gan 2010; Humbeck 2013). Using barley reconstructed metabolic network information (available at BarleyCyc; http://pmn.plantcyc.org/BARLEY/class-tree?object=Pathways) as a platform, high-throughput proteomic and metabolomic analyses were implemented to shed light on these processes in an important cereal crop, barley (*Hordeum vulgare* L.) (Mrízová et al. 2014b). Transcriptomics, proteomics, and metabolomics are tools that can provide insight into conditional changes in an organism by highlighting hierarchical regulation of enzyme activity and metabolism (Yizhak et al. 2010; Feist and Palsson 2008). Proteomic and metabolomic comparisons of near-isogenic barley germplasm with different senescence phenotypes were performed in this study. Specifically, the extensively characterized barley variety ‘Karl’, which has an established slower-senescing/lower grain protein content phenotype was compared with a near-isogenic line, ‘10_11’, that exhibits faster senescence and higher grain protein content (Heidlebaugh 2007; Heidlebaugh et al. 2008; Jukanti and Fischer 2008; Jukanti et al. 2008b). A system-wide analysis of barley senescence can be achieved by integrating genomic (Mayer et al. 2012) and transcriptomic data (Jukanti et al. 2008b) with newly gathered proteomic and metabolomic analyses. These data serve to enhance our understanding of senescence in barley and other economically important crop species.

Highly upregulated proteins found in ‘10_11’ during senescence likely serve functions in senescence regulation, signaling and nutrient remobilization. Conspicuously, proteins with obvious or putative functions in plant pathogen defense constituted the largest group of proteins upregulated in ‘10_11’. These proteins included membrane receptors (which may be involved in the detection of conserved microbial patterns), a cyclic nucleotide-gated ion channel, glucanases, pathogenesis-related and disease resistance proteins. Upregulation of defense-related genes during senescence has previously been found (Quirino et al. 2000a), and may indicate enhanced disease resistance in line ‘10_11, particularly during senescence. Some of the upregulated proteins may also be involved in crosstalk between senescence regulation and plant pathogen defense pathways; such proteins could be particularly valuable targets for crop improvement efforts. In addition to defense-related proteins, enzymes and proteins involved in protein degradation (a family S8 serine protease, a tripeptidyl peptidase and components of the ubiquitin-proteasome system) and in the metabolism of organic and amino acids (glutamine synthetase, citrate synthase) were upregulated in line ‘10_11’. This finding suggests enhanced protein degradation and nitrogen metabolism, confirming earlier biochemical and transcriptomic analyses of the utilized germplasm (Heidlebaugh et al. 2008; Jukanti and Fischer 2008; Jukanti et al. 2008a).

Metabolic profiles from ‘10_11’ and ‘Karl’ flag leaves were examined prior to, and during senescence by non-targeted mass spectrometric techniques (Chapter Three). Comparisons of metabolite levels between ’10_11' and ’Karl' revealed differences between early- and late-senescing germplasm. The highest number of significantly regulated features between ’10_11’ and ‘Karl’ was observed at anthesis (flowering) and significant differences decreased as senescence progressed. Putative compounds that were more abundant in flag leaves of line ‘10_11’ as compared to ‘Karl’ were associated with polyamine biosynthesis, gibberellin catabolism, methionine salvaging via the Yang cycle, and jasmonic acid biosynthesis, pointing to the importance of phytohormones in controlling the early-senescence phenotype of line ‘10_11’. The temporal trends that were exhibited by metabolite features were compared separately for ’10_11’ and ‘Karl’ throughout senescence. Globally the metabolic profiles of the two barley lines appeared very similar, as indicated by the distinct patterns followed by clusters of metabolites in both early and late-senescing germplasm. Overall conservation of metabolism between

‘Karl’ and ‘10_11’ indicates that the relatively few differentially regulated metabolites may have important roles in plant senescence and or pathogen defense.

Identification of enzymes and metabolites upregulated in line ‘10_11’ and belonging to the same metabolic pathways were made. Identified pathways included: methionine salvaging via the Yang cycle (methionine-adenosyltransferase; EC: 2.5.1.6) (Murr and Yang 1975; Sauter et al. 2013; Rausch and Wachter 2005; Kushad, Richardson, and Ferro 1983; Baur and Yang 1972), α-linoleate biosynthesis (long-chain-fatty-acid-CoA ligase; EC: 6.2.1.3) (Schmidt and Heinz 1990) and jasmonic acid biosynthesis (13-(*S*)-lipoxygenase; EC: 1.13.11.12) (Zimmerman and Vick 1970; Theorell, Holman, and Akeson 1947; Royo et al. 1996; Christopher, Pistorius, and Axelrod 1970; Bachmann et al. 2002), as each was accompanied with a significantly regulated enzyme. These results provided insight into differences in metabolic regulation between ’10_11’ and ‘Karl’ during senescence, confirming and expanding previous research indicating important roles of ethylene and jasmonates in senescence initiation and regulation (Sauter et al. 2013; Pommerrenig et al. 2011; Chou and Kao 1992; Gottwald et al. 2012; Parthier 1990). Further investigation of proteins, metabolites, and metabolic pathways that were differentially regulated between ’10_11’ and ‘Karl’ is essential to establish their role during senescence. The principal aims of these studies will be (1) to establish the molecular identities of putative metabolites of interest using commercially available authentic standards and MS patterns, (2) to compare the abiotic and biotic stress tolerance of ’10_11’ and ‘Karl’, and (3) to obtain a systems-level understanding of how modification of specific genes regulates preferred traits in commercially important crops. Such studies will also elucidate mechanisms of senescence regulation and nutrient metabolism in barley. Targeting and modifying such mechanisms can provide further insight about their specific functions during senescence.

In summary, this study provides essential new information characterizing the barley senescence process and its interaction with plant stress responses. Proteins identified in Chapter Two may be important novel targets whose manipulation can be used for ongoing crop improvement efforts aimed at enhancing barley quality and stress resistance. Metabolic pathways identified in Chapter Three can also serve as a platform for barley improvement and understanding of the senescence process. Taken together, integrating systems biology approaches can help to shed light on important processes of senescence in the important crop barley, and additionally, can offer guidance for applying these useful techniques in other crops.

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APPENDIX A

SUPPLEMENTAL INFORMATION

The supplemental information referenced in chapter two and three is available on DVD upon request. To request DVD copies contact your local public or university library to place an interlibrary loan request to Montana State University. For questions please call 406-994-3161.