REGULATION OF NAC TRANSCRIPTION FACTOR GENE EXPRESSION IN CAMELINA SATIVA LEAVES DURING EARLY SEED FILLING: INFLUENCE OF NITROGEN NUTRITION AND LEAF AGE

by

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ABSTRACT

Camelina [*Camelina sativa* (L.) Crantz] is an oilseed crop primarily used for biofuels and industrial lubricants. Nitrogen is the most quantitatively important plant mineral nutrient, with photosynthetic machinery representing the major nitrogen sink in leaf mesophyll cells. While camelina is less nitrogen intensive than other field crops, low nitrogen deficiency slows growth and accelerates senescence of older leaves, reducing plant biomass and yield. Increasing yield under reduced nitrogen inputs constitutes an important goal for modern agriculture, justifying research aimed at understanding physiological processes limiting nitrogen use efficiency. While numerous regulatory, metabolic, and transport processes influence plant nitrogen use, research presented here is focused on the role of NAC transcription factors in plant responses to nitrogen deficiency.

Camelina (var. 'Suneson') plants were grown under two nitrogen regimes termed 'low' (LN) and 'high' (HN). To identify genes and physiological processes governing plant responses to LN, a group of main stem leaves was analyzed during early seed filling using biochemical and transcriptomic approaches. Differential gene expression and gene enrichment analysis between treatments (LN vs. HN), and between mature-green and senescing leaves, identified numerous processes associated with photosynthesis (downregulated under LN, and with advancing senescence), autophagy, lipid, pigment, and nitrogen metabolism (upregulated with advancing senescence), characterizing leaf physiological state with respect to treatment and age. Based on both RNA-Seq and RT-qPCR, camelina orthologs of two well-known *Arabidopsis* transcription factors, NAC029/AtNAP and NAC092/ORESARA1, exhibited significant upregulation both under LN and with advancing leaf senescence. Overlaying camelina *NAC* gene expression data on a phylogenetic tree of *Arabidopsis* NAC proteins identified several clusters containing camelina *NAC* genes exhibiting higher expression under LN than HN on day 0, suggesting a role in the plant's response to LN.

These data suggest two possibilities: senescence was already more advanced in day 0 leaves from LN-grown plants, despite the *SAG12* expression data; or there is substantial functional overlap between NAC transcription factors involved in nitrogen deficiency and those regulating leaf senescence. A combination of biochemical approaches and genetic studies will be required to test these interpretations, which are not mutually exclusive.

CHAPTER ONE

LITERATURE REVIEW

Introduction

Camelina sativa is a reemerging biofuel crop from the Camelineae tribe in the Brassicaceae family. This species has an allohexaploid genome (n = 20) derived from hybridization between an auto-allotetraploid *Camelina neglecta*-like genome and *Camelina hispida*, resulting in three relatively stable sub-genomes (Brock et al., 2018). Consequently, up to three copies of each gene may be present where homeologs may have the same or different functions. Furthermore, having multiple copies of the same gene acting simultaneously may lead to a higher dosage of expression and consequently lead to different phenotypical traits (Mandáková & Lysak, 2022). In *C. sativa*, knockout mutants of a fatty acid desaturase *CsFAD2* adopt different traits depending on if one or all of the homeologs for this gene have been knocked out; knocking out all three genes resulted in lower seed quantity with higher amounts of monounsaturated fatty acids compared to the wild type whereas keeping a single copy resulted in higher seed quantity with similar levels of monounsaturated fatty acids (Miklaszewska et al., 2021). Homeolog copies of a gene may also act redundantly with each other or may adopt a new function (Aznar-Moreno & Durrett, 2017). *C. sativa* is native to the temperate dryland climates of Eastern Europe and is suitable for similar climates in North America, such as the northern Rockies and Plains. *C. sativa* is also closely related to *Arabidopsis thaliana* which is helpful in identifying important regulatory genes and pathways already established in the model species (Ghidoli et al., 2023).

Senescence Influences Oilseed Traits

While *C. sativa* has historically been used as a source of lamp fuels and food oil, it has recently emerged as an important candidate for biofuels, specifically for aviation (Ghidoli et al., 2023). Similar oilseed crops such as *Brassica napus* have relatively high amounts of triacylglycerols (TAGs) which are the most valuable source of biofuel in seeds (Miklaszewska et al., 2021). The importance of TAGs in oilseed production necessitates taking multiple approaches to understand and modify physiological processes during seed development. Senescence is a relevant developmental process because of its relationship with seed filling (Borrill et al., 2015).

Seed composition and yield reflect the onset and duration of senescence as there exists a tradeoff between nutrient remobilization and oil accumulation by the seed (Uauy et al., 2006) (Govindasamy et al., 2023). Earlier initiation of senescence favors seeds with higher protein content because earlier catabolism of leaf proteins (particularly chloroplast proteins) results in enhanced mobilization of nutrients to sink organs such as seeds (Kichey et al., 2006). However, because chloroplast proteins are among the first proteins to be degraded for nutrient remobilization, photosynthetic activity diminishes resulting in a smaller window of photosynthesis and carbon fixation (Izumi et al., 2017). The tradeoff between nutrient remobilization and carbon fixation in seed development exists partially because of how the carbon to nitrogen ratio can determine pathways and developmental processes. A study on xylem-phloem transfer of amino acids by (Zhang et al., 2010) found that mutants for *AAP2* (Amino Acid Permease 2) in *Arabidopsis* had increased levels of nitrogen in leaves but decreased levels of amino acids in seeds. This allowed for greater nitrogen assimilation and protein synthesis in leaves leading to higher amounts of Rubisco and photosynthetic activity while also favoring carbon assimilation and fatty acid synthesis in the seeds. Seeds of this mutant *Arabidopsis* line had increased amounts of fatty acids but decreased amounts of nucleic acids.

Nitrogen Use Efficiency

The onset and duration of senescence is dependent on internal and external factors including abiotic stressors such as nutrient deficiency (Bieker & Zentgraf, 2013). Different proteases and senescence associated pathways are initiated by different environmental cues. For example, the cysteine protease SAG12 is responsive to the natural progression of senescence rather than a specific abiotic or biotic stressor (Carrión et al., 2014). Nitrogen deficiency is an abiotic stressor of particular interest for multiple reasons. Nitrogen is one of the most important plant macronutrients, being a building block of amino acids, proteins, nucleic acids, and chlorophylls necessary for photosynthesis (Hajibarat & Saidi, 2022). However, despite its importance for plant growth and development, nitrogen available in the soil is never absorbed in its full capacity. In mesophyll cells of C_3 plants such as *C. sativa*, approximately 80% of N is utilized in Rubisco and light harvesting complexes, demonstrating an important role for maintaining photosynthesis (Izumi et al., 2010). Plants in N-deficient conditions exhibit early senescence and reduced vigor compared to plants grown with adequate N (A. N. Olsen et al., 2005). Because of its importance in maintaining plant growth and productivity, nitrogen is ubiquitously applied to crops to maximize yield.

Unfortunately, the use of nitrogen fertilizers has detrimental effects for the environment. Producing nitrogen fertilizers is very energy consuming and their application can lead to global and local pollution (Adviento-Borbe et al., 2013). The use of nitrogen fertilizers generates greenhouse gases and can contaminate local ecosystems from nitrogen runoff (Lee et al., 2021).

This phenomenon is even more detrimental when large amounts of nitrogen are supplied to a plant species documented as having efficient nitrogen uptake but poor nitrogen remobilization such as *Brassica napus* (Rossato et al., 2001) (Avice & Etienne, 2014). Furthermore, the onset and progression of senescence in response to nitrogen deficiency has been observed to be different compared to plants that receive adequate amounts of nitrogen. (Bieker et al., 2012) investigated how low supply of nitrogen may cause a disruption in the metabolic cascade leading to the onset of senescence. They found that indicators of senescence such as system wide allocation of seed storage proteins and reactive oxygen species (ROS) such as hydrogen peroxide were determined based on nitrogen availability. That study asserted that N deficiency could have a negative effect on nitrogen remobilization even if the onset of senescence occurs earlier for N deficient plants.

NAC Family Transcription Factors

The chronology of nutrient remobilization can be delineated with transcriptomic data sets that detail the time courses of *Senescence-Associated Genes* (*SAGs*). Gene expression analysis comparing leaves from different canopy levels and harvested at sequential time points after the onset of flowering can reveal how and when treatments such as nitrogen fertilization control the senescence process. Using this approach, the study presented here focuses on the induction of *NAC* genes. NAC transcription factors are encoded by a plant specific gene family involved in regulating developmental processes including senescence (Ooka et al., 2003b). NAC transcription factors are a diverse group; they form homo- or heterodimers that interface at the largely conserved N-terminal NAC domain (A. N. Olsen et al., 2005). The NAC domain is also responsible for binding target genes, while the C-terminal domain is variable and confers transcriptional regulation specific to each transcription factor. The C-terminal domain also exhibits intrinsic disorder which allows the protein to bind to other proteins for posttranslational regulation (A. N. Olsen et al., 2005). Based on phylogenetic relationships established using *Arabidopsis* NAC domain protein sequences, the NAC family has distinct subgroups (Jensen et al., 2010a). Subgroup III-3 is the best-characterized subset responsible for abiotic stress responses (Ooka et al., 2003b). However, other subgroups also confer abiotic stress responses, such as subgroups II-3, III-2, and V-1 which contain ORE1/ORS1, AtNAP, and JUNGBRUNNEN1 (JUB1), respectively (Addie N. Olsen et al., 2005). The first two are known positive regulators of senescence whereas JUB1 is a negative regulator (Stender et al., 2015a; Wu et al., 2012).

NAC transcription factors are regulated transcriptionally, post-transcriptionally, and post-translationally (A. N. Olsen et al., 2005). VND-INTERACTING2 (VNI2) is a NAC transcription factor involved in xylem vessel formation but has been recently discovered to bind to and inhibit ACTIVATION FACTOR 2 (ATAF2), a NAC transcription factor that upregulates *SAGs* (Nagahage et al., 2023). *ORE1/NAC092* has been documented to bind to and antagonize GLK1 and GLK2, which are transcription factors involved in chloroplast maintenance (Rauf et al., 2013a). Post-transcriptionally, miR164 targets transcripts of *ORE1* for degradation (Kim et al., 2009). However, NAC activity may be induced by different abiotic stressors.

ORE1 is the best documented positive regulator of senescence that is sensitive to nitrogen deficiency (Park et al., 2018b; Park et al., 2019). In *Arabidopsis thaliana*, nitrogen deficiency upregulates *ORE1* transcription (Park et al., 2018a). However, there are several genes that further regulate ORE1 at a post-translational level that are also sensitive to nitrogen deficiency.

NITROGEN LIMITATION ADAPTATION (NLA; an E3 ubiquitin ligase) and PHO2 (UBC24, an E2 conjugase) polyubiquitinate ORE1 during nitrogen starvation (Park et al., 2018b). NLA and UBC24 are antagonized by UBP12 and UBP13 which de-ubiquitinate ORE1 and confer stability also during nitrogen starvation (Park et al., 2019). The interactions between NLA/UBC24 and UBP12/UBP13 during nitrogen starvation demonstrate one mechanism of post-translational fine tuning. Another documented post-translational regulator of ORE1 during nitrogen deficiency is the mediator complex MED19a (Cheng et al., 2022). This protein exhibits liquid-liquid phase separation (LLPS) during nitrogen deficiency resulting in nuclear condensates that bind with ORE1 to enhance RNA polymerase II affinity (Cheng et al., 2022).

CHAPTER TWO

OBJECTIVES

Despite progress described in the previous section, the gene regulatory networks that determine the progression of senescence are still incompletely understood, especially in camelina. Abiotic stresses such as N deficiency can trigger either tolerance or avoidance responses, providing new possibilities of selecting for germplasm with improved nitrogen use efficiency. Furthermore, there is evidence that varying levels of nitrogen fertilization lead to different developmental profiles. This project aims to understand how *NAC* transcription factor gene expression is regulated in camelina leaves during early seed development, with a focus on the influence of nitrogen nutrition and leaf age. To achieve this goal, plants were grown under both low nitrogen (LN) and high nitrogen (HN) conditions, and senescence progression under both treatments was quantified using biochemical parameters in preparation for global gene expression profiling (RNA-Seq). Understanding the role of *NAC* genes in nitrogen deficiency-caused leaf senescence can identify novel targets for crop improvement, enabling the development of more nitrogen use efficient germplasm which maximizes leaf duration, carbon assimilation, and oil yield under limiting N availability.

CHAPTER THREE

MATERIALS AND METHODS

Plant Materials and Growing Conditions

The *Camelina sativa* variety ‘Suneson’ (Cs01) was grown in soil medium consisting of equal parts of loam soil, washed concrete sand, and sphagnum peat moss, containing ~28 ppm N from NO_3^- and NH_4^+. Three individual plants were grown in a triangular formation per eight-inch pot in a controlled growth chamber of the Plant Growth Center at Montana State University (Bozeman, MT, USA) between October and December 2024. Light was provided with Philips GreenPower LED Toplighting Module DR/W/LB lighting set to 16 hours of light per 24-hour cycle; light intensity (PAR) at leaf level was 300-500 µmol m^{-2} s^{-1}. The growth chamber temperature was set to 24 °C at 50-70 % relative humidity. Plants were watered twice per week and given one of two fertilizer treatments twice per week starting two weeks after germination.

Pots were placed in a randomized block design with four rows of 20 pots generated using the knitr package on R Studio (Xie, 2014). Two nitrogen fertilizer regimes were applied by modifying Hoagland’s solution. High nitrogen (HN) fertilizer contained 6.5 mM N whereas low nitrogen (LN) contained no nitrogen. Each pot received 250 mL of fertilizer solution per treatment (supplemental table 1).

Leaf Sampling

The experiment started one week after at least 90% of plants had opened one flower, to capture leaf senescence occurring during early seed development. Leaves from two canopy levels of the plant’s main stem were collected at the same time at each extraction date. The lower level included leaves 12-15, and the higher level included leaves 16-21, with leaf 1 representing the first true leaf. Three individual plants were randomly selected for a biological replicate, where leaves from all three plants were pooled together. Five biological replicates (15 plants) were harvested on each sampling date. Sampling occurred every three days at noon. Leaves were placed into 15 mL Falcon tubes, immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

Chlorophyll and Protein Analysis

Leaves were ground to a fine powder under liquid nitrogen, using a mortar and pestle. Chlorophylls were extracted with 80% acetone from liquid nitrogen powder and assayed spectrophotometrically according to (Strain et al., 1971). For protein extraction, leaves were homogenized using a chilled mortar and pestle in cold extraction buffer (25 mM Tris (pH 6.8), 1% polyvinylpolypyrrolidone, and 0.1% β-mercaptoethanol) at a ratio of 200 mg leaves per 800 μL buffer. The homogenate was centrifuged at 20,000 x g for 10 min at 4 °C. Aliquots of supernatant were boiled in Laemmli sample buffer (Laemmli, 1970). Soluble proteins were then separated on ExpressPlus^{TM} 4-12% acrylamide gels (GenScript Inc.; Piscataway, NJ, USA) in Tris-MOPS SDS-PAGE Running Buffer (GenScript). Following SDS-PAGE, proteins were incubated in staining solution (0.25% [w/v] Coomassie Blue in 30% [v/v] methanol / 10% [v/v] acetic acid), followed by destaining in the same solution without Coomassie Blue.

Protein concentrations were determined using Bradford reagent (Thermo Scientific, Rockford, IL, USA) where bovine serum albumin was used as a standard.

SAG12 Immunoblotting

Following SDS-PAGE, separated proteins were transferred to nitrocellulose membrane (BioTrace^{TM} NT nitrocellulose transfer membrane, Cytiva Life Sciences, Marlborough, MA, USA). The blots were blocked overnight at 4 °C in 20 mM Tris-buffered saline (150 mM NaCl), pH 7.4, containing 0.1% Tween-20 (TBST) and 5% (w/v) non-fat dry milk. Blots were then probed with SAG12 primary antibodies (Agrisera AB, Vännäs, Sweden) in a 1:2000 dilution. After 1 h incubation with the SAG12 antibodies, the blots were washed 3 x 10 min in TBST, then incubated for 1 h with HRP-conjugated secondary (goat anti-rabbit) antibody, diluted 1:50,000 before use (Abcam Inc., Waltham, MA, USA), and washed again 3 x 10 min in TBST. The blots were developed using SuperSignal^{TM} West Femto PLUS chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA). Images were obtained using GeneTools by Syngene on a GeneSys imaging platform (Syngene, Frederick, MD, USA).

RNA Extraction

RNA was extracted using the RNeasy Plant Mini Kit (Qiagen; Hilden, Germany) and treated with RNase-free on column DNase (Qiagen). RNA concentrations and RNA integrity numbers were obtained using a Qubit 4 Fluorometer (Thermo Fisher Scientific; Waltham, MA, USA), RNA Broad Range assay kit (Invitrogen, Waltham, MA, USA), and an Agilent 2100 Bioanalyzer RNA nano kit (Agilent, Santa Clara, CA, USA), respectively.

Quantitative real-time RT-PCR

Primers were designed using camelina sequence information from EnsemblPlants (https://plants.ensembl.org/Camelina_sativa/Info/Index), based on published camelina genome information (Kagale et al., 2014). Differentiating between homeologs was performed using multiple sequence alignments in Clustal Omega (Madeira et al., 2024). Primer design was performed on Primer3Web (Untergasser, 2012) and primers were obtained from Eurofins (https://eurofinsgenomics.com). Supplemental table 2 shows all utilized primers. Approximately 240 ng of RNA was used for each reverse transcription reaction using the iScript RT Supermix from Bio Rad (Hercules, CA, USA). RT-qPCR was performed on a CFX96 Real-Time PCR system from Bio-Rad resulting in 12ng/µL of cDNA. Each reaction utilized 12 ng of cDNA, 1 µL each of forward and reverse primer was added at a concentration ranging from 750 nM to 10µM (supplemental table 2), 5 µL of SsoAdvance SYBR Green Supermix (Bio-Rad), and nuclease-free water, with a final reaction volume of 10 µL. The cycling protocol was as follows: 30 s at 95 °C followed by 40 cycles consisting of xx s at 95 °C and annealing and extension temperatures based on primer melting temperature and amplicon size (Table 1). Melt-Curve analyses were set to 65 °C with 0.5 °C increment at 3 s/step to assess if primer pairs yielded a single product. PCR reactions were performed using three biological replicates per treatment/time point, and data are presented as means and 95 % confidence intervals. Gene expression was normalized using actin2 and PP2A (Table 1) as reference genes, and relative gene expression/transcript levels were calculated (Livak & Schmittgen, 2001). PCR product size was confirmed using gel electrophoresis with 1.6% agarose gels in buffer TBE stained with Biotium (Fremont, CA, USA) Gel Red Nucleic Acid Stain.

RNA Sequencing and Analysis

RNA-sequencing and bioinformatics analysis was performed by Novogene (Sacramento, CA, USA). Initial quality was assessed using FASTQC files where reads with adapter contamination, uncertain nucleotides that constitute over 10 % of either read, or low-quality nucleotides (Base Quality less than 5) constitute 50 % of the read were removed (Yan et al., 2013). Mapping and assembly was performed using HISAT2 v2.2.1 (Kim et al., 2019) against the *Camelina sativa* genome (Kagale et al., 2014) and Stringtie v2.2.3 (Pertea et al., 2015), respectively. Differential expression analysis was performed by DESeq2 v1.42.0 (Love et al., 2014) and enrichment analysis was performed using clusterProfiler v4.8.1 (Yu et al., 2012). *Arabidopsis* orthologs of camelina genes were identified using the 'Biomart' tool at EnsemblPlants (https://plants.ensembl.org).

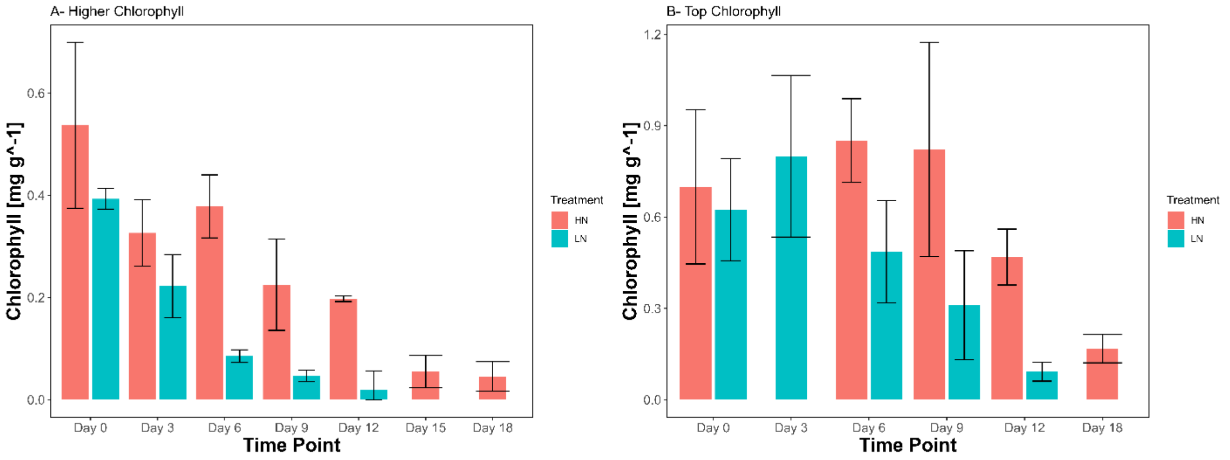
CHAPTER FOUR

RESULTS

Characterization of Camelina Leaf Senescence under low- and high- nitrogen Growth Conditions

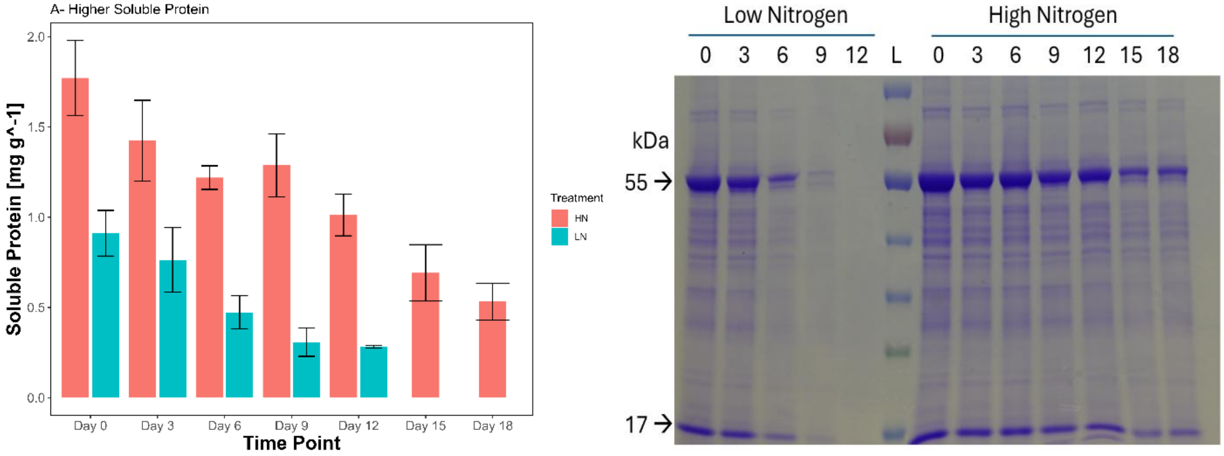
To understand how nitrogen nutrition and leaf age influence genes of interest, we measured chlorophyll concentrations, soluble protein abundance, and *SAG12* expression to quantify the level of senescence exhibited by each group.

Figure 1. Chlorophyll levels in senescing camelina leaves from plants grown under high nitrogen (HN) or low nitrogen (LN) conditions. Main stem leaf positions 12-15 (panel A) and leaf positions 16 and higher (panel B) were harvested starting with the onset of flowering (day 0), with leaf position 1 representing the first/oldest true leaf. For all treatment/time point combinations, mean values and 95% confidence intervals from three biological replicates are shown.



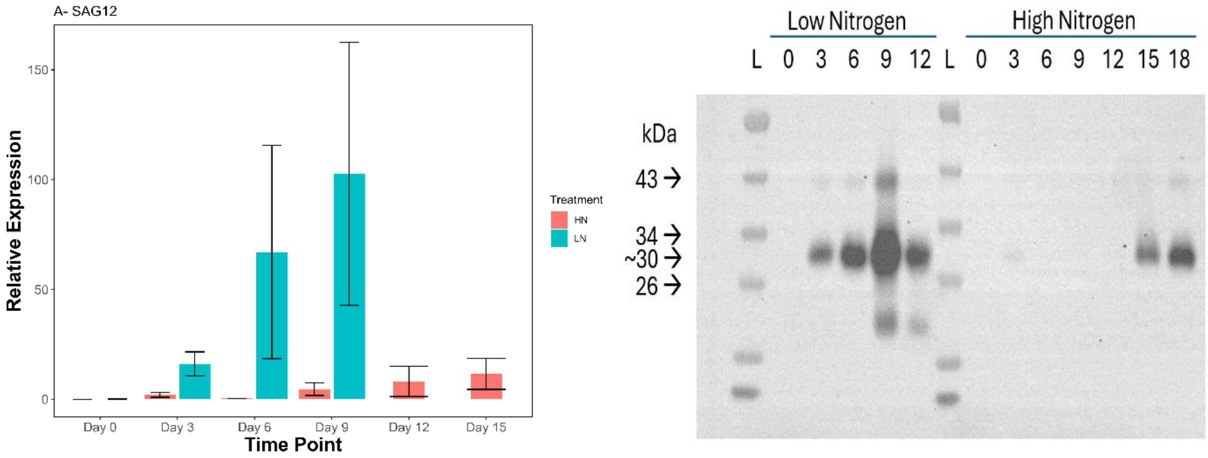
Chlorophyll concentrations were followed in two sets of leaves from both LN- and HN-grown plants and used as a proxy for advancing leaf senescence. In the first experiment, main stem leaves from positions 12-15 were pooled (Figure 1A), while positions 16 to 21 were studied in the second experiment, with leaf position 1 (fully senesced by the time of flowering) representing the first (oldest) true leaf. Analyses started one week after at least 90% of plants had opened one flower (see ‘materials and methods for details). Leaves 12-15 from LN-grown plants showed rapid chlorophyll degradation, with levels reaching < 0.1 mg g^{-1} FW by day 9. As expected, senescence progressed more slowly in leaves from HN-grown plants, with chlorophyll levels reaching values < 0.1 mg g^{-1} FW only by day 15 (Figure 1A). While most chlorophylls were also degraded by day 12 in the higher leaves (positions 16 to 21) of LN-grown pants, > 50 % was still present by day 12 in leaves from HN-grown plants (Figure 1B). As the study aimed to compare the influence of nitrogen nutrition on leaf senescence and early seed filling, all following analyses focused on leaf positions 12-15.

Figure 2. Protein levels in senescing camelina leaves from plants grown under high nitrogen (HN) or low nitrogen (LN) conditions. Soluble proteins were extracted from main stem leaf positions 12-15 starting with the onset of flowering (day 0), with leaf position 1 representing the first/oldest true leaf. Proteins were quantified using a Bradford assay (panel A) or separated by SDS-PAGE (panel B). Panel A shows means and 95% confidence intervals from three biological replicates; in panel B, gel lanes were loaded with proteins extracted from identical amounts of leaf fresh weight, with 36 leaves contributing to each analyzed protein extract.



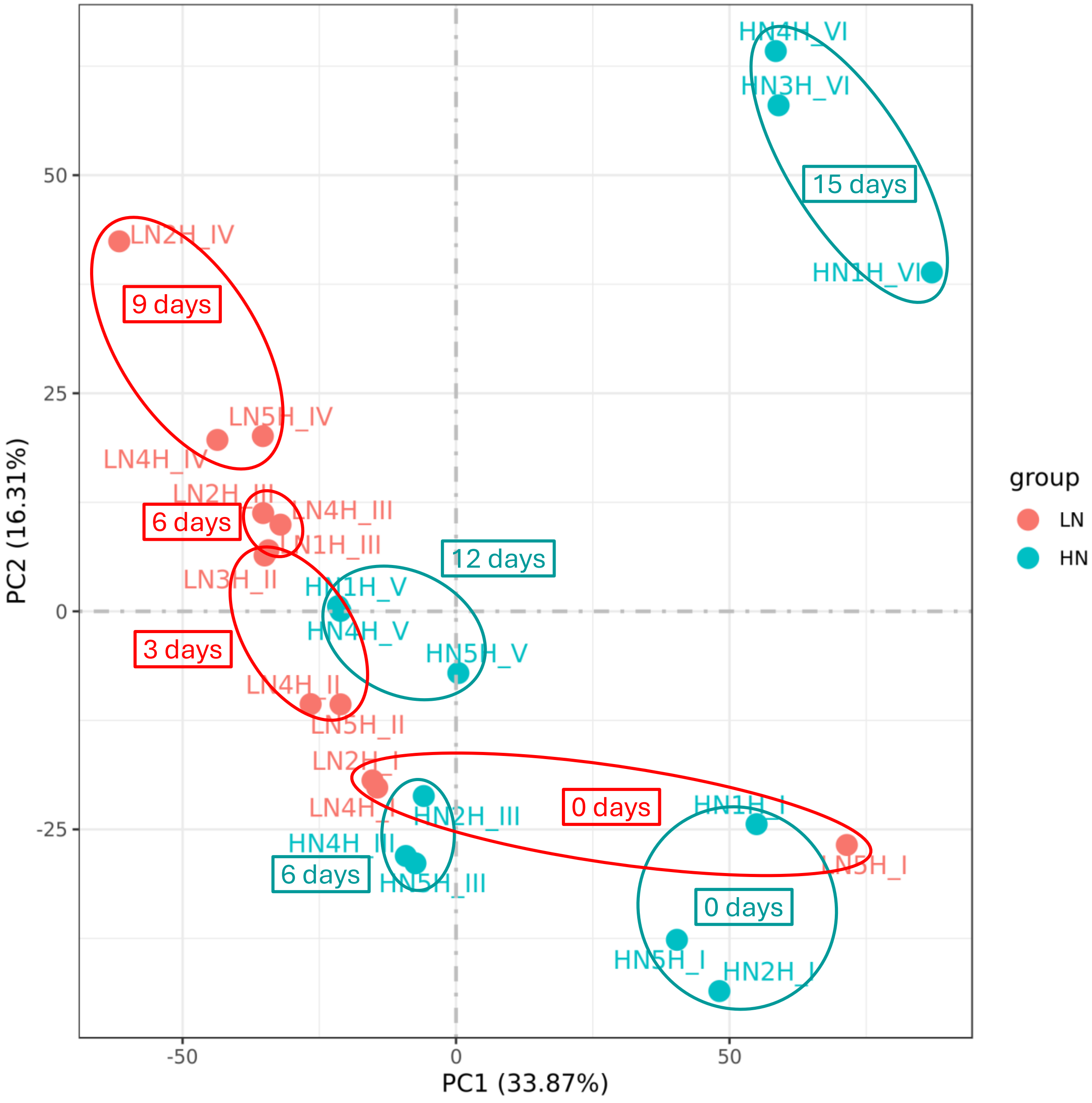
Soluble protein concentrations exhibited similar trends in both (LN, HN) treatment groups. While HN-treated plants had higher concentrations compared to LN-treated plants at all time points, both treatment groups exhibited a gradual decline over time (Figure 2A). In contrast to chlorophylls, soluble protein concentrations were almost twice as high in the HN-treated plants compared to LN treated plants at day 0. Concentrations < 0.75 mg g^{-1} FW were found in leaves from LN-treated plants by day 3, whereas concentrations remained above this threshold for HN-treated plants until day 12. Analysis of leaf soluble protein profiles by SDS-PAGE showed a rapid net degradation of large (~55 kDa) and small subunits (~17 kDa) of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in leaves from LN-grown plants, with very faint bands by day 9. In contrast, substantial amounts of Rubisco were detectable until day 18 in leaves from HN-grown plants (Figure 2B), confirming much slower progress of leaf senescence under HN.

Figure 3. Senescence-Associated Gene 12 (SAG12) gene expression (panel A) and SAG 12 protein levels (panel B) in senescing camelina leaves from plants grown under high nitrogen (HN) or low nitrogen (LN) conditions. RNA and proteins were extracted from main stem leaf positions 12-15 starting with the onset of flowering (day 0), with leaf position 1 representing the first/oldest true leaf. SAG12 relative gene expression (panel A) was determined by RT-qPCR, with expression levels for all treatment/time point combinations normalized to expression in leaves from HN-grown plants at day 0. Means and 95% confidence intervals of three biological replicates are shown. SAG12 protein levels (panel B) were determined by immunoblotting using a specific antibody; gel lanes were loaded with protein extracts obtained from identical amounts of fresh weight, with 36 leaves contributing to each protein extract.



Over the past 30 years, the *Arabidopsis SAG12* gene, encoding a family C1A (papain-like) cysteine protease, has emerged as the most-used marker of developmental leaf senescence (Balazadeh et al., 2008). Accordingly, we probed camelina leaves from LN- and HN-grown plants for *SAG12* gene expression using primers amplifying all three camelina *SAG12* homeologs (Figure 3A), and for protein levels (Figure 3B), using a commercially available antibody raised against *Arabidopsis* SAG12. The genes exhibited low expression for both treatments at day 0, suggesting that leaves had not started to senesce. Upregulation in HN-treated plants was slow, whereas upregulation in LN-treated plants occurred rapidly, with transcript levels reaching high values by days 6 and 9. SAG12 immunoblot analysis identified no proteins at day 0 for both treatments. Bands were detected by day 3 for LN-treated plants and were maximal by day 9; in contrast, essentially no signal was identified in leaves from HN-grown plants until day 15, confirming again much slower progression of senescence under HN. It should be noted that SAG12 and other cysteine proteases are synthesized as pre-proteins undergoing maturation (Grbic, 2003), which explains the presence of additional bands in leaves from LN-grown plants at day 9. Therefore, based on all senescence parameters analyzed (chlorophylls, total soluble protein levels, Rubisco, and SAG12 transcript and protein levels, day 9 leaves from LN-grown plants are strongly senescent, while senescence has also largely progressed by day 15 in leaves of HN-grown plants. These data set the stage for analyzing the influence of nitrogen nutrition on camelina leaf senescence during early seed development.

Figure 4. Principal component analysis (PCA) of RNA-Seq profiles (FPKM values) of senescing camelina leaves. RNA was extracted from main stem leaf positions 12-15 starting with the onset of flowering (0 days), with leaf position 1 representing the first/oldest true leaf. The three biological replicates (dots) obtained for each treatment/time point combination are grouped with ellipses; red-colored dots and ellipses refer to leaves from low nitrogen (LN)-grown plants, while teal-colored dots and ellipses refer to leaves from high nitrogen (HN)-grown plants. Time (days) is shown in rectangles for each group of three replicates.

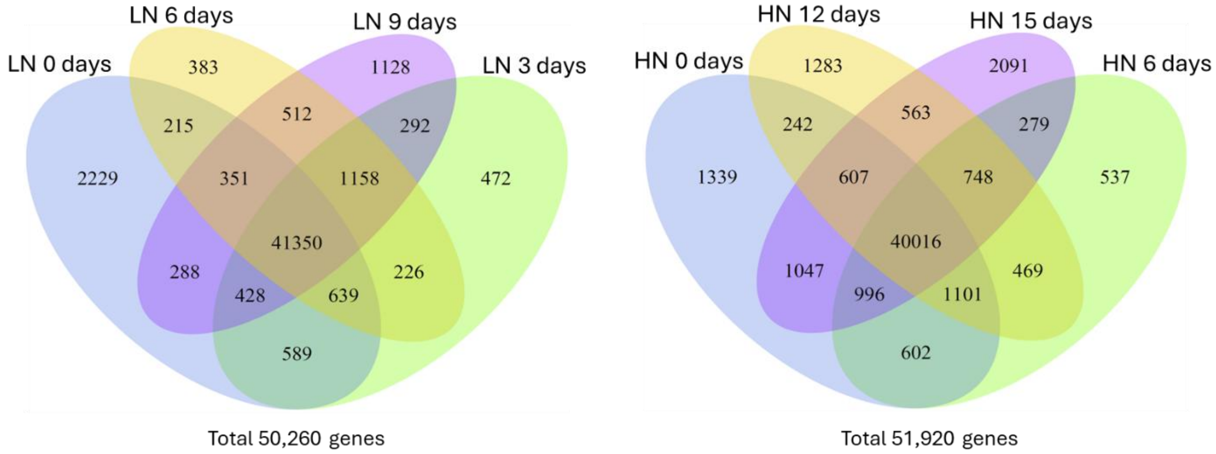


Comparative RNA Seq Analysis of Camelina Leaf Senescence Under low- and high-nitrogen Growth Conditions

To identify genes and functions controlling camelina leaf senescence under two different nitrogen regimes, global gene expression was profiled at 0, 3, 6, and 9 days in leaves from LN-grown plants, and at 0, 6, 12, and 15 days in leaves from HN-grown plants. Time points later than those analyzed did not allow extraction of high-quality RNA, likely due to the breakdown of cellular compartmentation with advanced senescence (Rolny et al., 2011).

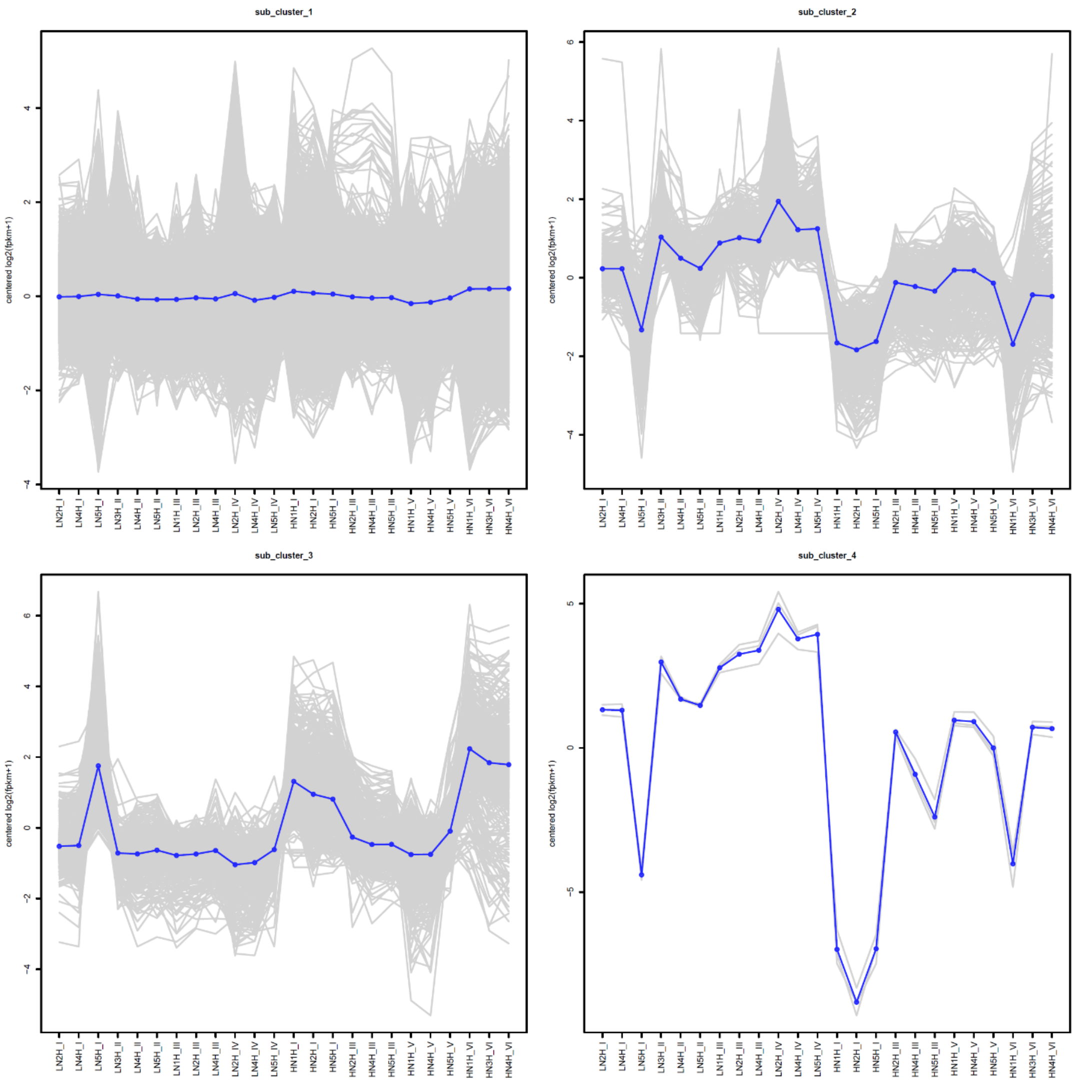
Principal component analysis (PCA) of gene expression profiles showed clustering of biological replicates for all treatment/time point combinations except for LN-treated plants on day 0. Leaf senescence progression under LN shows a clear shift in gene expression patterns with time (Figure 4). PCA also indicates that global gene expression profiles in leaves from HN-grown plants on day 12 are similar to profiles in leaves from LN-grown plants at 3 days, confirming the differences in senescence kinetics between treatment groups. Interestingly, gene expression in leaves from HN-grown plants at 15 days differs sharply from all other treatment / time point combinations, suggesting that senescence may progress differently under HN (Figure 4).

Figure 5. Venn diagrams of RNA-Seq profiling (FPKM values) of senescing camelina leaves. Panel A presents the number of genes that are uniquely expressed or co-expressed between different time points for leaves from low nitrogen (LN)-grown plants. Panel B presents the number of genes that are uniquely expressed or co-expressed between different time points for leaves from high nitrogen (HN)-grown plants. Gene numbers for each treatment/time combination are based on three biological replicates.



To further characterize global gene expression, overlap between developmental time points was analyzed (Figure 5). For the LN treatment, 82.3% of all expressed genes were shared between developmental time points. The first (pre-senescent) time point had the highest number of uniquely expressed genes (2,229), followed by the late-senescence time point (day 9) at 1,128. For the HN treatment, 77.1% of all expressed genes were shared between developmental time points. In contrast to the LN treatment, the most senesced leaves (day 15) had the highest number of uniquely expressed genes at 2,091, followed by mature-green leaves (day 0) with 1339 genes.

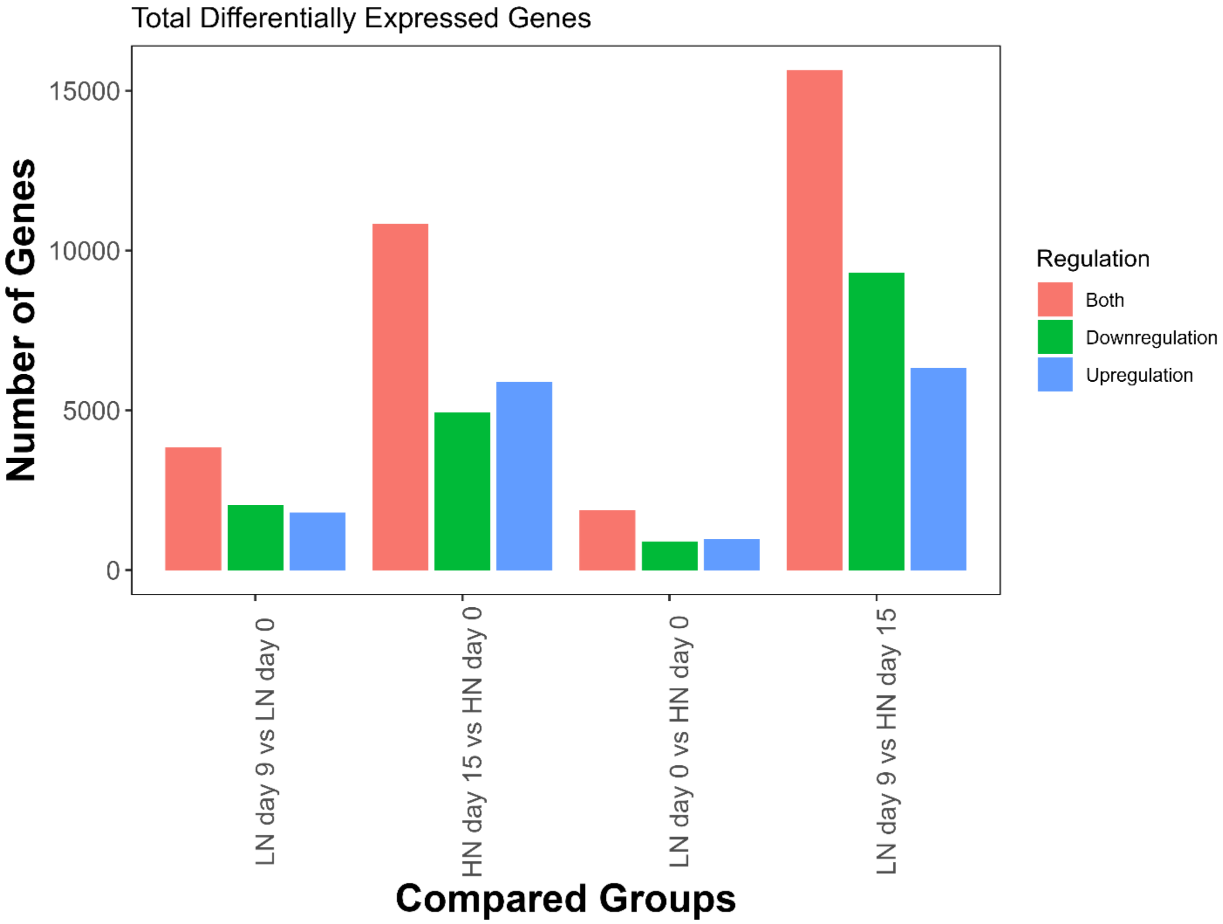
Figure 6. Cluster analysis of RNA-Seq profiling (FPKM values) of senescing camelina leaves. Panel A shows genes whose expression remains relatively unchanged across all treatment/time combinations tested; panel B clusters genes with higher expression under low nitrogen (LN) conditions; panel C clusters genes with higher expression under high nitrogen (HN) conditions, and panel D clusters three camelina SAG12 homologs. All (3) biological replicates for all treatment/time combinations are shown.



Hierarchical clustering of FPKM values was performed to identify clusters of genes with similar expression patterns across treatment/time combinations. Hierarchical clustering analysis of gene expression across all treatment/time point combinations identified four clusters (Figure 6). Cluster 1 (Figure 6A) contains genes whose expression is not changed consistently by the treatment/time point combinations evaluated. In contrast, expression of genes in cluster 2 increases over time under LN but remains lower under HN (Figure 6B). Cluster 3 contains genes whose expression trends higher under HN, particularly at the first (mature green leaves, 0 days) and last time points (15 days). Cluster 4 contains only three genes, namely the three homeologs of the *SAG12* gene (Figure 6D).

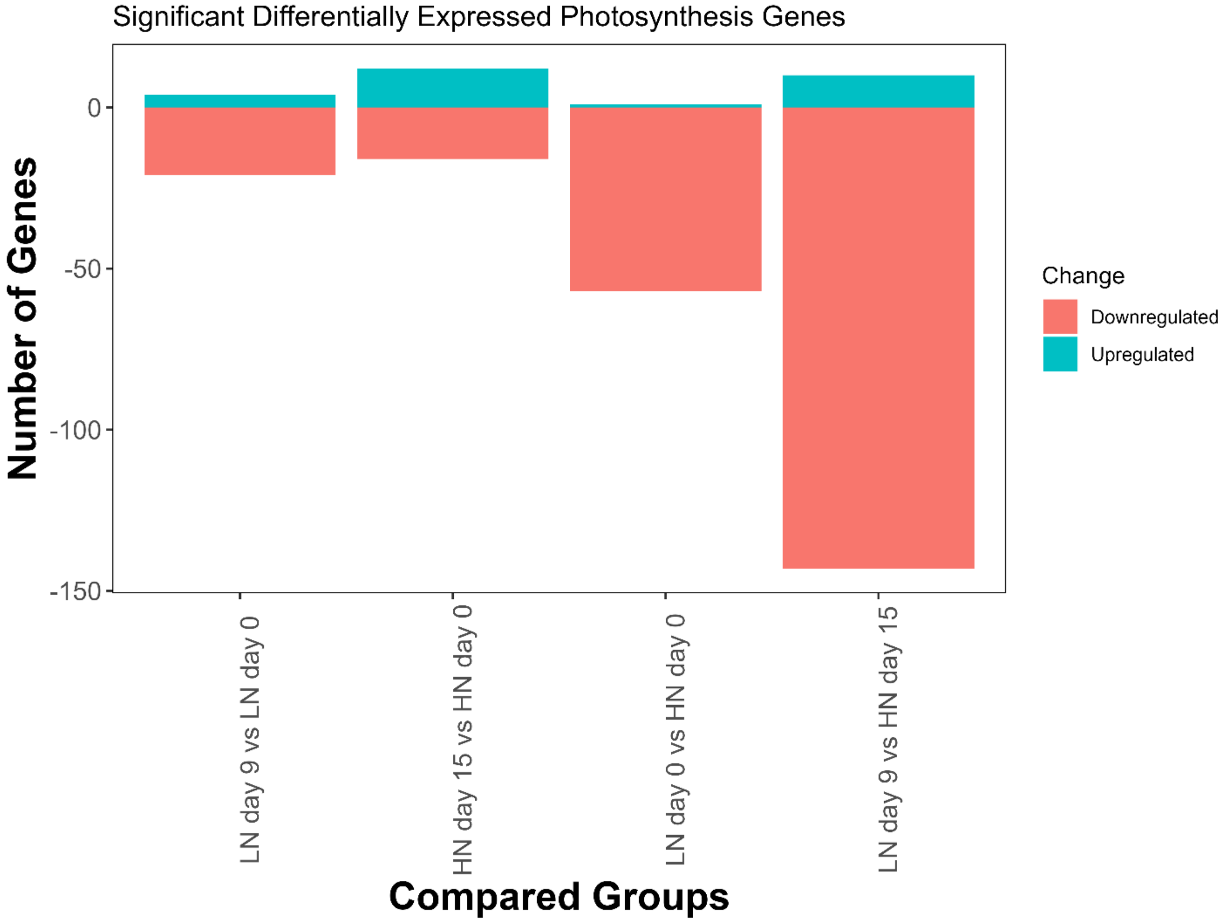
Global gene expression was further characterized by identifying the total numbers of genes that are differentially regulated between LN and HN treatments, or with advancing senescence (Figure 7). The comparison between the pre-senescent leaves from the two treatments yielded the smallest number of differentially regulated genes at 1,872. This comparison may identify genes regulated by nitrogen nutrition before senescence onset, including those involved in coping with low N availability. Higher numbers of differentially regulated genes were identified when comparing strongly senescent with mature-green leaves from LN-grown plants (3,845 genes), and especially when comparing strongly senescent leaves with mature-green leaves from HN-grown plants (10,828 genes). The highest number of differentially regulated genes (15,644) was found when comparing strongly senescent leaves from plants grown under LN or HN. This high number may reflect differences resulting from both nitrogen nutrition and senescence timing, with leaves obtained from LN-grown plants at 9 days exhibiting more advanced senescence than those from HN-grown plants at 15 days (Figures 2 and 3). The high number of differentially regulated genes may also reflect the possibility that different underlying mechanisms control senescence under LN and HN, as is suggested by PCA analysis (Figure 4).

Figure 7. Differential gene expression analysis. Numbers of genes that are significantly (adjusted p-value ≤ 0.05; log2-fold change ≥ 1.0) upregulated (teal columns) or downregulated (green columns) when comparing different treatment/time combinations are shown. Red columns represent the total number of differentially regulated genes (up- and downregulated) in these comparisons. Data are based on three biological replicates.



Genes involved in photosynthesis are typically downregulated with progressing leaf senescence, or in plant organs under low-nitrogen vs. nitrogen-sufficient conditions (Borrell et al., 2001). The influence of nitrogen deficiency on photosynthetic gene expression in mature-green leaves is clearly visible, with > 50 genes downregulated and only one gene upregulated when comparing leaves from LN- and HN-grown plants at day 0 (Figure 8). Photosynthetic genes are also mostly downregulated in the other comparisons; however, a larger fraction of upregulated genes is visible when comparing mature-green and senesced leaves from HN-grown plants (Figure 8), suggesting again that mechanisms underlying senescence progression under HN may differ from those under LN.

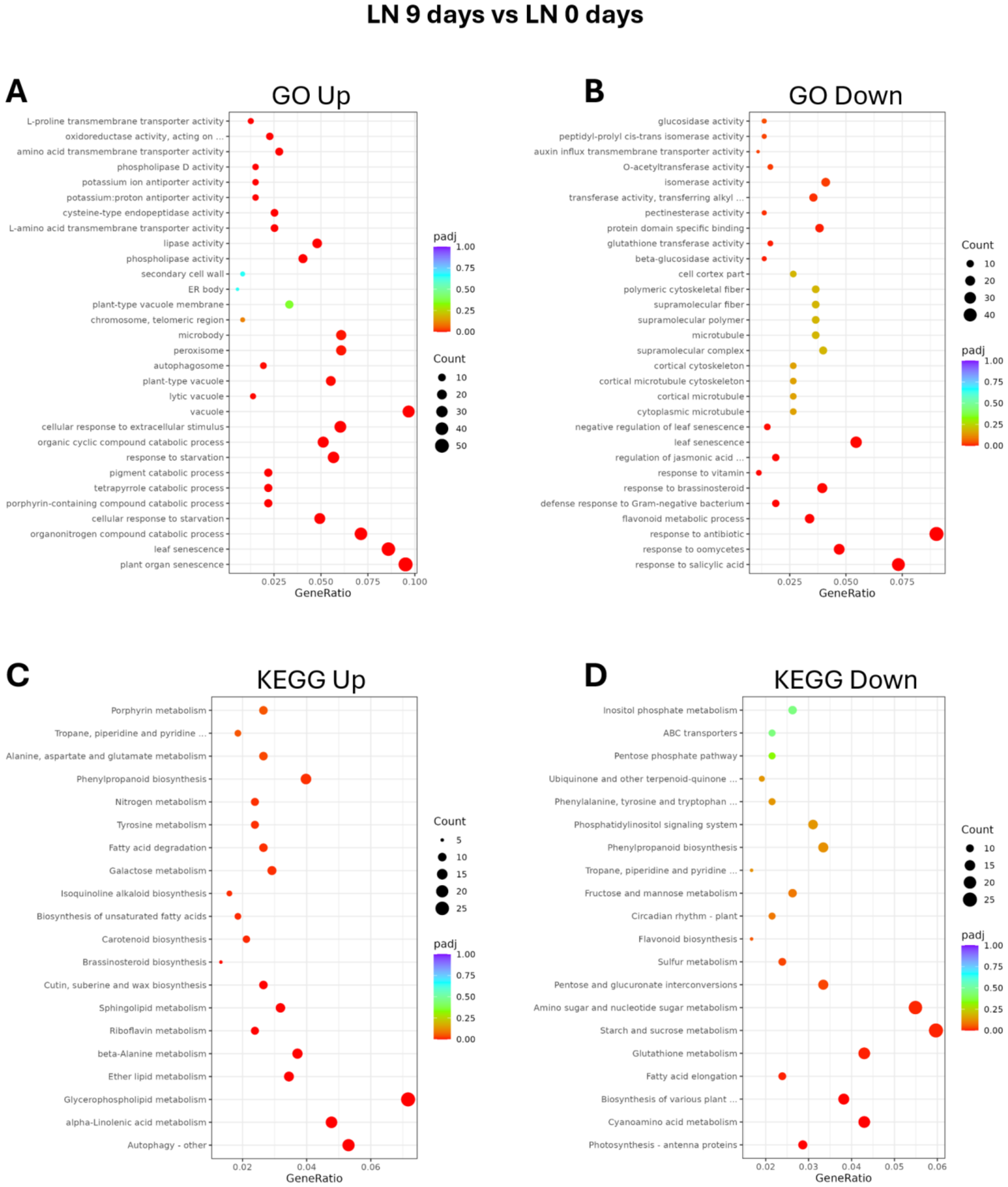
Figure 8. Differential gene expression analysis. Numbers of genes that are significantly (adjusted p-value ≤ 0.05; log2-fold change ≥ 1.0) upregulated (teal-colored, values > 1) or downregulated (red-colored, values <1) when comparing different treatment/time combinations are shown. Data are based on three biological replicates.



GO and KEGG Analysis of Camelina Leaf Senescence Under low- and high- nitrogen Growth Conditions

Sets of differentially expressed genes resulting from comparisons between treatments (i.e., mature green vs. senescing leaves) and/or between LN and HN treatments were analyzed for enrichment of Gene Ontology (GO) terms (Gene Ontology et al., 2023) in the three categories ‘biological process’, ‘cellular component’, and ‘molecular function’ (Figures 9-13).

Figure 9. RNA-Seq comparison of leaves from low nitrogen (LN)-grown plants at 0 and 9 days. GO term enrichment analysis (panels A and B) and KEGG term enrichment analysis (panels C and D) are shown, with terms enriched among upregulated genes (higher expression at 9 days) presented in panels A and C, and terms enriched among downregulated genes (lower expression at 9 days) displayed in panels B and D.



LN Day 9 vs LN Day 0

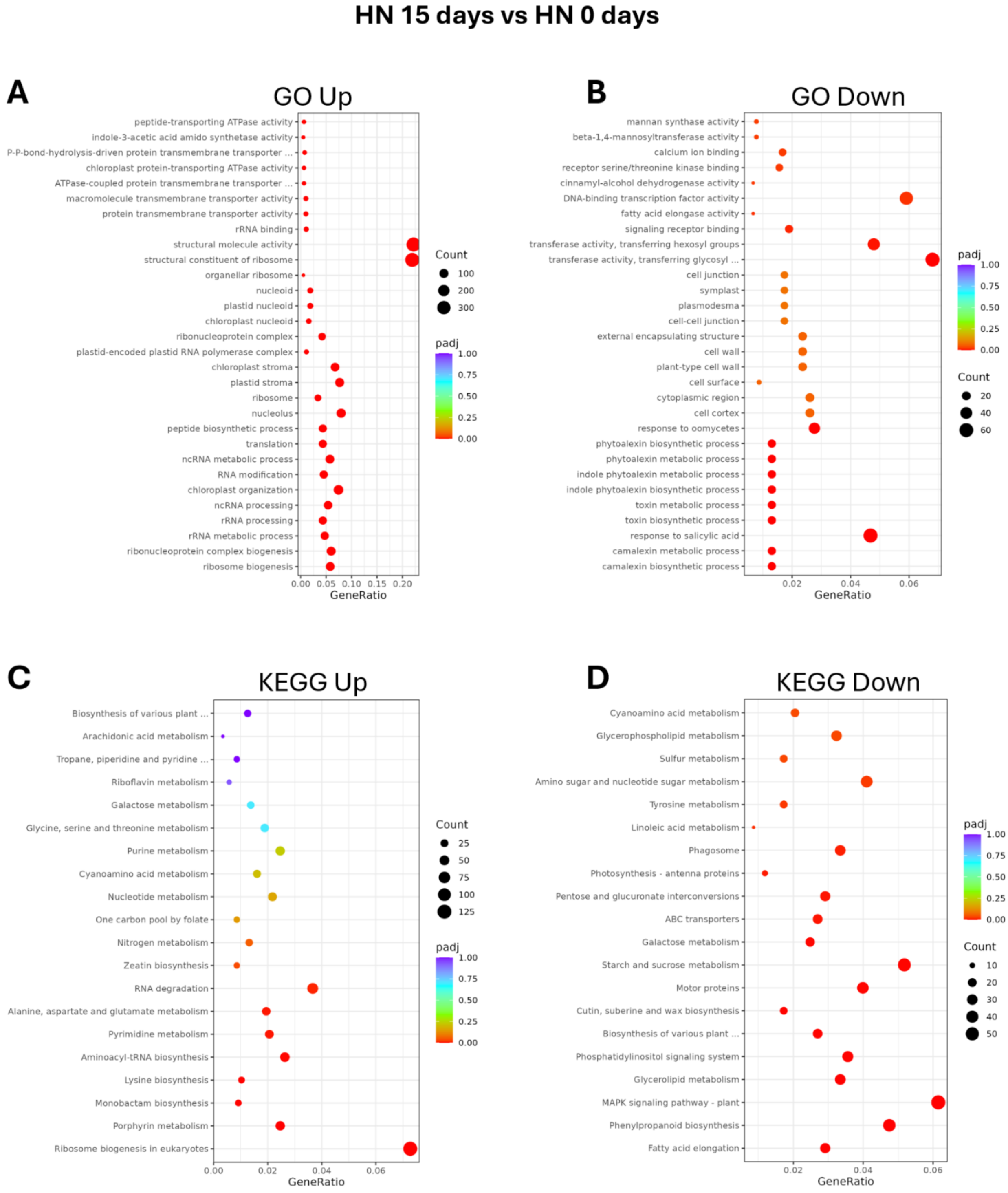
Comparing leaves from LN-grown plants at advanced senescence (9 days) with mature-green leaves (day 0) (Figure 9), biological process terms related to senescence, nutrient starvation, and chlorophyll degradation were prominent among upregulated genes (i.e., genes with higher expression at 9 days). For cellular components, significant terms include those relating to autophagy and (lytic) vacuoles, while peroxisome/microbody likely points to lipid metabolic processes including β-oxidation of fatty acids and the glyoxylate cycle. Molecular functions are enriched in lipid and protein degradation and transport processes, with amino acid transporters likely including those involved in nitrogen remobilization from senescing leaves.

Among genes which were downregulated at 9 days vs. 0 days, GO ‘biological process’ terms associated with leaf senescence, negative regulation of leaf senescence and hormone (jasmonic acid, brassinosteroid) action were prominent. The ‘molecular function’ category contained multiple terms related to cell wall metabolism, primarily in the degradation of carbohydrates such as pectin. Protein folding and transportation processes were also enriched. There were no statistically significant terms in the ‘cellular component’ category.

Sets of differentially expressed genes were also analyzed for enrichment of KEGG pathways (Kanehisa et al., 2016) (https://www.genome.jp/kegg/). Among genes upregulated in leaves harvested at 9 days vs. 0 days in LN-grown plants, pathways such as autophagy, α-linolenic acid metabolism, and glycerophospholipid metabolism were all shown to be highly enriched. Other highly significant pathways included alkaloid metabolism and lipid metabolic processes including fatty acid degradation and ether lipid metabolism. Nitrogen metabolism was also present, which may indicate remobilization from source to sink tissue.

Among the downregulated genes, significant enrichment was found for starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, and cyanoamino acid metabolism. Photosynthesis - antenna proteins was also one of the few very significant pathways downregulated from LN day 0 to day 9. Downregulation of glutathione metabolism may point to reduced defense against oxidative stress, while fatty acid elongation, in contrast to fatty acid catabolic processes, was also downregulated.

Figure 10. RNA-Seq comparison of leaves from high nitrogen (HN)-grown plants at 0 and 15 days. GO term enrichment analysis (panels A and B) and KEGG term enrichment analysis (panels C and D) are shown, with terms enriched among upregulated genes (higher expression at 15 days) presented in panels A and C, and terms enriched among downregulated genes (lower expression at 15 days) displayed in panels B and D.



HN Day 15 vs HN Day 0

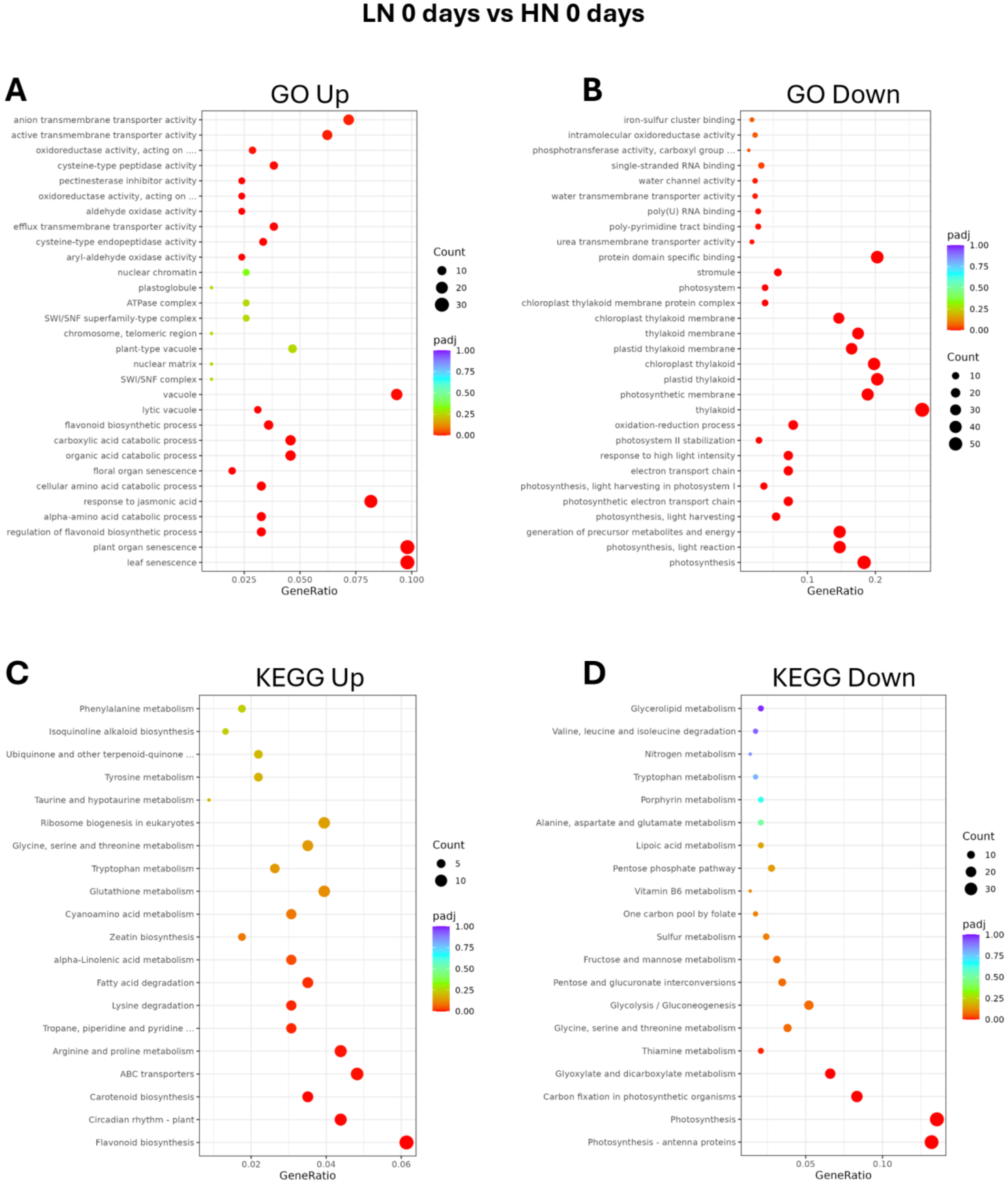
Comparing leaves from HN-grown plants at advanced senescence (15 days) with mature-green leaves (day 0) (Fig. 10), genes in the 'biological processes' category related to protein synthesis and chloroplast disassembly were upregulated. RNA synthesis and modification were also identified enriched terms. Enrichment of the 'cellular component' category identified the nucleolus and ribosome, which points to increased translational activity. Genes related to components of the nucleoid were also upregulated which indicates translational activity also being upregulated in the chloroplast. Enrichment of the 'molecular function' category includes terms related to the synthesis of phytohormones such as auxin, but also transmembrane transportation of proteins.

Genes that were downregulated when comparing day 15 to day 0 in the 'biological processes' category identified numerous terms related to the synthesis of biotic and abiotic stress defense. Other enriched terms not portrayed in this figure include terms related to negative regulation of leaf development and leaf senescence as observed in supplementary table 5. While not statistically significant, the 'cellular component' category was enriched for multiple terms relating to the cell wall. Enrichment in the 'molecular function' category includes terms relating to the movement and synthesis of oligosaccharides. This likely points to decreased plant productivity with advancing senescence.

KEGG pathway enrichment analysis of senescent leaves (day 15) compared to mature green leaves (day 0) given HN treatment identified several pathways relating to the metabolism of amino acids and components of chlorophyll. Nitrogen metabolism was also an enriched pathway. While this likely indicates the upregulation of pathways that recycle amino acids in senescence, lysine biosynthesis is also enriched. Ribosome biogenesis being an enriched pathway in senescent leaf tissue could indicate synthesis of specific proteins needed during advancing leaf senescence.

Enriched pathways identified in analyzing downregulation of day 15 compared to day 0 prominently included the metabolism of sugars as well as lipids such as glycerolipids which points to membrane degradation. Photosynthesis (antenna proteins) was also enriched among downregulated genes.

Figure 11. RNA-Seq comparison of leaves from low nitrogen (LN)-grown and high nitrogen-grown (HN) plants at 0 days. GO term enrichment analysis (panels A and B) and KEGG term enrichment analysis (panels C and D) are shown, with terms enriched among upregulated genes (higher expression in LN-grown plants) presented in panels A and C, and terms enriched among downregulated genes (lower expression in LN-grown plants) displayed in panels B and D.



LN Day 0 vs HN Day 0

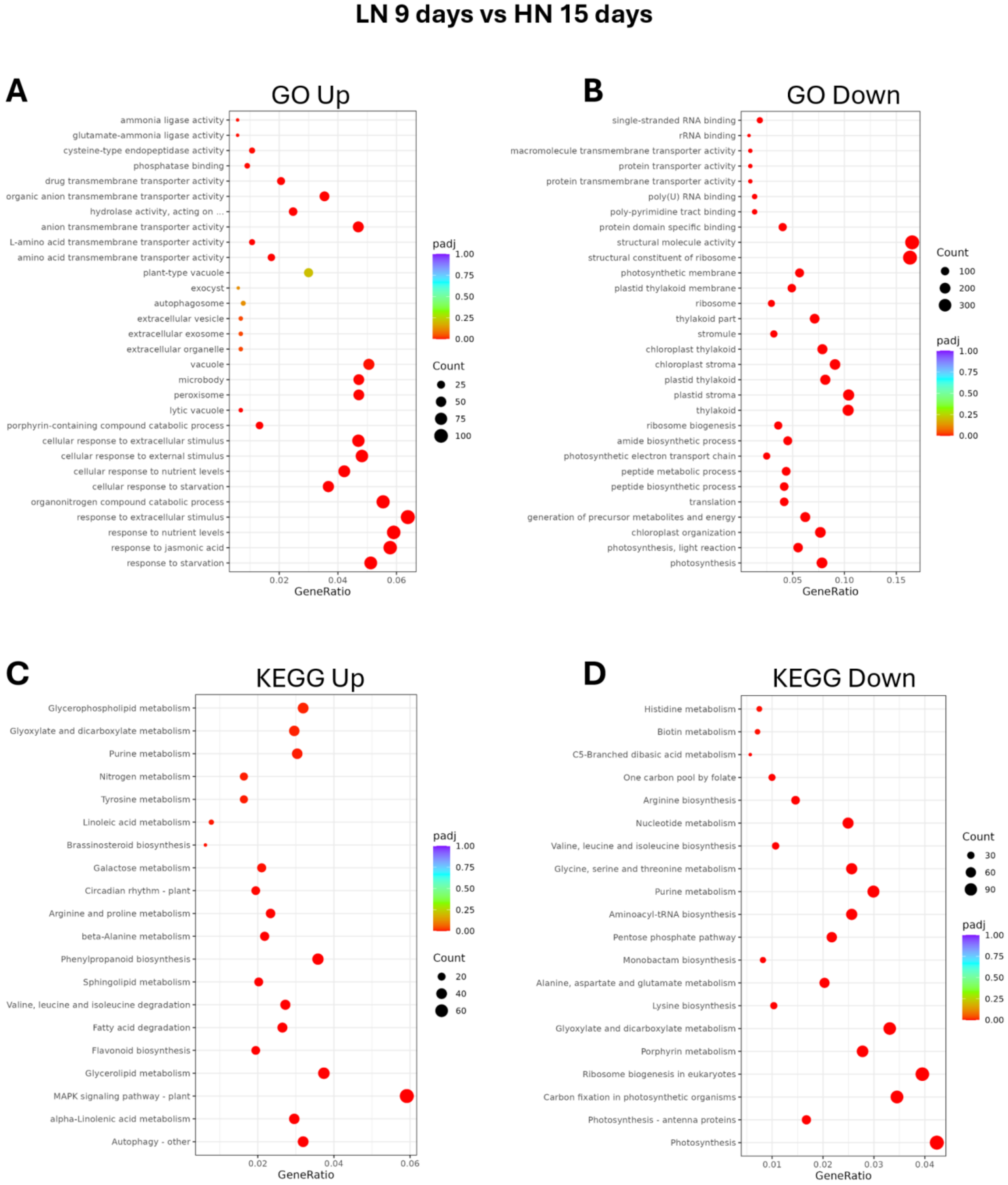
Comparing the two (LN, HN) treatment groups at day 0 for GO terms that are enriched among genes that are upregulated under LN identified multiple GO terms related to senescence, despite the fact that *SAG12* expression was similar under LN and HN at that time point. This suggests that nitrogen deficiency and senescence lead to upregulation of overlapping sets of genes. Other enriched processes include the degradation of amino acids and the synthesis of flavonoids. The GO 'cellular component' category contained only two statistically significant terms, namely lytic vacuole and vacuole which are likely related to autophagy. Molecular function terms related to cysteine peptidase activity and transmembrane transport were also enriched and point to enhanced protein degradation/turnover and nutrient remobilization under low N conditions.

GO terms enriched among downregulated genes of LN leaves compared to HN leaves at day 0 included those related to photosynthesis and photosynthetic activity. These terms included photosynthesis, light harvesting, and electron transport. The 'cellular component' category also demonstrated enrichment for photosynthesis related terms including thylakoid, photosynthetic membrane, and photosystem. Enriched terms included in the 'molecular function' category include protein domain specific binding, urea transmembrane transporter activity, and poly-pyrimidine tract binding which points to higher transcriptional activity in the HN group. This is further supported by terms like single-stranded RNA binding.

Nine statistically significantly enriched KEGG pathways were identified at day 0 of LN leaves compared to HN leaves extracted at the same time point. Terms related to the biosynthesis of phytochemicals such as flavonoids, carotenoids, and several alkaloids were prominent. Genes belonging to the metabolism of several amino acids such as arginine, proline, and lysine were also upregulated. ABC transporters was also enriched, which points to enhanced detoxification or secondary metabolite transport.

Five statistically significantly enriched pathways were identified among the genes that are downregulated under LN as compared to HN at day 0. These included photosynthesis, antenna proteins, carbon fixation, glyoxylate and dicarboxylate metabolism and thiamine metabolism, mostly reflecting reduced photosynthesis under LN, as is also suggested by lower soluble protein and Rubisco levels (Figure 2).

Figure 12. RNA-Seq comparison of leaves from low nitrogen (LN)-grown plants at 9 days and high nitrogen (HN)-grown plants at 15 days. GO enrichment analysis (panels A and B) and KEGG enrichment analysis (panels C and D) are shown, with terms enriched among upregulated genes (higher expression at LN 9 days) presented in panels A and C, and terms enriched among downregulated genes (lower expression at LN 9 days) displayed in panels B and D.



LN Day 9 vs HN Day 15

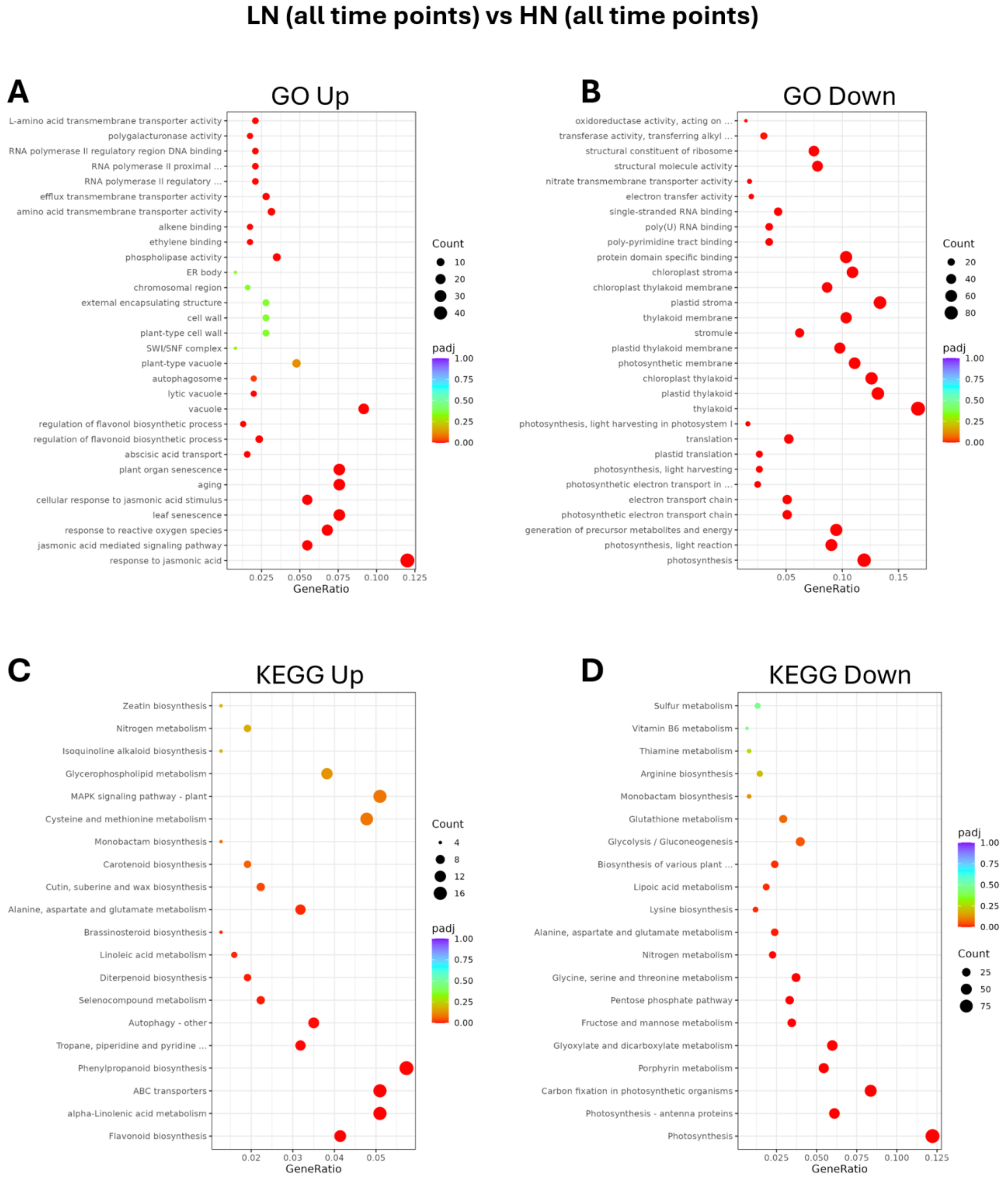
Upregulated genes between strongly senescent leaves (day 9) given LN treatment and senescent leaves (day 15) given HN treatment were analyzed for GO enrichment in the categories ‘biological processes’, ‘cellular component’, and ‘molecular function’. Response to nutrient deficiencies and external stimuli were prominent terms found in the ‘biological process’ category. Other significantly enriched terms include autophagy, transmembrane transport activity, and terms related to aging as seen in supplementary table 9. Significant enrichment of terms in the ‘cellular component’ category includes vacuole and peroxisome which indicates protein and lipid catabolism. Molecular function includes activities related to transmembrane transport and catalytic activity, both of which point to nutrient remobilization. Lipid and nucleic acid metabolism were also enriched as seen in supplementary table 9.

Genes downregulated between day 9 LN and day 15 HN leaves analyzed for GO enrichment notably include biological processes related to photosynthesis and chloroplast maintenance. While both compared treatments are considered senescent, SAG12 data indicates a more advanced senescence stage for leaves from LN-treated plants. Other significant enrichment terms found in the ‘biological processes’ category include terms related to amide and protein synthesis. Cellular component enrichment terms included numerous components of the chloroplast. Components of the light dependent reactions were also enriched as seen in supplementary table 9. Enrichment in the ‘molecular function' category prominently included terms related to protein modification and transport. Transcriptional activity was also enriched as seen in supplemental table 9.

KEGG pathway enrichment analyzing upregulated genes in senescent leaves (day 9) given LN treatment compared to senescent leaves (15 days) given HN treatment identified numerous terms pointing to protein and lipid degradation. Metabolism of amino acids and sugars were also enriched terms for the LN treated plants. Pathways involved in the disassembly of proteins and lipids were also enriched, including autophagy and, while not statistically significant but present in supplementary table 10, peroxisomes.

KEGG pathway enrichment analyzing downregulated genes for senescent leaves (day 9) of LN treated plants to senescent leaves (day 15) of HN treated plants contains several terms related to photosynthetic activity. Pathways related to amino acid and purine metabolism are also enriched. The biosynthesis of several alkaloids was also prominently enriched as seen in supplementary table 10.

Figure 13. RNA-Seq comparison of leaves from low nitrogen (LN) and high nitrogen-grown plants across all time points. GO term enrichment analysis (panels A and B) and KEGG term enrichment analysis (panels C and D) are shown, with terms enriched among upregulated genes (higher expression in LN-grown plants) presented in panels A and C, and terms enriched among downregulated genes (lower expression in LN-grown plants) displayed in panels B and D.



LN vs HN

Genes that are differentially regulated when comparing all LN leaf samples (0-9 days) with all HN leaf samples (0-15 days) were also analyzed for enrichment of GO and KEGG terms. Among genes upregulated under LN, significantly enriched terms included those related to senescence and aging, but also included terms related to programmed cell death as seen in supplementary table 11. Biological processes involved in nitrogen starvation (supplemental table 11) and reactive oxygen species signaling were also significantly enriched. Statistically significant enriched terms in the ‘cellular component’ category primarily include components related to protein and lipid metabolism. Molecular function enrichment included terms related to the mobilization of nutrients and transmembrane transport activity. Other statistically significant terms in the ‘molecular function' category shown in supplementary table 11 included protease and lipase activity.

Significant enrichment terms identified in analyzing genes that are downregulated between the LN and HN treatments largely include terms related to photosynthetic activity. The ‘biological processes’ category was enriched in categories related to photosynthesis and light dependent reactions. Enriched terms in the ‘cellular component’ category prominently include components of the chloroplast including thylakoids. Molecular function enrichment also contains terms related to photosynthesis such as electron transport. In addition, nitrate transport is enriched among the downregulated genes, as may be expected in plants growing under N deficiency.

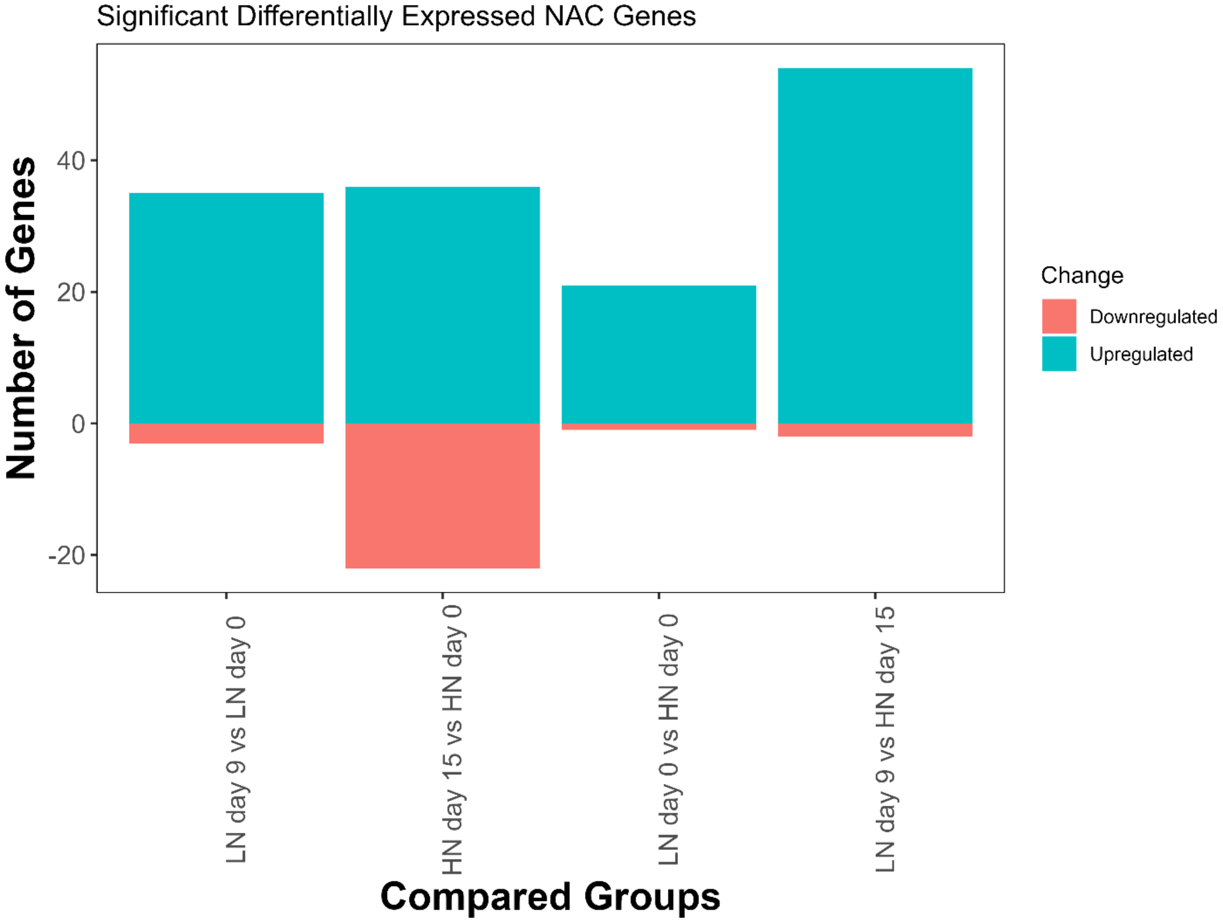
KEGG pathway enrichment analysis of genes that were upregulated under LN vs. HN identified α-linolenic acid metabolism and the biosynthesis of secondary metabolites including cutin/suberine, flavonoids and phenylpropanoids, possibly indicating routing of excess carbon into these compounds. Among genes that were downregulated under LN vs HN across all time points, enrichment of KEGG pathways associated with photosynthesis/carbon fixation, amino acid/nitrogen metabolism, and carbohydrate metabolism were prominent.

Analysis of NAC Gene Expression in Senescing Camelina Leaves from Plants Grown Under low- and high-nitrogen Conditions

While global gene expression analysis described in the previous sections provides a snapshot of camelina's response to nitrogen deficiency and of camelina senescence under low and sufficient nitrogen, this project aims to specifically enhance our understanding of the role of *NAC* transcription factor genes in these processes. RNA-Seq data were therefore analyzed for differential expression of all genes annotated as NAC transcription factors, or as having a NAC domain. Response to senescence was determined by comparing gene expression at early and late time points in leaves from LN-grown plants (0 days and 9 days) and HN-grown plants (0 days and 15 days). To establish if there is a correlation with nitrogen treatment, comparisons between the pre-senescent time points of both treatments (i.e., LN 0 days and HN 0 days) were analyzed. Differential expression between the two treatments at day 0 point could point to an involvement in plant responses to nitrogen nutrition.

To initiate the analysis, a list of every gene annotated as a NAC transcription factor or containing a NAC domain that exhibited at least one significant change (adjusted p-value ≤ 0.05; $\log_2$-fold change ≥ 1.0) in the comparisons outlined above was collected (Table 1). LN leaves harvested on day 9 compared to HN leaves extracted on day 15 showed the highest number of upregulated genes (Figure 14). Overall, 83 genes were identified, showing significant changes in at least one comparison. For 14 NAC transcription factors present in the *Arabidopsis* genome, three camelina orthologs were identified as differentially regulated in at least one comparison (Table 1). Almost half the *NAC* genes identified either only had one or two homeologs present, indicating that (a) the other homeolog(s) were not differentially regulated in our analysis, or the presence of fewer than three homeologs in the genome. Similar to global transcriptomic data, the comparison between the pre-senescent groups contained the fewest changes among *NAC* transcription factor genes (Figure 14, Table 1).

Figure 14. Differential expression of NAC genes. Numbers of genes that are significantly (adjusted p-value ≤ 0.05; log2-fold change ≥ 1.0) upregulated (teal-colored, values ≥ 1) or downregulated (red-colored, values ≤ 1) when comparing different treatment/time combinations are shown. Data are based on three biological replicates.



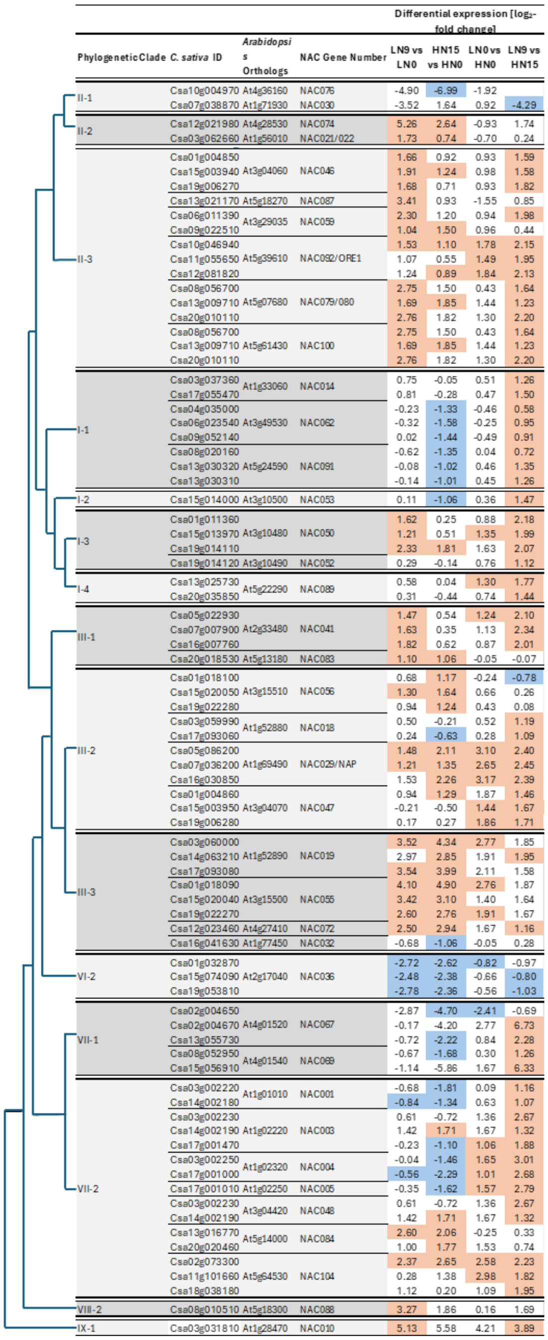
Analysis of the 83 identified NAC transcription factors (Table 1) was also performed against a phylogenetic tree of *Arabidopsis* orthologs established by (Jensen & Skriver, 2014). (Figure 14). Phylogenetics clusters *Arabidopsis* NAC transcription factors into 10 groups, with several of the larger ones divided further into subgroups (see introduction).

Figure 15. NAC genes exhibiting differential expression during leaf senescence and/or in response to nitrogen treatment. Camelina gene IDs, Arabidopsis gene IDs, NAC gene numbers (based on the Arabidopsis numbering system (Ooka et al., 2003a), and names (if available; from arabidopsis.org (Reiser et al., 2024) are shown. Gene expression (FPKM) values are means of three biological replicates. Significant differential expression (adjusted p-value ≤ 0.05) is indicated with red color for upregulation and blue color for downregulation. If no color is shown, log2-fold changes are not significant. NAC genes whose expression was confirmed with RT-qPCR are printed in red font. FPKM values are represented on a color gradient where blue is indicative of low expression, yellow is indicative of moderate expression, and red is indicative of high expression.

Gene designators				Gene expression (FPKM)								Differential expression [log$_2$-fold change]			
C. sativa ID	*A. thaliana* ID	NAC TF#	Name	LN0	LN3	LN6	LN9	HN0	HN6	HN12	HN15	LN9 vs LN0	HN15 vs HN0	LN0 vs HN0	LN9 vs HN15
Csa03g002220	AT1G01010	**NAC001**	NTL10	6.88	4.43	4.27	4.22	6.45	6.31	7.20	1.89	-0.68	-1.81	0.09	1.16
Csa14g002180				8.43	6.53	5.18	4.63	5.45	6.39	7.74	2.21	-0.84	-1.34	0.63	1.07
Csa03g002230	AT1G02220	**NAC003**	XVP	0.53	0.70	0.62	0.85	0.22	0.27	0.58	0.13	0.61	-0.72	1.36	2.67
Csa14g002190				1.80	3.61	3.78	4.88	0.58	1.78	2.89	1.95	1.42	1.71	1.67	1.32
Csa17g001470				23.0	26.8	22.3	19.5	11.1	26.4	21.8	5.36	-0.23	-1.10	1.06	1.88
Csa03g002250	AT1G02230	**NAC004**		7.75	11.37	7.92	7.55	2.52	8.07	3.31	0.94	-0.04	-1.46	1.65	3.01
Csa17g001000				42.8	40.6	34.7	28.8	21.5	39.6	22.4	4.52	-0.56	-2.29	1.01	2.68
Csa17g001010	AT1G02250	**NAC005**		6.87	5.75	5.88	5.39	2.35	4.98	3.58	0.79	-0.35	-1.62	1.57	2.79
Csa03g031810	AT1G28470	**NAC010**	SND3	0.05	0.25	0.33	2.03	0.00	0.01	0.00	0.13	5.13	5.58	4.21	3.89
Csa03g037360	AT1G33060	**NAC014**	NTL2	2.76	2.74	3.29	4.69	1.98	2.61	2.43	1.97	0.75	-0.05	0.51	1.26
Csa17g055470				2.30	3.19	2.97	3.98	1.67	1.86	3.16	1.42	0.81	-0.28	0.47	1.50
Csa03g059990	AT1G52880	**NAC018**	NAM;NARS2	20.2	23.8	26.1	28.5	14.2	21.4	32.8	12.6	0.50	-0.21	0.52	1.19
Csa17g093060				36.9	40.5	43.0	43.5	30.8	42.5	48.6	20.5	0.24	-0.63	0.28	1.09
Csa03g060000	AT1G52890	**NAC019**		3.76	8.90	10.4	43.4	0.57	0.99	1.44	11.8	3.52	4.34	2.77	1.85
Csa14g063210				17.6	40.7	55.9	138	4.75	13.9	19.1	35.4	2.97	2.85	1.91	1.95
Csa17g093080				8.51	20.1	29.0	99.6	2.01	3.46	6.09	32.9	3.54	3.99	2.11	1.58
Csa03g062660	AT1G56010	**NAC021/022**	NAC1	2.30	4.49	5.44	7.78	3.85	6.43	10.89	6.60	1.73	0.74	-0.70	0.24
Csa05g086200	AT1G69490	**NAC029**	AtNAP	66.7	115	171	185	7.90	40.1	68.2	35.2	1.48	2.11	3.10	2.40
Csa07g036200				155	254	327	358	25.0	112	169	65.8	1.21	1.35	2.65	2.45
Csa16g030850				46.6	84.7	109	135	5.25	29.7	52.3	25.9	1.53	2.26	3.17	2.39
Csa07g038870	AT1G71930	**NAC030**	VND7	0.32	0.12	0.00	0.03	0.16	0.06	0.00	0.51	-3.52	1.64	0.92	-4.29
Csa16g041630	AT1G77450	**NAC032**		5.61	4.23	3.22	3.54	5.94	7.28	4.44	2.92	-0.68	-1.06	-0.05	0.28
Csa01g032870	AT2G17040	**NAC036**		40.1	14.9	9.71	6.01	71.2	25.8	13.1	11.9	-2.72	-2.62	-0.82	-0.97
Csa15g074090				47.4	21.1	14.8	8.35	74.8	29.7	13.7	14.8	-2.48	-2.38	-0.66	-0.80
Csa19g053810				29.7	11.1	7.74	4.26	43.9	15.1	9.08	8.77	-2.78	-2.36	-0.56	-1.03
Csa05g022930	AT2G33480	**NAC041**		84.9	151	191	234	36.3	92.9	140	54.6	1.47	0.54	1.24	2.10
Csa07g007900				47.0	78.1	108	145	21.8	47.9	66.0	28.8	1.63	0.35	1.13	2.34
Csa16g007760				40.2	81.6	107	142	22.3	42.4	61.7	35.4	1.82	0.62	0.87	2.01
Csa01g004850	AT3G04060	**NAC046**		4.39	6.09	8.38	13.9	2.35	5.02	8.48	4.59	1.66	0.92	0.93	1.59
Csa15g003940				2.26	3.45	4.28	8.58	1.17	2.95	5.15	2.86	1.91	1.24	0.98	1.58
Csa19g006270				2.42	3.91	4.40	7.78	1.30	3.32	4.36	2.19	1.68	0.71	0.93	1.82
Csa01g004860	AT3G04070	**NAC047**	SHG;SHYG	10.4	17.8	18.1	20.1	2.90	12.8	19.8	7.32	0.94	1.29	1.87	1.46
Csa15g003950				28.3	28.0	27.0	24.4	10.5	22.4	27.3	7.67	-0.21	-0.50	1.44	1.67
Csa19g006280				13.7	16.0	16.7	15.5	3.84	14.5	14.2	4.77	0.17	0.27	1.86	1.71
Csa03g002230	AT3G04420	**NAC048**		0.53	0.70	0.62	0.85	0.22	0.27	0.58	0.13	0.61	-0.72	1.36	2.67
Csa14g002190				1.80	3.61	3.78	4.88	0.58	1.78	2.89	1.95	1.42	1.71	1.67	1.32
Csa01g011360	AT3G10480	**NAC050**		6.62	13.3	14.1	20.5	3.66	10.8	11.4	4.50	1.62	0.25	0.88	2.18
Csa15g013970				13.6	24.5	28.7	31.4	5.40	19.3	22.8	7.94	1.21	0.51	1.35	1.99
Csa19g014110				2.92	6.31	7.10	14.9	0.97	4.31	7.93	3.52	2.33	1.81	1.63	2.07
Csa19g014120	AT3G10490	**NAC052**	SGS1	6.84	6.73	6.99	8.26	4.05	7.35	6.39	3.79	0.29	-0.14	0.76	1.12
Csa15g014000	AT3G10500	**NAC053**	AIF;NTL4	16.5	19.5	17.9	17.8	13.1	17.2	14.7	6.46	0.11	-1.06	0.36	1.47
Csa01g018090	AT3G15500	**NAC055**	NAC3	6.29	20.0	26.5	108	0.95	3.22	5.44	29.2	4.10	4.90	2.76	1.87
Csa15g020040				15.4	39.9	52.6	166	5.93	14.6	16.1	52.7	3.42	3.10	1.40	1.64
Csa19g022270				18.9	39.4	48.2	114	5.08	14.6	22.1	35.5	2.60	2.76	1.91	1.67
Csa01g018100	AT3G15510	**NAC056**	NAC2;NARS1	9.55	14.7	15.8	15.3	11.4	20.4	23.8	26.6	0.68	1.17	-0.24	-0.78
Csa15g020050				10.1	16.2	18.8	24.9	6.48	19.5	28.9	20.9	1.30	1.64	0.66	0.26
Csa19g022280				6.72	12.7	12.5	13.0	5.09	15.0	16.1	12.4	0.94	1.24	0.43	0.08
Csa06g011390	AT3G29035	**NAC059**	NAC3;ORS1	2.78	5.47	11.1	13.9	1.48	3.80	7.42	3.51	2.30	1.20	0.94	1.98
Csa09g022510				6.01	12.2	18.0	12.2	3.12	11.8	10.7	9.09	1.04	1.50	0.96	0.44

Gene designators				Gene expression (FPKM)								Differential expression [log$_2$-fold change]			
C. sativa ID	*A. thaliana* ID	NAC TF#	Name	LN0	LN3	LN6	LN9	HN0	HN6	HN12	HN15	LN9 vs LN0	HN15 vs HN0	LN0 vs HN0	LN9 vs HN15
Csa04g035000	AT3G49530	**NAC062**	NTL6;NTM1	13.3	11.9	10.4	11.3	18.6	19.6	10.5	7.6	-0.23	-1.33	-0.46	0.58
Csa06g023540				9.69	8.92	6.99	7.74	11.7	12.4	8.26	4.04	-0.32	-1.58	-0.25	0.95
Csa09g052140				9.68	9.14	7.67	9.77	13.7	14.0	7.46	5.23	0.02	-1.44	-0.49	0.91
Csa02g004650	AT4G01520 AT4G01540	**NAC067/068**	NTM1	0.25	0.05	0.04	0.03	1.42	0.51	0.28	0.06	-2.87	-4.70	-2.41	-0.69
Csa02g004670				0.49	0.82	0.51	0.43	0.07	0.17	0.28	0.00	-0.17	-4.20	2.77	6.73
Csa13g055730				15.1	12.6	10.1	9.13	8.56	12.3	5.84	1.90	-0.72	-2.22	0.84	2.28
Csa08g052950	AT4G01550	**NAC069**	NTL13;NTM2	21.9	19.4	19.7	13.6	17.9	17.9	12.7	5.74	-0.67	-1.68	0.30	1.26
Csa15g056910				0.37	0.16	0.08	0.17	0.12	0.06	0.05	0.00	-1.14	-5.86	1.67	6.33
Csa12g023460	AT4G27410	**NAC072**	ATRD26;RD26	10.9	20.7	29.7	62.3	3.51	10.9	17.4	27.8	2.50	2.94	1.67	1.16
Csa12g021980	AT4G28530	**NAC074**	KIR1;KIRA1	0.04	0.17	0.51	1.91	0.09	0.32	0.24	0.56	5.26	2.64	-0.93	1.74
Csa10g004970	AT4G36160	**NAC076**	VND2	0.06	0.00	0.02	0.00	0.26	0.03	0.04	0.00	-4.90	-6.99	-1.92	
Csa08g056700	AT5G07680	**NAC079/080**	ATNAC4;NAC4	0.52	1.11	1.27	3.68	0.40	0.61	0.70	1.18	2.75	1.50	0.43	1.64
Csa13g009710				1.43	2.17	2.69	4.69	0.54	1.12	1.45	2.00	1.69	1.85	1.44	1.23
Csa20g010110				0.43	0.97	1.51	3.03	0.18	0.39	0.23	0.66	2.76	1.82	1.30	2.20
Csa20g018530	AT5G13180	**NAC083**	VNI2	16.1	19.0	23.0	34.1	16.7	12.4	24.3	35.9	1.10	1.06	-0.05	-0.07
Csa13g016770	AT5G14000	**NAC084**		0.20	0.45	0.62	1.42	0.26	0.62	0.82	1.13	2.60	2.06	-0.25	0.33
Csa20g020460				1.37	1.81	2.61	2.82	0.49	1.69	1.23	1.71	1.00	1.77	1.53	0.74
Csa13g021170	AT5G18270	**NAC087**		0.15	0.50	0.53	1.79	0.50	0.40	0.84	0.98	3.41	0.93	-1.55	0.85
Csa08g010510	AT5G18300	**NAC088**		0.18	1.41	1.28	2.12	0.18	0.63	1.67	0.66	3.27	1.86	0.16	1.69
Csa13g025730	AT5G22290	**NAC089**	FSQ6;NTL14	6.07	7.50	7.08	9.01	2.49	4.97	6.56	2.64	0.58	0.04	1.30	1.77
Csa20g035850				5.97	8.16	8.02	7.49	3.64	5.59	7.05	2.77	0.31	-0.44	0.74	1.44
Csa08g020160	AT5G24590	**NAC091**	TIP	8.80	6.93	6.58	5.68	8.61	9.80	6.60	3.47	-0.62	-1.35	0.04	0.72
Csa13g030320				12.7	10.6	11.0	11.9	9.3	14.0	10.7	4.69	-0.08	-1.02	0.46	1.35
Csa13g030310				14.7	12.9	11.7	13.1	10.8	15.7	11.9	5.49	-0.14	-1.01	0.45	1.26
Csa10g046940	AT5G39610	**NAC092**	ORESARA1	48.2	87.2	129	139	14.2	55.6	73.5	31.6	1.53	1.10	1.78	2.15
Csa11g055650			ORE1	53.4	88.5	123	112	19.3	59.7	81.9	29.0	1.07	0.55	1.49	1.95
Csa12g081820			NAC2;NAC6	16.7	31.3	41.3	39.7	4.74	21.6	22.1	9.07	1.24	0.89	1.84	2.13
Csa02g068390	AT5G61430	**NAC100**	NAC5	8.45	9.02	10.2	13.3	4.52	7.23	10.1	4.50	0.66	-0.05	0.91	1.56
Csa11g097230				5.56	6.76	8.38	13.2	1.92	5.63	8.54	3.92	1.24	0.99	1.56	1.75
Csa18g034760				4.11	6.54	7.48	13.2	2.62	5.10	10.1	3.64	1.67	0.43	0.68	1.85
Csa02g073300	AT5G64530	**NAC104**	XND1	5.01	9.29	12.8	26.2	0.86	6.10	5.49	5.55	2.37	2.65	2.58	2.23
Csa11g101660				4.77	4.50	4.00	5.76	0.61	2.76	2.24	1.63	0.28	1.38	2.98	1.82
Csa18g038180				2.70	5.19	5.20	5.97	1.31	2.78	2.17	1.55	1.12	0.20	1.09	1.95
Csa19g017210	AT3G12977	**NAC1-like**	NAC1L	0.10	0.01	0.20	0.46	0.00	0.00	0.41	0.31	2.01	6.34	4.85	0.55

Figure 16. Phylogenetic analysis of differentially expressed NAC genes. NAC genes showing differential expression (based on Table 1) are organized according to a previously established phylogenetic tree of Arabidopsis NAC genes. Differential expression (log2-fold changes) is shown in blocks next to NAC gene numbers, using the same organization, coloring scheme, and cutoff values as Table 1. Presence of values for more than one gene (i.e., blocks with two or three rows of values) next to an Arabidopsis gene indicates presence of more than one camelina homeologs for that gene. NAC genes whose expression was confirmed with RT-qPCR are printed in red font.



				Differential expression [$\log_2$-fold change]			
Phylogenetic Clade	*C. sativa* ID	*Arabidopsis* Orthologs	NAC Gene Number	LN9 vs LN0	HN15 vs HN0	LN0 vs HN0	LN9 vs HN15
II-1	Csa10g004970	At4g36160	NAC076	-4.90	-6.99	-1.92	
	Csa07g038870	At1g71930	NAC030	-3.52	1.64	0.92	-4.29
II-2	Csa12g021980	At4g28530	NAC074	5.26	2.64	-0.93	1.74
	Csa03g062660	At1g56010	NAC021/022	1.73	0.74	-0.70	0.24
II-3	Csa01g004850	At3g04060	NAC046	1.66	0.92	0.93	1.59
	Csa15g003940			1.91	1.24	0.98	1.58
	Csa19g006270			1.68	0.71	0.93	1.82
	Csa13g021170	At5g18270	NAC087	3.41	0.93	-1.55	0.85
	Csa06g011390	At3g29035	NAC059	2.30	1.20	0.94	1.98
	Csa09g022510			1.04	1.50	0.96	0.44
	Csa10g046940	At5g39610	NAC092/ORE1	1.53	1.10	1.78	2.15
	Csa11g055650			1.07	0.55	1.49	1.95
	Csa12g081820			1.24	0.89	1.84	2.13
	Csa08g056700	At5g07680	NAC079/080	2.75	1.50	0.43	1.64
	Csa13g009710			1.69	1.85	1.44	1.23
	Csa20g010110			2.76	1.82	1.30	2.20
	Csa08g056700	At5g61430	NAC100	2.75	1.50	0.43	1.64
	Csa13g009710			1.69	1.85	1.44	1.23
	Csa20g010110			2.76	1.82	1.30	2.20
I-1	Csa03g037360	At1g33060	NAC014	0.75	-0.05	0.51	1.26
	Csa17g055470			0.81	-0.28	0.47	1.50
	Csa04g035000	At3g49530	NAC062	-0.23	-1.33	-0.46	0.58
	Csa06g023540			-0.32	-1.58	-0.25	0.95
	Csa09g052140			0.02	-1.44	-0.49	0.91
	Csa08g020160	At5g24590	NAC091	-0.62	-1.35	0.04	0.72
	Csa13g030320			-0.08	-1.02	0.46	1.35
	Csa13g030310			-0.14	-1.01	0.45	1.26
I-2	Csa15g014000	At3g10500	NAC053	0.11	-1.06	0.36	1.47
I-3	Csa01g011360	At3g10480	NAC050	1.62	0.25	0.88	2.18
	Csa15g013970			1.21	0.51	1.35	1.99
	Csa19g014110			2.33	1.81	1.63	2.07
	Csa19g014120	At3g10490	NAC052	0.29	-0.14	0.76	1.12
I-4	Csa13g025730	At5g22290	NAC089	0.58	0.04	1.30	1.77
	Csa20g035850			0.31	-0.44	0.74	1.44
III-1	Csa05g022930	At2g33480	NAC041	1.47	0.54	1.24	2.10
	Csa07g007900			1.63	0.35	1.13	2.34
	Csa16g007760			1.82	0.62	0.87	2.01
	Csa20g018530	At5g13180	NAC083	1.10	1.06	-0.05	-0.07
III-2	Csa01g018100	At3g15510	NAC056	0.68	1.17	-0.24	-0.78
	Csa15g020050			1.30	1.64	0.66	0.26
	Csa19g022280			0.94	1.24	0.43	0.08
	Csa03g059990	At1g52880	NAC018	0.50	-0.21	0.52	1.19
	Csa17g093060			0.24	-0.63	0.28	1.09
	Csa05g086200	At1g69490	NAC029/NAP	1.48	2.11	3.10	2.40
	Csa07g036200			1.21	1.35	2.65	2.45
	Csa16g030850			1.53	2.26	3.17	2.39
	Csa01g004860	At3g04070	NAC047	0.94	1.29	1.87	1.46
	Csa15g003950			-0.21	-0.50	1.44	1.67
	Csa19g006280			0.17	0.27	1.86	1.71
III-3	Csa03g060000	At1g52890	NAC019	3.52	4.34	2.77	1.85
	Csa14g063210			2.97	2.85	1.91	1.95
	Csa17g093080			3.54	3.99	2.11	1.58
	Csa01g018090	At3g15500	NAC055	4.10	4.90	2.76	1.87
	Csa15g020040			3.42	3.10	1.40	1.64
	Csa19g022270			2.60	2.76	1.91	1.67
	Csa12g023460	At4g27410	NAC072	2.50	2.94	1.67	1.16
	Csa16g041630	At1g77450	NAC032	-0.68	-1.06	-0.05	0.28
VI-2	Csa01g032870	At2g17040	NAC036	-2.72	-2.62	-0.82	-0.97
	Csa15g074090			-2.48	-2.38	-0.66	-0.80
	Csa19g053810			-2.78	-2.36	-0.56	-1.03
VII-1	Csa02g004650	At4g01520	NAC067	-2.87	-4.70	-2.41	-0.69
	Csa02g004670			-0.17	-4.20	2.77	6.73
	Csa13g055730			-0.72	-2.22	0.84	2.28
	Csa08g052950	At4g01540	NAC069	-0.67	-1.68	0.30	1.26
	Csa15g056910			-1.14	-5.86	1.67	6.33
VII-2	Csa03g002220	At1g01010	NAC001	-0.68	-1.81	0.09	1.16
	Csa14g002180			-0.84	-1.34	0.63	1.07
	Csa03g002230	At1g02220	NAC003	0.61	-0.72	1.36	2.67
	Csa14g002190			1.42	1.71	1.67	1.32
	Csa17g001470			-0.23	-1.10	1.06	1.88
	Csa03g002250	At1g02320	NAC004	-0.04	-1.46	1.65	3.01
	Csa17g001000			-0.56	-2.29	1.01	2.68
	Csa17g001010	At1g02250	NAC005	-0.35	-1.62	1.57	2.79
	Csa03g002230	At3g04420	NAC048	0.61	-0.72	1.36	2.67
	Csa14g002190			1.42	1.71	1.67	1.32
	Csa13g016770	At5g14000	NAC084	2.60	2.06	-0.25	0.33
	Csa20g020460			1.00	1.77	1.53	0.74
	Csa02g073300	At5g64530	NAC104	2.37	2.65	2.58	2.23
	Csa11g101660			0.28	1.38	2.98	1.82
	Csa18g038180			1.12	0.20	1.09	1.95
VIII-2	Csa08g010510	At5g18300	NAC088	3.27	1.86	0.16	1.69
IX-1	Csa03g031810	At1g28470	NAC010	5.13	5.58	4.21	3.89

Analyzing groups given the same treatment for the number of upregulated and downregulated genes provided further insight into how senescence, as well as nitrogen treatment, influences expression patterns of NAC transcription factors (Figure 15). Most of the identified significant fold changes were upregulations representing 83.4% of total differential expression showing higher expression with advancing senescence for both treatment groups. Comparisons between late-senescence plants treated with LN to late-senescence plants treated with HN (days 9 and 15 respectively) contain the highest number of upregulated NAC transcription factors. Comparisons between late-senescence and pre-senescent plants treated with HN contained the second highest number of NAC transcription factors being upregulated but also showed the largest number of downregulated genes. Analysis across all comparison groups establishes NAC transcription factors as being primarily upregulated.

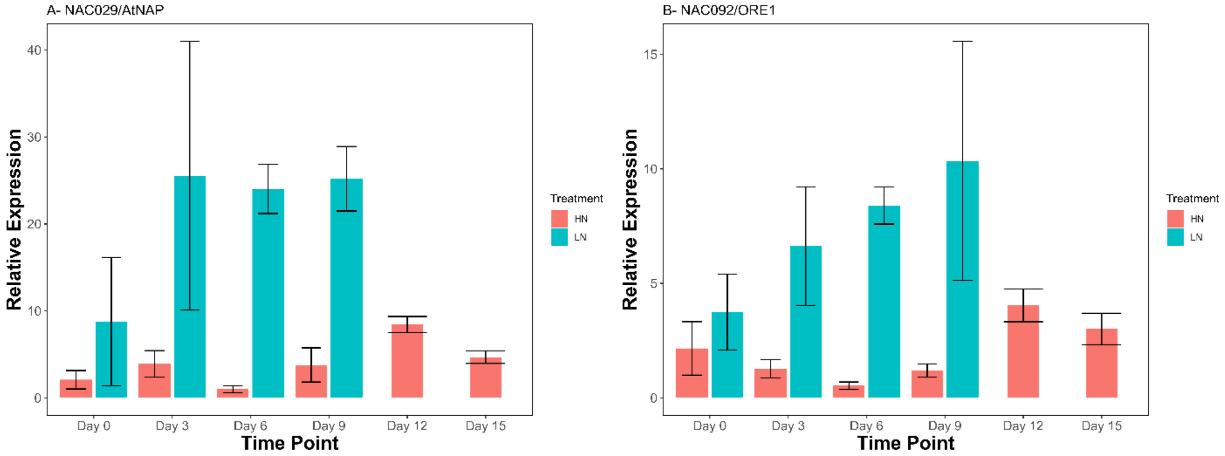
In our analysis, differentially expressed *NAC* genes fell into 15 subgroups, but larger numbers were found in subgroups II, III, and VII; all of which contain genes known for being responsive to abiotic stress conditions (Stender et al., 2015a). Seven out of the ten NAC transcription factors whose differential expression was confirmed by RT-qPCR (adding additional analysis time points for HN) were found in subgroups II and III, which contained genes that were upregulated during advancing senescence. Subgroup VII contained the most genes that were downregulated as senescence progressed under HN. *NAC036*, which was the only NAC transcription factor that was downregulated over time under both LN and HN, was identified in subgroup VI-2 which contained no other genes with identified significant changes. Other NAC transcription factors of interest were found in subgroups I-1 and I-3, where the two genes found in the separate subgroups exhibited entirely different expression patterns.

Characterization of Gene Expression of Individual NAC Transcription Factors

To confirm RNA-Seq data and increase time resolution for leaves obtained from HN-grown plants, gene expression of camelina orthologs of *Arabidopsis NAC092/ORE1*, *NAC029/AtNAP*, *NAC019, NAC055, NAC036, NAC062, NAC041, NAC046, NAC050,* and *NAC079* was quantified using RT-qPCR. In all instances, utilized primers amplify all camelina orthologs of the listed *Arabidopsis* genes; sequence identities among these camelina genes are too high to distinguish homeologs with standard RT-qPCR protocols.

AtNAP/NAC029 and *ORE1/NAC092* Regulation During Camelina Leaf Senescence Under LN and HN Conditions

Figure 17. RT-qPCR gene expression analysis of NAC029/AtNAP (panel A) and NAC092/ORESARA1 (panel B). RNA was extracted from main stem leaf positions 12-15 starting with the onset of flowering (0 days), with leaf position 1 representing the first/oldest true leaf. Values are means and 95% confidence intervals from three biological replicates and are relative to gene expression in leaves from HN-grown plants at 0 days. Primers utilized for PCR amplified all camelina NAC029 and NAC092 homologs.



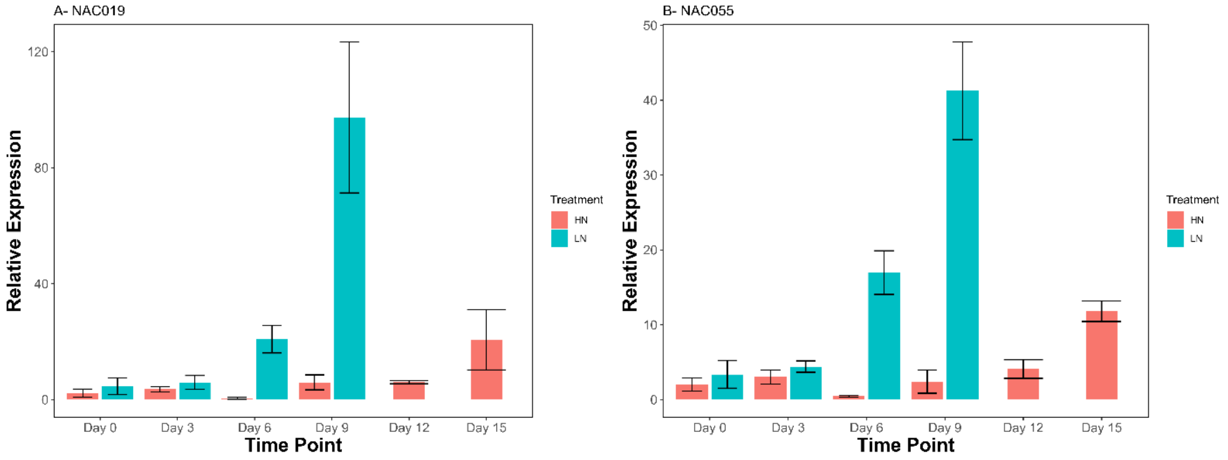
Of the ten *Arabidopsis NAC* transcription factor homologs evaluated with RNA-Seq and RT-qPCR data, two were identified in both analyses as having significant expression differences between LN and HN leaves extracted on day 0. *NAC029* and *NAC092* (AtNAP and ORESARA1/ORE1, respectively) have both been studied in *Arabidopsis* for their regulation of senescence (Ali et al., 2018). *NAC029* has been explored for its general control over the onset of senescence whereas *NAC092* has been documented as playing a direct role in nitrogen deficiency induced senescence (Chun et al., 2023).

RNA-Seq differential expression data identified statistical significance between all comparison groups for *NAC029*. Importantly, the largest differences were in groups comparing LN treated plants to HN treatment (Table 1). Upregulation between senescent leaves (day 9) and mature green leaves (day 0) both treated with LN exhibited the smallest change with two homeologs being statistically significant. RT-qPCR data, using primers amplifying all camelina *NAC029* homeologs, displayed similar trends to the RNA-Seq data (Figure 16). Gene expression increased with advancing leaf senescence under both LN and HN conditions, with earlier increase and overall higher expression under LN.

Camelina *NAC092*/*ORE1* homeologous genes exhibited the highest upregulation when comparing the most senesced leaves obtained from LN- and HN-grown plants (9 and 15 days, respectively) (Table 1). When comparing late-senescence to mature green leaves for both LN and HN, Csa10g046940, a *NAC092* homeolog, was the only significantly regulated gene. Interestingly, this same homeolog also exhibited the largest change in the comparisons between LN and HN groups, although the other two homeologs were also statistically significant. This suggests that, based on gene expression analysis, not all camelina *NAC092* homeologs are equally important during senescence and plant adaptation to low nitrogen. RT-qPCR data, again using primers amplifying all three homeologs, confirm increased gene expression with senescence, but only at later time points (12 and 15 days) for leaves from HN-grown plants. As observed for *AtNAP/NAC029* homeologous genes, *NAC092/ORE1* gene expression was higher under LN than HN conditions, suggesting a role for both transcription factors in plant responses to LN.

NAC019 and NAC055 Regulation During Camelina Leaf Senescence Under LN and HN Conditions

Figure 18. RT-qPCR gene expression analysis of NAC019 (panel A) and NAC055 (panel B). RNA was extracted from main stem leaf positions 12-15 starting with the onset of flowering (0 days), with leaf position 1 representing the first/oldest true leaf. Values are means and 95% confidence intervals from three biological replicates and are relative to gene expression in leaves from HN-grown plants at 0 days. Primers utilized for PCR amplified all camelina NAC019 and NAC055 homologs.



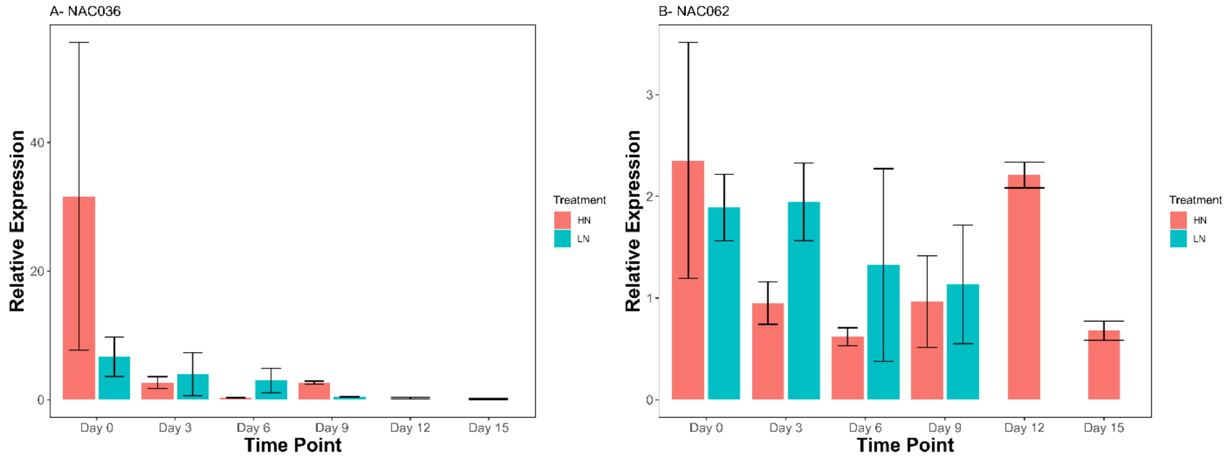
NAC019 and *NAC055* are known positive regulators of leaf senescence with overlapping stress response functions (Takasaki et al., 2015) but have not been studied for their roles in nitrogen deficiency. Comparisons using RNA-Seq identified the strongest *NAC019* upregulation occurring with advancing senescence under both LN and HN conditions (i.e., HN 15 days vs. HN 0 days, and LN 9 days vs. LN 0 days) (Figure 17). Comparisons of LN day 0 to HN day 0 and LN day 9 to HN day 15 exhibited only one homeolog with statistical significance each, both

being different homeologs. RT-qPCR data exhibited similar trends, again with higher expression in senescing leaves from LN- than from HN-grown plants.

NAC055 RNA-Seq differential expression data identified trends which were very similar to *NAC019*, where upregulation within the same treatment (LN or HN) over time had the largest changes. Comparing both groups at day 0 identified two homeologs with higher expression under LN, namely Csa01g018090 and Csa19g022270. RT-qPCR data indicates substantial upregulation over time, again with substantially higher gene expression levels in senescing leaves from LN- than from HN-grown plants.

NAC036 and *NAC062* Regulation During Camelina Leaf Senescence Under LN and HN Conditions

Figure 19. RT-qPCR gene expression analysis of NAC036 (panel A) and NAC062 (panel B). RNA was extracted from main stem leaf positions 12-15 starting with the onset of flowering (0 days), with leaf position 1 representing the first/oldest true leaf. Values are means and 95% confidence intervals from three biological replicates and are relative to gene expression in leaves from HN-grown plants. Primers utilized for PCR amplified all camelina NAC036 and NAC062 homologs.



The majority of *NAC* transcription factors studied thus far act as positive regulators of leaf senescence, and their transcript levels are upregulated with advancing senescence (Bengoa Luoni et al., 2021). However, a few act as negative regulators of this developmental process.

NAC036 comparisons of groups within the same treatment but extracted at different times exhibited the largest changes in gene expression based on RNA-Seq differential expression data. In contrast to TFs discussed in the previous sections, mature green leaves from both LN- and HN- grown plants exhibited higher expression of *NAC036* than senescing leaves. Comparing day 0 between both treatments identified only one statistically significant homeolog whereas comparisons of days 9 and 15 of the LN and HN treated plants exhibited two homeologs that were significantly different. Interestingly, the statistically significant homeologs did not overlap between the two comparisons. RT-qPCR followed the same trend of sharing a negative correlation with senescence, but expression was higher in the HN day 0 group compared to any other group, including LN day 0.

NAC062 expression also exhibited a negative correlation with the progression of senescence, but RNA-Seq indicated significant decreases in transcript levels under HN only. This is corroborated by RT-qPCR when comparing leaves from HN-grown plants at 0 and 15 days. Interestingly, both RNA-Seq and RT-qPCR indicate lower expression levels in leaves obtained from HN-grown plants at 15 days than LN-grown plants at 9 days, in contrast to differential regulation observed for other NAC TFs. Therefore, based on transcript-level evidence, both *NAC036* and *NAC062* homeologs are likely to be involved in delaying rather than advancing the senescence process.

NAC041, NAC046, NAC050, and *NAC079*Regulation During Camelina Leaf Senescence Under LN and HN Conditions

Figure 20. RT-qPCR gene expression analysis of NAC041 (panel A) and NAC046 (panel B). RNA was extracted from main stem leaf positions 12-15 starting with the onset of flowering (0 days), with leaf position 1 representing the first/oldest true leaf. Values are means and 95% confidence intervals from three biological replicates and are relative to gene expression in leaves from HN-grown plants at 0 days. Primers utilized for PCR amplified all camelina NAC041 and NAC046 homologs.

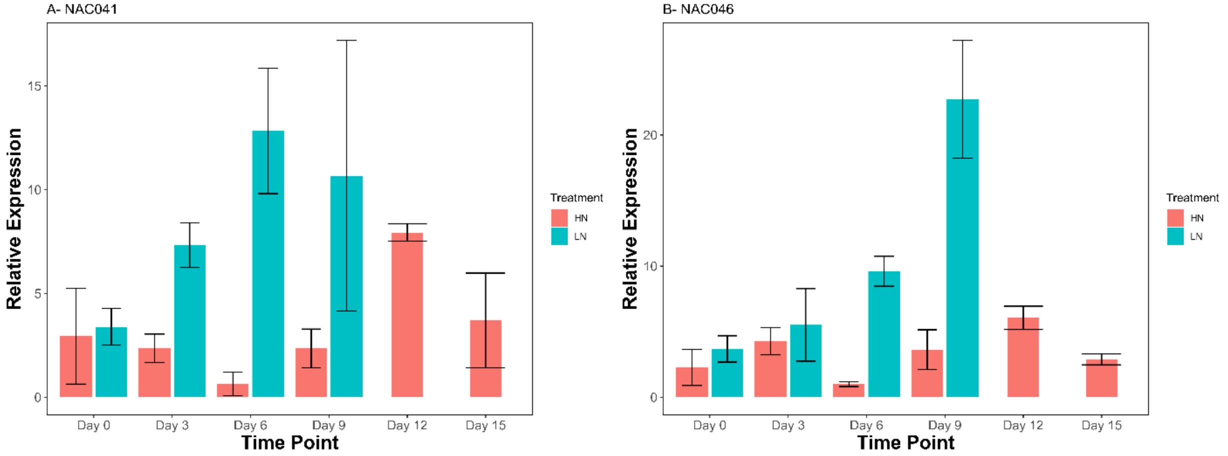
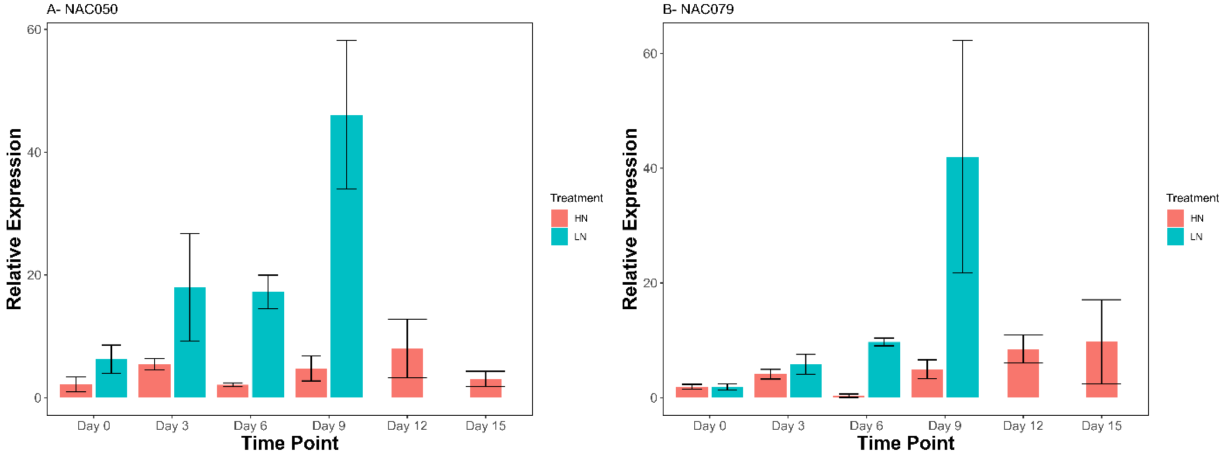


Figure 21. RT-qPCR gene expression analysis of NAC050 (panel A) and NAC079 (panel B). RNA was extracted from main stem leaf positions 12-15 starting with the onset of flowering (0 days), with leaf position 1 representing the first/oldest true leaf. Values are means and 95% confidence intervals from three biological replicates and are relative to gene expression in leaves from HN-grown plants at 0 days. Primers utilized for PCR amplified all camelina NAC050 and NAC079 homologs.



Camelina genes homologous to four additional *Arabidopsis NAC* transcription factors, and for which three homeologs could be identified, were analyzed in detail. These transcription factors are most responsive to the level of senescence exhibited by leaves harvested at 9 days from LN-grown plants which, according to their chlorophyll contents, *SAG12* gene expression, and SAG12 protein levels are more senesced than any other group (Figures 2 and 3). *NAC041* has been investigated for its role in conferring salt stress tolerance but has not previously been investigated with respect to leaf senescence or nitrogen deficiency (Fan et al., 2020). RNA-Seq differential expression data indicate significant upregulation of three camelina *NAC041* homeologs when comparing LN day 9 to LN day 0 and LN day 9 to HN day 15. Comparing both treatment groups on day 0 identified only one homeolog upregulated in the LN group that was statistically significant, but this homeolog is not more induced in other comparisons. Comparisons between HN day 15 and HN day 0 reported no statistically significant upregulation. RT-qPCR data reported the highest expression on day 6 of the LN treatment. Within the LN treatment group, there was a positive relationship between progression of senescence and expression except for slight downregulation from day 6 to day 9. However, within the HN treatment group, there were no consistent changes over time.

NAC046 has been investigated for its role in programmed cell death in the root cap of *Arabidopsis* (Oda-Yamamizo et al., 2016). While not directly implicated in having a role in senescence or nutrient deficiency, *NAC046* has been linked to cell death-associated genes (O'Shea et al., 2017). RNA-Seq differential expression data identified significant upregulation of three homeologs between LN day 9 and LN day 0, and between LN day 9 and HN day 15. One significantly upregulated homeolog was identified when comparing HN day 15 and HN day 0.

There were no significantly regulated homeologs when comparing LN day 0 to HN day 0. RT-qPCR data demonstrates the highest level of expression occurring in LN day 9. Leaves from LN-treated plants exhibited gradually higher expression over time where expression at day 6 is higher than any time point in the HN treated plants. In contrast, leaves from HN-grown plants exhibited little upregulation of *NAC046* expression over time.

Expression of camelina *NAC050* homeologs follows trends that are very similar to *NAC046*. Group comparisons analyzed with RNA-Seq data identified three homeologs as significantly upregulated between LN day 9 and LN day 0, as well as between LN day 9 and HN day 15. When comparing HN day 15 to HN day 0, there was one homeolog with significant upregulation. Comparing LN day 0 to HN day 0 also identified a single homeolog with statistical significance. However, these homeologs were not shared between the two comparisons. LN day 9 exhibited the highest levels of expression when analyzing RT-qPCR data. LN samples exhibited gradual upregulation over time whereas HN samples did not.

The role of *NAC079* in senescence regulation and response to nitrogen deficiency has not been previously investigated. As for *NAC046* and *NAC050*, RNA-Seq data identified significant upregulation of three *NAC079* homeologs when comparing LN day 9 to LN day 0 and HN day 15. One significantly upregulated homeolog was also identified when comparing HN day 15 to HN day 0. There were no significantly regulated homeologs between LN day 0 and HN day 0. RT-qPCR data substantiated these findings; LN day 9 had the highest level of expression of all treatment/time combinations tested, but upregulation was also detected in HN day 15 as compared to HN day 0. For both LN and HN, upregulation over time was gradual, with a steeper increase under LN. Neither RNA-Seq nor RT-qPCR observed any significant differences in expression between the two treatment groups at day 0, suggesting that *NAC079* homeologs are not directly responsive to nitrogen treatment.

CHAPTER FIVE

DISCUSSION

This study analyzes *NAC* transcription factor gene expression in camelina leaves during early seed development, with a focus on the influence of nitrogen nutrition and leaf age. NAC transcription factors influence a diverse set of gene regulatory networks involved in plant development and response to the environment (Diao et al., 2020). Importantly, many *NAC* genes are involved in stress responses such as drought and salinity (Chen et al., 2014); these NAC transcription factors have been previously found in subgroup III in *Arabidopsis*. However, the NAC transcription factors involved in nitrogen deficiency induced senescence have yet to be identified and characterized in camelina. While many NAC transcription factors act redundantly, understanding expression patterns based on nitrogen nutrition could elucidate which *NAC* genes should be further investigated. Camelina's hexaploid genome complicates the analysis; for genes with multiple homeologs, functions may have diversified, or particular homeologs may be more relevant for responses to nitrogen nutrition than others.

We analyzed leaf senescence time series in plants grown under contrasting nitrogen regimes to differentiate between the influences of nitrogen nutrition and leaf age on gene expression. RNA-Seq and RT-qPCR were applied to assess how both factors influenced the expression of *NAC* genes globally and individually, identifying orthologs characterized in *Arabidopsis* to connect relevant findings with the scientific literature.

To define the progression of leaf senescence, we quantified chlorophyll concentrations, protein concentrations, and SAG12 transcript and protein levels. Analyzed leaves exhibited a gradual loss in chlorophyll and soluble proteins over time when treated with HN but demonstrated a far sharper decrease when treated with LN (Figures 1 and 2). Importantly, both treatment groups were mature-green (pre-senescent) on the first analysis date, based on chlorophyll values and SAG12 transcript/protein abundance. This allowed the examination of differential gene expression between nitrogen treatments, largely eliminating the confounding influence of accelerated senescence occurring under LN at later dates.

Transcriptomics (RNA-Seq) was also used for a global assessment of the influence of nitrogen nutrition and advancing leaf senescence on leaf function (Figures 4-13). These analyses demonstrated upregulation of genes typically associated with leaf senescence, particularly in LN-treated plants. In contrast, photosynthetic genes exhibited strong downregulation, both when comparing advanced senescence with mature green stages, and when comparing leaves from LN- with leaves from HN-grown plants (Figures 8, 9, and 11).

Global NAC Transcriptomic Expression and Classification

The phylogenetic relationships of over 100 *Arabidopsis* NAC transcription factors have previously been established, based on (N-terminal) NAC domain protein sequences (Jensen et al., 2010a). Overlaying camelina *NAC* gene expression data onto the *Arabidopsis* NAC transcription factor phylogenetic tree facilitates interpretation of our data by enabling direct comparison with previous studies in the model species. In addition to the NAC domain, NAC proteins have highly variable intrinsically disordered domains located in the C terminal region; this intrinsic disorder allows for fine tuning in a NAC transcription factor’s regulatory elements (Jensen et al., 2010b). Intrinsically disordered domains can assume different conformations during interactions (Bah et al., 2014). Short sequence motifs called molecular recognition features (MoRFs) may be present within an intrinsically disordered domain which retains a conserved function and facilitates predictable interactions in gene regulatory networks (Stender et al., 2015b).

Despite NAC family subgroups being organized based on the less variable NAC domain, some subgroups have conserved regulatory elements based on the presence of specific MoRFs (Jensen et al., 2010a). Subgroup III contains many genes referred to as stress responsive NAC transcription factors (SNACs) (Jensen et al., 2010a). SNACs can be categorized based on their response to abiotic stress and senescence where *SNAC-B* genes are implicated in age- and abiotic stress-induced senescence and *SNAC-A* genes have roles in plant response to abiotic stress. (Takasaki et al., 2015) further investigated the behaviors of *SNAC-As* and found that these genes can be further classified as being responsive to ABA but less responsive to ethylene, which would indicate a role in ABA-induced leaf senescence. (Jensen et al., 2010a) observed similar expression patterns in the subgroup III abiotic stress responsive genes to subgroup VII-2 and transmembrane-anchored subgroups I-1 and I-2. (O'Shea et al., 2015) substantiated this research by examining common features of intrinsically disordered domains between subfamilies. They found that MoRFs can predict interacting gene regulatory elements and determine common expression patterns within and between subgroups. *In silico* intrinsically disordered profiles were used to identify subgroup III-2 as having conserved MoRFs, which were also similar to subgroup VII-2 NAC proteins. Subgroup II-3 contains NAC092 and NAC046 which are both involved in senescence regulation (Chun et al., 2023). While subgroup II-3 does not contain conserved MoRFs across the entire subgroup, it contains conserved MoRFs in the transcription factors directly involved in senescence regulation. Available literature provides functional information for subgroups I, II, III, and VII, but our analysis identified additional subgroups with *NAC* genes showing significant expression changes in camelina (Figure 14). Three camelina genes orthologous to *NAC036* that are downregulated with advancing senescence are found in subgroup VI-2. Subgroups VIII-2 and IX-1 also contained *NAC* genes upregulated during senescence, but only one camelina gene was identified as differentially regulated in each case. Overall, the combination of transcriptomics and phylogenetic analysis identifies gene clusters that have previously been identified as important for abiotic stress responses and senescence in *Arabidopsis*, but additional work is needed to elaborate the importance of camelina *NAC* genes in response to nitrogen deficiency.

Expression of Camelina *NAC* genes With well-characterized *Arabidopsis* Orthologs

The most biologically relevant NAC transcription factor studied in *Arabidopsis* to nitrogen deficiency induced senescence is NAC092 (ORESARA1). NAC092 belongs to the NAC subgroup II-3 although it contains MoRFs implicated in senescence regulation. Three homeologs of *NAC092* were observed to be upregulated in the LN treatment group compared to the HN treatment group. Although many *NAC* genes were found to be upregulated accompanying senescence, *NAC092* homeologs demonstrated stronger upregulation when comparing LN samples to HN samples (Table 1). This occurred even when the two treatment groups are pre-senescent (day 0). NAC092 regulates numerous *Senescence-Associated Genes* under nitrogen deficiency and binds directly to transcription factors of other families that promote photosynthesis and carbon fixation. Investigation of NAC092 antagonizing chloroplast maintenance by (Rauf et al., 2013b) identified G2-like transcription factors GLK1 and GLK2 as being targets of NAC092. GLK1 and GLK2 regulate chloroplast maintenance and their downregulation by the NAC092 protein causes an inflection point in photosynthesis and senescence. Furthermore, research performed using *Arabidopsis* described how nitrogen treatment influences *NAC092* and its downstream genes on a transcriptomic, translational, and post translational level. Research on nitrogen deficiency induced senescence in *Arabidopsis* by (Kim et al., 2009) found that *NAC092* transcripts are targeted for degradation by *miR164* during nitrogen deficient conditions. (Cheng et al., 2022) identified MEDIATOR SUBUNIT 19a (MED19a) as a mediator complex that forms a nuclear condensate under nitrogen deficient conditions which binds directly to NAC092 proteins. Absence of this condensate, even in *NAC092* overexpression mutants, prevented downstream expression of genes associated with NAC092. Further analysis of this regulatory system under nitrogen deficient conditions conducted by (Cheng et al., 2023) identified *ELF18-INDUCED LONG NONCODING RNA 1* (*ELENA1*) RNA transcripts acting as a negative regulator of NAC092 downstream interactions. NAC092 requires binding to MED19a to regulate nitrogen deficient response genes which can be interrupted by *ELENA1* transcripts that dissociate the two entities. *ELENA1* is transcribed in the roots under nitrogen deficient conditions and travels to shoot tissue. NAC092 proteins are degraded during nitrogen- sufficient conditions by *NITROGEN LIMITED ADAPTATION* (*NLA*) which polyubiquitinates NAC092 with *PHO2* (*UBC24*) as the E2 conjugase as observed by (Park et al., 2018a). This regulation is reversed by *UBP12*/*UBP13* which deubiquitinates NAC092 under nitrogen deficient conditions when studied in *Arabidopsis* by (Park et al., 2019). NAC092 homeologs in camelina likely share similar regulatory networks governed by nitrogen treatment, but transcriptomic data do not suggest clear differences in gene expression levels or regulation between the three identified homeologs.

NAC029 (AtNAP) and its orthologs have been investigated in several species for their role in positively regulating senescence. AtNAP belongs to subgroup III-2 which is rich in abiotic stress signaling NAC transcription factors. Upregulation of three camelina *AtNAP* homeologs was observed for all comparisons in the more senescent group (Table 1). Upregulation was strongest when LN treated leaves were compared to HN treated leaves where expression in LN treated leaves was consistently higher in RT-qPCR data (Figure 16). While literature investigating NAC029 and its gene regulatory network relating to senescence is rich, little is known about its role in nitrogen deficiency induced senescence. (Kim et al., 2014) identified common regulators upstream of NAC029 that are shared with NAC092 and NAC055 in ETHYLENE-INSENSITIVE2 induced leaf senescence, implying a role for ethylene signaling preceding NAC029 activation in *Arabidopsis*. Studies by (Seok et al., 2017) identified NAC029 induction to also be caused by ABA treatments and salt stress. However, studies investigating the relationship between a NAC029 ortholog and nitrogen deficiency were only performed in *Brassica napus* by (Koeslin-Findeklee et al., 2014). Their findings suggest that nitrogen deficiency in multiple cultivars was identifiable based on *NAC029* expression, which agrees with our findings of *NAC029* expression in LN treated plants (Figure 16). While regulation of AtNAP by nitrogen deficiency are has received less attention than that of NAC092, it is conceivable that NAC029 is activated by nitrogen deficiency induced senescence. RT-qPCR data suggest that pre-senescence leaves treated with LN have higher *NAC029* expression compared to day 15 leaves treated with HN (Figure 16). Taking into account their differences in *SAG12* expression and protein accumulation (Figure 3), the expression patterns of *NAC029* deviate from upregulation occurring linearly with advancing senescence, pointing to a possible role for this transcription factor in plant responses to N deficiency.

NAC Transcription Factors from Subgroup III

NAC transcription factors in the same phylogenetic (sub)group may have similar functions (i.e. response to abiotic stress) but may differ in in the exact conditions that cause their upregulation. Besides NAC029/AtNAP, NAC019, NAC055, and NAC072 all belong to NAC subgroup III-3 and have been studied together for their overlapping gene regulatory networks regarding abiotic stress signaling (Tran et al., 2004). Three homeologs of NAC019 and NAC055 were observed to be upregulated with advancing senescence where RT-qPCR data suggests upregulation to be leaf age-dependent (Figure 17). However, only a single camelina *NAC072* ortholog was observed to be upregulated. While all three *Arabidopsis* genes have been studied for their roles in drought and salinity stress, ANAC072 also has a role in cold and desiccation stress response (Fujita et al., 2004) that is different from the other two whereas ANAC019 and ANA055 have been implicated in JA signaling following pathogen infections (Bu et al., 2008). Highly similar promoter regions were observed by (Tran et al., 2004), although downstream gene regulatory networks were observed to deviate (Hickman et al., 2013). Both NAC transcription factors activated senescence, but *anac019* mutants implicated ANAC019 as being also involved in flavonoid and anthocyanin biosynthesis. ANAC055 had a different regulatory network leading to senescence, as it was involved in the regulation of chloroplast genes that are responsive to chitin (reference). Similar pathways may be activated in camelina leading to senescence where homeologs of NAC019 and NAC055 were observed to be responsive to nitrogen treatment.

Several NAC transcription factors belonging to subgroups III-2 and III-3 have been implicated in their regulatory roles in abiotic stress signaling (Takasaki et al., 2015). However, the roles of subgroup III-1 NAC transcription factors are less understood. Three camelina *NAC041* homeologs were upregulated in LN treated camelina leaves as observed in RNA-Seq and RT-qPCR data (Figures 14 and 19). While upregulation in late senescent HN treated leaves was minimal, LN treated leaves exhibited increased expression over time. In a study examining an ortholog to *Arabidopsis NAC041*, (Fan et al., 2020) identified *BrNAC041* as functioning as a GA repressor by binding to and repressing GA biosynthetic genes in Chinese flowering cabbage. GA and ABA share an antagonistic relationship where accumulation of GA inhibits ABA accumulation. It is conceivable that NAC041 homeologs identified in camelina may act as positive regulators of ABA accumulation in LN treated leaves, which would advance senescence.

NAC Transcription Factors from Subgroup II

While conserved MoRFs were not identified across the entire subgroup II-3, the intrinsic disordered domain retained similar properties across NAC proteins of this subgroup involved in abiotic stress response (Stender et al., 2015a). NAC transcription factors that exhibit upregulation during advancing leaf senescence may share pathways and directly interact with each other. (Oda-Yamamizo et al., 2016) identified *Arabidopsis* ANAC046 as a positive regulator of chlorophyll degradation. Chlorophyll degradation and loss of photosynthetic activity is a common symptom of advancing senescence, a symptom that is shared by the expression of other NAC transcription factors including NAC092, which is found in subgroup II-3 in the NAC family tree. Furthermore, NAC046 and NAC092 have been identified in sharing similar pathways by associating with the same chlorophyll catabolic genes in age dependent senescence

(Oda-Yamamizo et al., 2016). However, they have been observed as not acting redundantly to each other as they are regulated by different mechanisms such as miR164 causing degradation of *NAC092* but not of *NAC046* transcripts (Kim et al., 2009). Additionally, dark-induced senescence is accompanied by upregulated expression of *NAC046* with minimal influence on *NAC092*. However, induction of *NAC046* in LN treated leaves (Figure 19) indicates that NAC046 is responsive to nitrogen treatment in camelina along with NAC092. (Oda-Yamamizo et al., 2016) also observed upregulation of other senescence-promoting NAC transcription factors such as *NAC055* and *NAC019* in Arabidopsis NAC046-overexpressing lines. NAC055 and NAC019 likely act downstream of NAC046 and may associate through heterodimer formation characteristic of NAC transcription factors (Oda-Yamamizo et al., 2016).

Three camelina homeologs of *NAC079* were also observed to upregulate with advancing senescence under LN, and when comparing the most advanced senescence stages under LN and HN (Table 1, Figure 20). While this implicates NAC079 as being responsive to nitrogen treatment and senescence progression, statistically significant differential expression was not observed when comparing both treatments at the pre-senescent stage, and only one homeolog demonstrated significant upregulation when comparing day 15 leaves treated with HN to day 0 leaves of the same treatment. Furthermore, subgroup II-3 is a functionally diverse subgroup of NAC transcription factors where functions may be less predictable. However, NAC079 has been implicated in rice to inhibit ethylene biosynthesis and signaling genes, but this was identified by (Li et al., 2025) when investigating resistance against sheath blight and not abiotic stress conditions. This could point to NAC079 as having some role in ethylene signaling, but the stress factors inducing these genes are still uncertain.

NAC Transcription Factors from other Subgroups

While a majority of NAC transcription factors are upregulated with advancing senescence, and/or under nitrogen deficiency, some show the opposite pattern. Downregulation of three camelina *NAC036* homeologs was observed with advancing senescence in camelina leaves; also, expression was higher under HN than LN, as based on RT-qPCR (Figure 18). In this context (Zhu et al., 2020) demonstrated that a *Citrus* ortholog of NAC036, CrNAC036, negatively regulates a key gene in abscisic acid biosynthesis. As abscisic acid is a well-known positive regulator of the senescence process, this finding suggests a role for CrNAC036 in senescence timing, with decreasing gene expression allowing increased ABA biosynthesis. A similar mechanism may be important in camelina, with NAC036 homeologs controlling levels of this senescence hormone. An additional interesting finding by (Zhu, Luo et al., 2020) is the fact that the phylogenetic analysis places CrNAC036 and Arabidopsis NAC036 in the same cluster as JUNGBRUNNEN1 (NAC042), a well-known negative regulator of leaf senescence (Jensen et al., 2010a)).

Downregulation of three camelina *NAC062* homeologs was observed over time in camelina leaves under HN (Table 1). While differential regulation was minimal when comparing pre-senescent leaves between treatment groups, attenuation of expression was observed over time within treatment groups, which were also substantiated by RT-qPCR (Figure 18). Abiotic stress factors were tested by (Yang et al., 2021) in *Arabidopsis* when investigating *AtNAC062* for its regulation and role in endoplasmic reticulum stress. In that study, *AtNAC062* was upregulated during multiple abiotic stressors including salinity and cold stress but notably played a role in the unfolded protein response. The unfolded protein response caused by ER stress has been documented as being responsive to nutrient deficiency. While RNA-Seq differential expression data displayed minimal changes in expression between treatment groups, RT-qPCR data suggests that expression was generally higher in LN treated plants (Figure 18). This could suggest a role in nitrogen deficiency causing ER stress in camelina and *NAC062* playing a role in the plant's response.

Upregulation of three *NAC050* homeologs was observed with advancing senescence under LN (Table 1; Figure 20). There are no reports documenting a role of NAC subgroup I-3, or specifically of NAC050, in abiotic stress signaling or senescence. However, upregulation of three camelina *NAC50* homeologs could implicate the encoded proteins in senescence timing under LN.

CHAPTER SIX

CONCLUSION

A large number of *NAC* genes demonstrated statistically significant differential expression when comparing senescence stages (Table 1). Most of these genes were upregulated with progressing senescence, with only a few (e.g., *NAC036*) demonstrating downregulation. Biochemical parameters together with SAG12 expression and protein levels indicated that leaf senescence had not yet started on day 0 under either LN or HN treatment, suggesting that differential expression seen at this time point is primarily due to leaf nitrogen status, and not differences in senescence progression. Using this constraint, camelina orthologs of several *Arabidopsis NAC* genes were identified as being responsive to nitrogen nutrition. These include *NAC092* (*ORESARA1*) and *NAC029* (*AtNAP*) which are both well-documented for their roles in senescence, with a substantial body of literature also analyzing NAC092 regulation by nitrogen (Cheng et al., 2022). Additional camelina *NAC* genes, which, based on transcriptomics (Table 1) and RT-qPCR (Figures 16-20), exhibit significantly higher expression under LN at day 0 include orthologs of *Arabidopsis NAC003*, *NAC004*, *NAC005* in subgroup VII-2; *NAC019*, *NAC041*, *NAC047*, and *NAC055* in subgroup III, *NAC050* and *NAC089 in subgroup I*, *NAC100* in subgroup II, and *NAC104*; for several of them, more than one homeolog is upregulated. In contrast, only two *NAC* genes exhibit downregulation under LN; these include orthologs of *Arabidopsis NAC036* (which is also downregulated with advancing senescence) in subgroup VI-2, and *NAC067/068* (subgroup VII-I) (Figure 14). While this analysis suggests a role for these genes under N limitation, providing candidates for the improvement of plant nitrogen use, most of them are also upregulated with advancing leaf age, particularly those in subgroup III which contains *AtNAP*/*NAC029*. These data suggest two possibilities: (a) senescence was already more advanced in day 0 leaves from LN-grown plants, despite the low *SAG12* expression; or (b) there is substantial functional overlap between NAC transcription factors involved in nitrogen deficiency and those regulating leaf senescence. A combination of biochemical approaches (e.g., identification of transcription factor target genes) and genetic studies (knockout or knockdown) will be required to test these possibilities.

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