Oilseed rape (*Brassica napus* L.) transcriptome response to senescence, nitrogen deficiency and elevated CO_2

Dissertation zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie der Freien Universität Berlin

vorgelegt von
Vajiheh Safavi Rizi

December 2014

Die vorliegende Arbeit wurde in der Zeit von Oktober 2009 bis Dezember 2014 unter der Leitung von Prof. Dr. Reinhard Kunze am Institut für Biologie - Angewandte Genetik angefertigt.

1. Gutachter: Prof. Dr. Reinhard Kunze

Freie Universität Berlin

Institut für Biologie – Angewandte Genetik

2. Gutachter: Prof. Dr. Wolfgang Schuster

Freie Universität Berlin

Institut für Biologie – Angewandte Genetik

Disputation am 29.04.2015

Acknowledgements

Despite being far from my homeland, Iran, I found Germany as a second home where I could add new dimensions to my life.

Foremost, I would like to express my sincere gratitude to my supervisor, Prof. Dr. Reinhard Kunze, for giving me the opportunity to work on this interesting topic in his group. His support, patience and kindness were motivating me through the whole project. I thank him for his practical advises and fruitful discussions.

I am very thankful to my second supervisor Prof. Dr. Wolfgang Schuster.

I am grateful for working in a multicultural group which provided the possibility to improve myself in different directions. I thank all of my colleagues for being so supportive and friendly. Especial thanks to Dr. Christine Rausch and Dr. Alexis Kasaras for their invaluable support thorough my study. I would like to thank Jana Oberländer and Kevin Mielich for their kind efforts in corrections and translating the abstract in German. Thanks to Dr. Wolfram Brenner for his helpful comments. Jana, I especially thank you for our scientific tee times!

I also would like to thank Charlotte Thomas for her contribution in sorting out the oilseed rape samples and Dr. Elke Diedrichsen for sharing her knowledge about oilseed rape.

A special mention for the members of the research unit, FOR948, for their detailed discussions from different angles during our meetings.

I owe a great depth of gratitude to all the people who were supporting me from the beginning to this end.

I like to express my deepest gratitude and appreciation to my lovely family, mother, father and my two brothers, who were supportive and encouraged me during the whole time. I am truly blessed to have them in my life.

Table of contents

1 Introduction

1.1 General aspects of leaf senescence

Senescence is the last step of leaf development and is a very active and regulated process (Lim P.O. et al. 2007). Leaf senescence is regulated in an elaborate way to maximize plant fitness by optimization of nutrient remobilization from senescing leaves. Onset, progression and completion of leaf senescence are controlled by differential expression of many genes (Woo et al. 2013). Plants show two types of senescence including replicative (mitotic) senescence and post-mitotic senescence. Replicative senescence of a cell indicates loss of ability for further cell division during aging. Post-mitotic senescence is a degenerative process which happens after cellular maturation or differentiation and results in cell death. Leaf senescence mainly refers to post-mitotic senescence which occurs at the leaf organ level (Lim P. O. et al. 2003).

Lack of synchronization is one of the major limitations of the study of developmental leaf senescence. The cells within a single leaf are not at the same physiological and molecular status and usually senescence starts from tips and margins of the leaf and proceeds towards the base (Buchanan-Wollaston V. et al. 2005). Between different cell types mesophyll cells undergo final cell death earlier while veins remain active longer to ensure maximum export of nutrients (Buchanan-Wollaston V. et al. 2005, Lim P.O. et al. 2007) . Zentgraf et al., (2004), compared the effect of plant age and leaf age on senescence-associated gene expression profiles of rosette leaves in *Arabidopsis thaliana*. They harvested individual rosette leaves of a 6-week-old plant and also the same leaves from plants of different ages, ranging from 5 to 8 weeks. Clustering of gene expression pattern revealed that genes which are up-regulated with respect to the age of the entire plant, showed completely different expression profiles with respect to the age of the individual leaves within one rosette. Authors suggested that gene expression is governed by different parameters: i) the age of the individual leaf and ii) the age and developmental stage of the whole plant (Zentgraf et al. 2004).

Using a high-resolution temporal transcript profiling of senescing Arabidopsis leaves, Breeze et al. (2011) reported the order of genetic events during senescence. Switch from anabolic to catabolic processes is one of the earliest steps occurring in senescence. Protein synthesis and amino acid metabolism are down-regulated while reactive oxygen related genes and the genes involved in response to water stress together with autophagy genes are up-regulated. In the next step, expression of the genes involved in abscisic acid (ABA), jasmonic acid (JA) signaling and cytokinin mediated signaling are repressed similar to chlorophyll and carotenoid biosynthesis.

These changes are followed by down-regulation of carbon utilization and higher expression of cysteine-aspartate proteases, carotene metabolism related genes and pectin esterases. Reduction of photosynthetic activity and degradation of the photosynthetic apparatus with enhanced activity of lipid catabolism, ethylene signaling and increased level of genes encoding cytoskeletalproteins occur at the latest phases of senescence. Leaf senescence is reversible under certain conditions in a specific time span after initiation which results in re-greening of the leaves (Parlitz et al. 2011). There is not a unique conclusion about reversibility of induced senescence in Arabidopsis. No recovery was observed after dark incubation of individual attached leaves of Arabidopsis plants grown under constant light (Parlitz et al. 2011, Weaver and Amasino 2001). However, addition of nitrogen supply to Arabidopsis plants undergoing nitrogen deficiency induced leaf senescence leads to re-greening of Arabidopsis leaves (Balazadeh et al. 2014, Schildhauer et al. 2008). Thus, senescence is a programmed process controlled by an elegant molecular system but the complex molecular mechanism involved in the regulation of senescence initiation and progression has not yet been fully discovered. However, the transcriptome studies have shown massive gene expression alterations during senescence (Breeze et al. 2011, Buchanan-Wollaston V et al. 2003, van der Graaff et al. 2006). These data indicate that there is a complex regulatory network involved in regulation of leaf senescence.

1.2 Regulation of leaf senescence

Leaf senescence can be regulated by both environmental cues and endogenous signals such as development and plant growth regulators (Guo and Gan 2005). Large scale transcriptome profiling identified thousands of genes which are up- or down-regulated during senescence (Breeze et al. 2011, van der Graaff et al. 2006). Different genes including transcription regulators and signaling components functioning in hormone and stress response together with metabolism regulators are involved in regulation of leaf senescence (Buchanan-Wollaston V et al. 2003). Members of different transcription factor families including WRKY, NAC, MADS, MYB, bZIP and bHLH have been shown to play role in leaf senescence regulation (Buchanan-Wollaston V et al. 2003, Buchanan-Wollaston V. et al. 2005, Liu et al. 2011). WRKY53 is a well-known example of WRKY transcription factors which has been shown to be involved in regulating of senescence specific gene expression (Miao Y. et al. 2004). Plant hormones such as ethylene, ABA, SA , JA and small molecules, such as oxygen, induce leaf senescence while cytokinin, auxin, nitric oxide as well as small molecules, such as Ca^{2+}, delay leaf senescence which indicates the importance of phytohormones in leaf senescence regulation (Li Z. et al.

2012). Even though the role of gibberellic acid (GA) and brassionsteroids (BRs) during senescence is not well known, it has been shown that several GA and BRs related genes display regulation changes during senescence (van der Graaff et al. 2006). For example the GA-responsive xyloglucan endotransglycosylase *AtMERI5* is massively up-regulated during senescence. Moreover, in the aforementioned study, the BR receptor, *AtBRL3,* is strongly up-regulated in later stages of natural and induced (darkness, darkness treated detached leaf) leaf senescence (van der Graaff et al. 2006).

Epigenetic processes are also involved in the regulation of leaf senescence. Changes in the chromatin structure during senescence, differential histone and DNA methylation have been shown to be involved in senescence regulation. In addition small RNAs play role in the regulation of senescence associated genes (Humbeck 2013). For example, ORE1, a NAC transcription factor regulating downstream senescence-associated genes, such as *SAG12*, is repressed by the microRNA (miR164) via cleavage of ORE1 mRNA (Kim J. H. et al. 2009). In the aforementioned study, authors showed that miR164 mutation results in senescence acceleration and moreover they observed that the expression of microR164 in wild-type plants decreases with increasing leaf age in an ethylene-dependent manner (Kim J. H. et al. 2009).

To get more insight in to different types of senescence, in the following section, molecular players involved in natural and induced leaf senescence as well as similarities and differences between these two types of senescence are discussed.

1.3 Natural leaf senescence vs. induced leaf senescence

Beside developmental age which results in natural leaf senescence, different biotic and abiotic stress conditions such as dark treatment, leaf detachment and nutrient starvation can results in premature induced leaf senescence. A comparative transcriptome study between *Arabidopsis thaliana* developmental leaf senescence (NS), darkening-induced senescence of individual leaves attached to the plant (DIS), and senescence in dark-incubated detached leaves (DET) was conducted by (van der Graaff et al. 2006). This study reveals many novel senescence-associated genes with distinct expression profiles and also high number of regulated genes which are shared between these three types of senescence. For instance, they identified transporter genes which show expression changes in all three or individual type of senescence. This indicates that remobilization of catabolites during senescence is under the control of different genetic programs (van der Graaff et al. 2006). Buchanan et al., 2005, revealed the gene expression similarities and discrepancies between developmental leaf senescence and artificially induced leaf senescence by dark treatment or starvation. Reduction in cytokinin levels and

activation of salicylic acid pathway were observed only during developmental senescence. Asparagin metabolism, fatty acid degradation and a putative alternative flavonoid pathway were shown to be involved only in controlling starvation induced senescence. Moreover, Expression of ABA response genes together with jasmonic acid and ethylene dependent genes were observed in all three types of senescence (Figure 1). These results indicate that even though, natural leaf senescence and induced leaf senescence by darkness, starvation and leaf detachment share large number of regulated genes but they show distinct expression profiles indicating the complexity of the senescence regulatory network (Buchanan-Wollaston V. et al. 2005, van der Graaff et al. 2006).

1.3.1 Nitrogen deficiency induced leaf senescence

The connection between nitrogen (N) deficiency and senescence induction has been already documented (Aguera et al. 2010, Gregersen et al. 2013, Schildhauer et al. 2008). Nitrogen is an important macronutrient and a structural component of proteins, nucleic acids, cofactors and secondary metabolites and is thus essential for plant growth and development (Scheible et al. 2004). Nitrogen deficiency results in extensive physiological and biochemical changes in plants in order to adapt to nitrogen limitation (Peng et al. 2007). It has been shown that sunflower plants (*Helianthus annuus*) grown under limited N supply show lower photosynthetic activity and stronger senescence symptoms compared to plants grown under adequate N condition. In the same study, a higher ratio of hexose to sucrose at the beginning of the senescence in N-starved plants indicated that availability of nitrogen affects sugar-related senescence induction (Aguera et al. 2010). Nitrogen deficiency-induced primary leaf senescence can be reversed by addition of N supply in a time dependent manner. Recently, Balazadeh et al., (2014), showed that leaf senescence induced by nitrogen starvation in hydroponically grown Arabidopsis plants can be reversed when nitrogen is resupplied at the onset of senescence. Authors concluded that plants undergoing senescence retain the capacity to sense and respond to the availability of nitrogen nutrition. In the same study, *SAG12* was highly reduced (~500-fold), while expression of *SEN1*, an N depletion-repressed gene was markedly increased after 3 days of N resupply. (Balazadeh et al. 2014).

Nitrate fertilization at the onset of senescence in *Hordeum vulgare*, can delay leaf senescence associated changes such as decrease in chlorophyll content and photosystem-II efficiency. Moreover, two nitrogen metabolism marker genes including plastidic *GLUTAMINE SYNTHASE* (*GS2*) and *LYSINE-KETOGLUTARATE REDUCTASE/SACCHAROPINE DEHYDROGENASE*

(LKR/SDH), whose expression is down- and up-regulated during senescence, respectively, show retardation in the expression after fertilizer application (Schildhauer et al. 2008).

Under low N supply, plants have to economize on this valuable resource. One way is recycling N from older leaves during senescence for reuse in sink organs that require N for growth. Efficient N remobilization is very crucial when N supply and availability in the soil are low and therefore root N uptake is limited (Balazadeh et al. 2014).

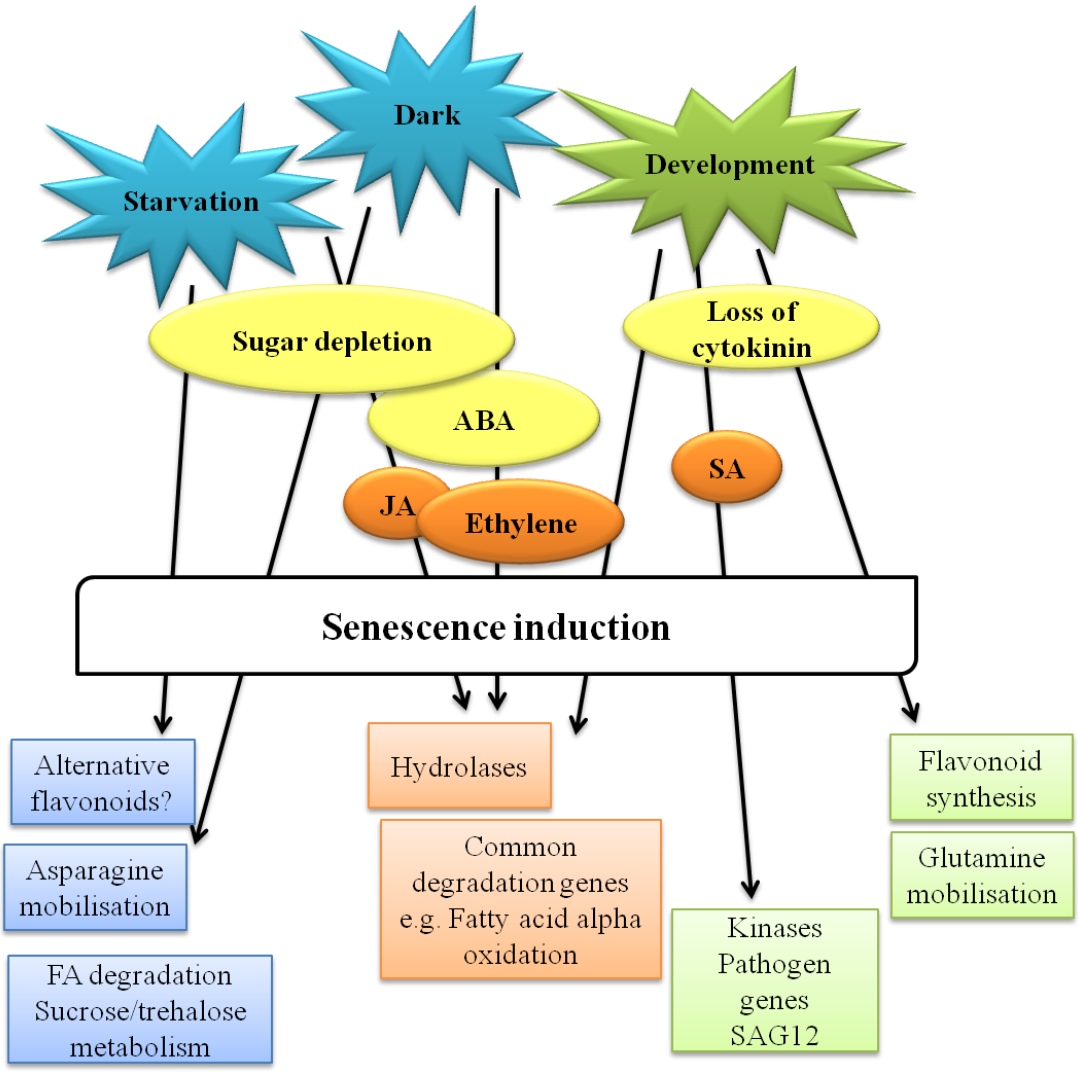


Figure 1. Schematic model illustrating the similar and alternative pathways involved in the three types of senescence. Genes expressed only in developmental senescence (green boxes), starvation-induced senescence resulting from both dark treatment and ageing suspension cultures (blue boxes) and all three types of senescence (orange boxes) are depicted in this model adapted from (Buchanan-Wollaston V. et al. 2005).

1.4 N remobilization during senescence

Nitrogen remobilization is a key factor determining plant nitrogen use efficiency (NUE). ^{15}N tracing studies in cereals, oilseed rape and legumes revealed that N uptake and N_2 fixation decreases during seed filling which indicates that onset of grain filling is a critical phase for nitrogen remobilization (Salon et al. 2001). Leaf protein degradation, especially plastid proteins, during senescence provides an important nitrogen source for the growing leaves and seeds (Masclaux-Daubresse et al. 2010). Figure 2 represents the main enzymatic steps involved in nitrogen remobilization from older leaves (source) to younger leaves (sink).

Functional analyses of senescence associated genes (SAGs) have revealed three major protein degradation pathways occurring in senescing leaves: the ubiquitin/proteasome pathway, the autophagy/vacuolar pathway and the chloroplast degradation pathway (Liu et al. 2008). Rubisco accounts for 50 and 20% of the total soluble protein in the leaves of C3 and C4 plants, respectively. Coordinated and controlled dismantling of chloroplasts occurs at early stages of senescence. Rubisco and other photosynthetic proteins are the main source of N for remobilization during senescence (Martinez et al. 2008). Chloroplast proteases (ClpP) have been categorized in three types based on their subcellular compartments: stroma, thylakoid-membrane (FtsH and Lon) and lumen (Adam and Clarke 2002). Stroma proteases are ATP dependent while thylakoid-membrane and lumina proteases are ATP-independent. Chloroplast proteases are thought to be crucial for the remobilization of free amino acids during senescence (Adam et al. 2006). It has been shown that in tobacco, aspartic protease CND41 is involved in Rubisco degradation allowing subsequent translocation of N during senescence (Kato et al. 2004). However, this is not the case in senescing Arabidopsis leaves where no chloroplast protease has been shown to be involved in Rubisco degradation and it is thought that ROS is a potential initiator of Rubisco degradation in this plant (Desimone et al. 1996, Ishida et al. 1998).

To analyze metabolic markers related to N assimilation and N remobilization pathways, Diaz et al., (2008) studied five recombinant inbred lines (RILs) of *Arabidopsis thaliana*, exhibiting differential leaf senescence phenotypes (from early senescing to late senescing) under nitrogen (N)-limiting conditions. In each line, leaf aging resulted in a decrease of total N, free amino acid, and soluble protein contents. Moreover, the expression of markers for N remobilization such as cytosolic glutamine synthetase, glutamate dehydrogenase, and CND41-like protease was enhanced. This increase occurred earlier and more rapidly in early-senescing lines than in late-senescing lines. ^{15}N tracing analysis during vegetative stage of development showed that N remobilization from the source leaves to the sink leaves was more efficient in the early-

senescing lines indicating that there was a correlation between N remobilization rate and leaf senescence severity at the vegetative stage. However, ^{15}N tracing study at the reproductive stage showed that the rate of N remobilization from the rosettes to the flowering organs and seeds is similar in early- and late-senescing lines. This indicates that at the reproductive stage, N remobilization efficiency is related to the ratio between the biomasses of the sink and the source organs and not senescence phenotypes (Diaz et al. 2008).

In oilseed rape senescing leaves, down-regulation of a protease inhibitor, BnD22, during nitrogen remobilization phase is accompanied by up-regulation of several proteases, including SAG12 which indicates that protease activation is possibly controlled during leaf development in relation with N remobilization (Desclos et al. 2009, Wicker et al. 2007). NRT1.7, a member of NRT1 family, is a pH dependent bidirectional nitrate transporter. It is expressed in old leaves phloem tissue and its expression is induced by N limitation. It has been shown that NRT1.7 is involved in nitrate remobilization from older leaves to younger leaves. This indicates that source-to-sink remobilization of nitrate is mediated by the phloem. Arabidopsis *nrt1.7* mutants showed growth retardation under nitrate limitation. Analysis with ^{15}N revealed that *nrt1.7* mutants are unable to transport N from older leaves to younger leaves and there were more accumulation of nitrate in older leaves of the mutant plants which further confirmed the role of NRT1.7 in nitrate remobilization (Fan et al. 2009). Another Arabidopsis nitrate transporter involved in remobilization is NRT2.4 which plays a double role in roots and shoots of nitrogen-starved plants. It is a high affinity transporter which its expression is induced upon nitrogen starvation. It has been observed that *nrt2.4* mutant lines had lower phloem sap nitrate contents but did not show altered growth or development which may indicates that decreased nitrate content at this level is not limiting for adaptation to N deficiency. Moreover, most likely there are other redundant transporter systems compensating the function of NRT2.4 in the mutant plants (Kiba et al. 2012).

There is a difference in nitrogen remobilization during senescence between perennial and annual plants. Perennial plants are able to grow and reproduce in multiple years. Many perennial plants enter dormancy during winter and recycle nutrients, such as nitrogen, to below ground structures for the next growing season (Schwartz C. and Amasino 2013). In monocarpic annual plants, which only flower once, the main function of leaf senescence is to provide nutrients for seed formation before death of the whole plant (Davies and Gan 2012) and therefore, N remobilization from old leaves to the upper part of the plant is crucial for seed production. One example of economically important annual plants is oilseed rape which has low nitrogen

remobilization efficiency. Thus, oilseed rape is an interesting plant model for studying N remobilization associated with leaf senescence.

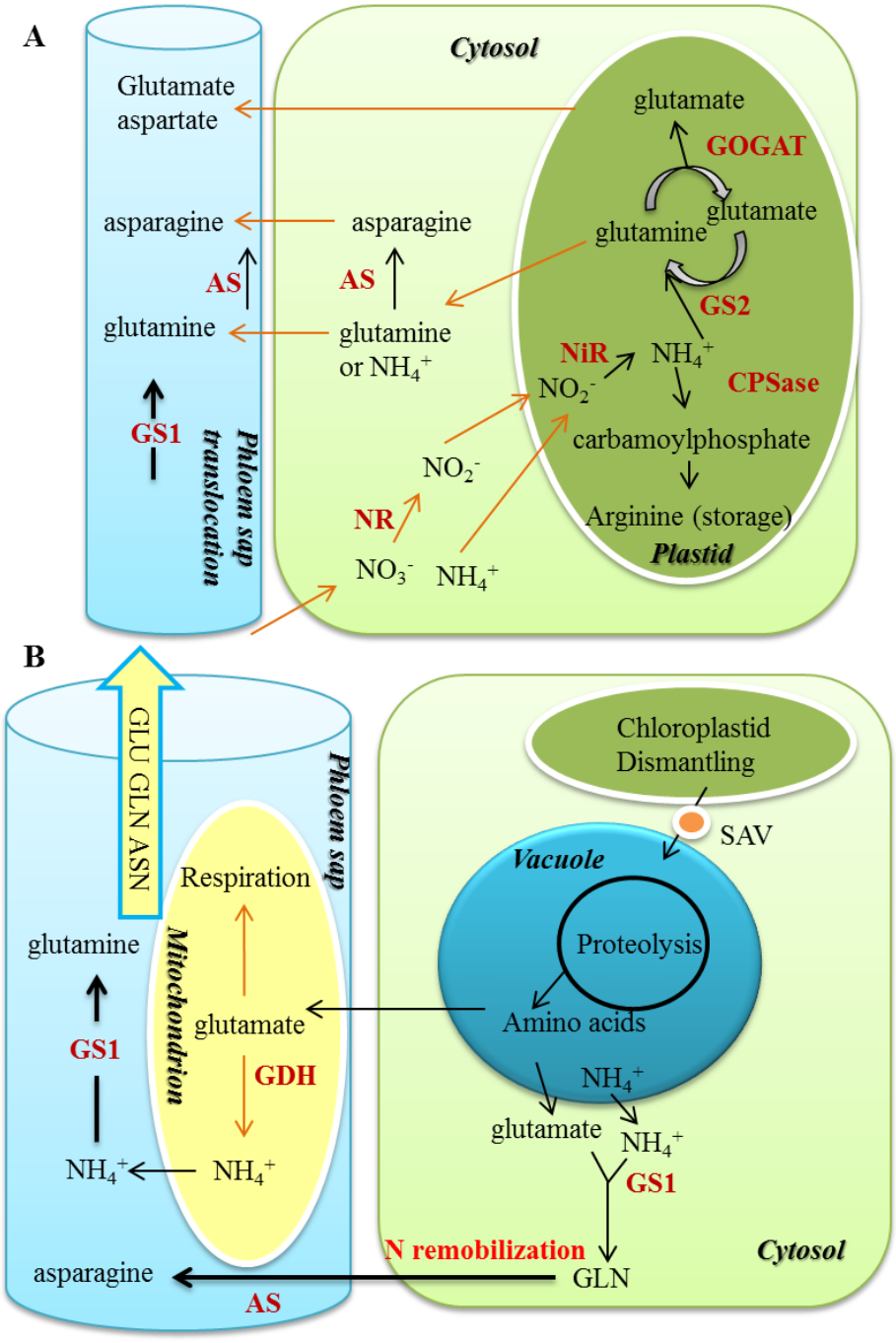


Figure 2. Key enzymes involved in nitrogen management in (A) young and (B) senescing leaves. (A) Nitrate reductase (NR) and asparagine synthetase (AS) are localized in the cytosol, and nitrite reductase (NiR), glutamine synthetase 2 isoenzyme (GS2), glutamate synthase (GOGAT) and carbamoylphosphate synthetase (CPSase) within the plastids of mesophyll cells. Glutamine synthetase isoenzyme 1 (GS1) and AS are located in the cytosol of companion cells. (B) Senescence-associated events include chloroplast degradation and translocation of plastid proteins to the central vacuole via enescence-associated vacuole (SAV) trafficking. Amino acid recycling occurred in mitochondria and cytosol of mesophyll cells and companion cells. Glutamate dehydrogenase (GDH), GS1 and AS are the major enzymes involved in the synthesis of glutamine, glutamate and asparagine in the phloem. Adapted from (Masclaux-Daubresse et al. 2010).

1.4.1 Senescence and N remobilization in oilseed rape

Oilseed rape (*Brassica napus*) is considered as a valuable crop plant which is cultivated for oil and biofuel production. Because of its high capacity for nitrate uptake from the soil, it is also grown as a catch crop to reduce N leaching during autumn and winter (Malagoli et al. 2005). Top 5 oilseed rape producing countries are Canada (16.7), China (14.2), India (7.2), Germany (5.3) and France (4.9). Numbers are million ton in 2012-2013 (http://faostat3.fao.org). Figure 3 provides an overview of the oilseed rape production in the world (2012-2013).

Figure 3. An overview of oilseed rape production in the world (2012-2013). (Modified from http://faostat3.fao.org).

However, despite high nitrate uptake capacity, oilseed rape has low nitrogen use efficiency (NUE). NUE is defined as utilization of available N in different forms in order to maximize grain yield (Gupta N. et al. 2012). One critical factor determining the NUE is the timing of leaf senescence initiation and the efficiency of N remobilization to the sink organs (Gombert et al. 2006). In oilseed rape the onset of senescence at lower nodes and leaf abscission occurs long before flowering and pod filling. As a consequence, nitrogen is inefficiently remobilized from these leaves. Approximately 200 kg fertilizer N ha^{-1} is required for optimal seed yield. At the time of abscission, the lower leaves still have a high N content (2.5-3% of dry weight), resulting in releasing up to 100 kg N ha^{-1} to the soil during winter (Dejoux et al. 2000, Jackson and Overpeck 2000, Musse et al. 2013). Nitrogen remobilization is significantly more efficient in younger leaves in the upper canopy that senesce during pod development. At the time of abscission their nitrogen content is only 1% of dry weight (Malagoli et al. 2005). N distribution

in oilseed rape depends on plant growth stage. Before flowering, 76% and 24% of N are available in leaves and stem, respectively (Schjoerring et al. 1995). After progression of pod formation, N is translocated from leaves to the pod walls and finally to seeds which results in accumulation of 76% of total plant N in the seed during maturity phase.

Low NUE results in demand for higher N fertilization (Gombert et al. 2006) while only 50% of the fertilizer N is recovered by oilseed rape (Desclos et al. 2009). Therefore, improving NUE not only increases N remobilization from source leaves and prevents N leaching from fallen leaves to the soil; it can also improve negative economic and environmental effects of fertilizer application in excessive amounts.

Desclos et al., (2009) studied leaf proteomic changes which appeared during N remobilization that were associated or not associated with senescence of oilseed rape in response to contrasting nitrate availability. Remobilization of N and leaf senescence status were followed using ^{15}N tracing, chlorophyll level, total protein content and a molecular indicator based on expression of SAG12/Cab genes. Three phases associated with N remobilization were distinguished. Proteomics revealed that 55 proteins involved in metabolism, energy, detoxification, stress response, proteolysis and protein folding were significantly induced during N remobilization. Four proteases were specifically identified. FtsH, a chloroplastic protease, was induced transiently during the early stages of N remobilization. Considering the dynamics of N remobilization, chlorophyll and protein content, the pattern of FtsH expression indicated that this protease could be involved in the degradation of chloroplastic proteins. Aspartic protease increased at the beginning of senescence and was maintained at a high level, implicating this protease in proteolysis during the course of leaf senescence. Two proteases, proteasome beta subunit A1 and SAG12, were induced and continued to increase during the later phase of senescence, suggesting that these proteases are more specifically involved in the proteolysis processes occurring at the final stages of leaf senescence (Desclos et al. 2009). These data indicate that specific proteins are playing role at different stages of leaf senescence. However, our knowledge about signaling processes and gene expression changes which is behind the protein expression and especially those ones involved in initiating the developmental or N-induced senescence process in oilseed rape is still scarce.

Since N deficiency promotes an even earlier onset of leaf senescence it may affect N remobilization and thus NUE. Moreover, one of the frequent effects of nitrogen deficiency is limiting crop yields (Frink et al. 1999). Therefore, it is crucial to measure the plant N status in order to optimize N fertilization regimes. In the following section the current approaches to determine plant N level in the field, are discussed and compared.

1.5 Marker genes of N Status in oilseed rape

The nitrogen nutrition index (NNI) is a precise indicator of nitrogen status but it is not so practical because it requires time-consuming measurements and destructive plant sampling at a precise growth stage (Prost and Jeuffroy 2007). Since, lower N content in the leaves results in lower chlorophyll content (Leakey et al. 2009) a common alternative approach to estimate plant N status is measuring leaf chlorophyll content (using SPAD chlorophyll meter) (Miao Y. X. et al. 2009). Chlorophyll meter is a non-destructive, convenient, leaf clip-on device that determines the relative amount of chlorophyll present in plant leaves. However, this method must be performed at the leaf level and therefore, it is very laborious and time consuming to be applied across the large fields (Prost and Jeuffroy 2007).

Remote sensing techniques, based on measuring the reflected radiation from plant canopies, have the potential of evaluating the N status of many plants (Daughtry et al. 2000). Crop canopy sensors are efficient for applications in large scales in the field compared to the SPAD meter (Cao et al. 2013). It is necessary that crop canopy sensors be supplied with their own light sources to avoid limitation by environmental light conditions (Yao et al. 2012). Cao et al., (2013) developed a multispectral crop canopy sensor for rice plants which can calculates the correlations of aboveground biomass and plant N uptake across growth stages. However, this method has its own limitations. One limiting point is the reflectance from the soil which has been shown that significantly affects sensor measurement (Daughtry et al. 2000). Moreover, symptoms of N deficiency occurs more at older, lower leaves and gradually proceeds towards upper part of the canopy and it is not known to what extent lower part of the plant contribute in the whole canopy sensor measurement. Both abovementioned methods are useful when visible symptoms of N deficiency (lower chlorophyll) is detectable and therefore, they are rather late diagnostic tools. For precise N management, it is important to be able to detect N deficiency at earlier stage, before the visible symptoms of senescence can be observed. Since molecular changes occurs before phenotypical changes can be observed, identification of gene expression markers which their expression level is dependent on plant N status can be useful tool to estimate plant N level at earlier stage of N deficiency. Such N-responsive expression markers have already been identified in maize (Yang X. S. et al. 2011). However, despite their importance, they have not yet been reported for other crop plants such as oilseed rape. In oilseed rape, the already known senescence molecular marker, *SAG12*, has been shown to be required for the proteolysis processes occurring at the ultimate stages of leaf senescence when

chlorophyll content is already declined and yellowing of the leaves is visible (Desclos et al. 2009). Therefore, it is not a useful marker for determining N status at earlier stages of leaf senescence. Oilseed rape low nitrogen use efficiency (NUE) is correlated with low N remobilization efficiency (NRE) from source to sink leaves during the vegetative phase (Avice and Etienne 2014). This results in higher fertilizer applications to achieve maximum yield. Using N fertilizers in excess amount is harmful to the environment, especially aquatic ecosystems (algal bloom formation). It is necessary to consider identification of N sensitive expression markers at earlier stage of vegetative phase to improve N management in the crop plants such as oilseed rape through optimization of fertilizer application. Figure 4 shows the trend in application of different types of fertilizers (from http://faostat3.fao.org). As it can be seen, the amount of N fertilizers shows a trend of increase from 2002-2009. Therefore, it is urgent to minimize the amount of fertilizers to protect the environment. Moreover, this will also have financial benefits for crop producers.

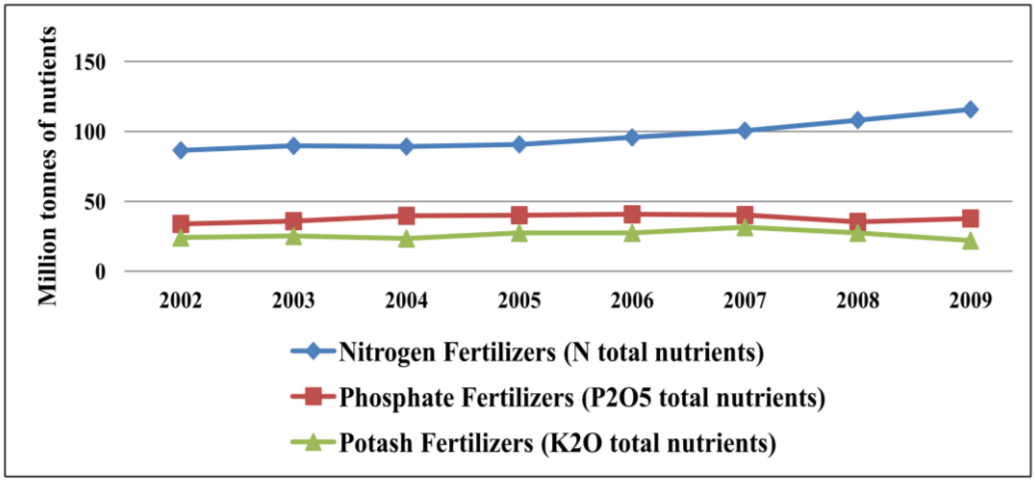


Figure 4. Fertilizer application amount (Million tones) from 2002-2009. (source: http:// faostat3 .fao.org).

1.6 Carbon and nitrogen balance signaling

Understanding the cell response during nitrogen deficiency induced senescence is crucial for improving nitrogen use efficiency and optimizing N fertilization. One important aspect is considering the interaction of N with other essential nutrients, especially carbon (C) due to the interconnection of N and C metabolism. A tight coordination between cellular C and N metabolism is necessary to sustain optimal growth and development of the plant. Furthermore,

C/N balance is also critical for the response to elevated atmospheric CO_2 (Zheng 2009). It has been shown that elevated CO_2 leads to early senescence induction in sunflower (*Helianthus annuus* L.) due to the reduction in N availability and suppression of the key N assimilation enzymes such as nitrate reductase (NR) (De la Mata et al. 2013). In the following sections the interaction between N and C as well as the effect of elevated CO_2 (as C source) on the plants are discussed.

Carbon is one of the important nutrients which have close inter-relationship with N. C and N are both crucial elements for fundamental cellular functions (Zheng 2009). Maintaining C/N adjustment is one of the important factors for optimal plant growth. Higher C/N ratio causes reduction in N uptake and finally lower grain quality in cereals because of lower protein content which has negative health and economic consequences (Kant et al. 2012).

It has been shown that more than half of the Arabidopsis transcriptome is regulated by C, N and the C/N balance (Zheng 2009). Three different proteins including a putative nitrate transporter (NTR2.1), a putative glutamate receptor (GLR1.1) and a putative methyltransferase (OSU1) play important roles in the C/N balance. OSU1 is similar to QUA2/TSD2 which acts in cell wall biogenesis which may indicate that there is a link between cell wall property and the C/N balance signaling (Zheng 2009). Over expression of a maize transcription factor involved in C-metabolism, *DOF1*, in Arabidopsis and rice resulted in higher N uptake and assimilation under low N supply (Fischer et al. 2013, Kurai et al. 2011, Yanagisawa et al. 2004). DOF1 regulates expression of keto acids needed for N assimilation (Fischer et al. 2013). Another example is SNRK1 protein kinase that plays a key role in the global control of plant carbon metabolism (Halford et al. 2003). *SNRK1*overexpression in tomato plants leads to higher N-uptake, C assimilation and promoted fruit ripening (Wang X. L. et al. 2012). These results indicate the effect of C metabolism related genes on the N uptake and assimilation.

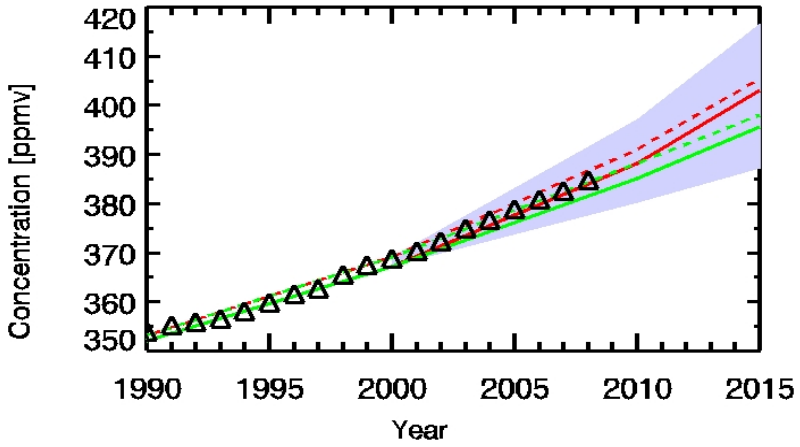
Elevated CO_2 laboratory experiments under different N concentrations, have shown that N deficiency inhibits the promoting effect of elevated CO_2 on shoot growth (Sun J. et al. 2002). Long term (6 years) elevated CO_2 study with different plant species have revealed that N deficiency is a major limitation of sustainability of plant ecosystem response to elevated CO_2 (Reich et al. 2006). Therefore, providing adequate N input in order to keep an optimal C/N balance in plants seems to be important to raise the CO_2 utilization efficiency at the ecosystem level (Zheng 2009).

Analysis of plant responses to elevated CO_2 reveal a clear reduction of N levels and correspondingly, protein levels in plant tissues of C3 species with the exception of legumes (Taub et al. 2008). The reduced N concentration caused by elevated CO_2 could be recovered

neither by increased N supply (e.g. fertilization) in wheat nor by Source-sink manipulations (e.g. panicle removal) in rice (Fangmeier et al. 1999, Shimono et al. 2010). Other macro and micro nutrient also show changes in response to elevated CO_2 but in a lesser extent than N. Upon different growth conditions, potassium and phosphorous amount showed decrease or increase. Lower amount of calcium, magnesium, sulfur, iron, zinc, manganese and aluminum was observed in wheat grains under elevated CO_2 (Hogy and Fangmeier 2008).

1.7 Plant response to elevated CO_2

Increasing emissions from burning of fossil fuels and deforestation resulted in elevated level of atmospheric CO_2 during the last two centuries. Atmospheric CO_2 increased from 280 ppm in pre-industrial era to 379 ppm in 2005 and it may continue to 730-1020 ppm at the end of the 21st century (De Souza et al. 2008). Figure 5 represents the atmospheric CO2 concentration from 1990 to 2015 (source: http://www.ipcc-data.org/).



Figur 5. **Observed and projected atmospheric CO_2 concentrations from 1990 to 2015**. Triangles show annual average marine surface air CO_2 concentrations for the period 1990 to 2008 downloaded from the NOAA ESRL web site (www.esrl.noaa.gov) in September 2009. The red and green lines shows annual average CO_2 concentrations for the SRES A1B (red) and B2 (green) marker emissions scenarios projected using the reference version of the Bern carbon cycle model (solid) and the ISAM model (dashed). These two scenarios give the upper and lower limits of the 6 illustrative/marker scenarios in the period plotted. The blue shading shows the range from low to high climate - carbon cycle feedbacks. All SRES projections are taken from Annex B of the 2001 IPCC Working Group I Report (Source: http://www.ipcc-data.org/).

Elevated level of CO_2 is associated with effects on plant responses such as metabolism, photosynthesis rate and development. In the following sections, the focus is on the effects of elevated CO_2 on different aspects of plant response including photosynthesis, yield, stomatal conductance and transcriptome response.

- Photosynthesis and yield

CO_2 concentration is a limiting factor for photosynthesis (Larkum 2010). There is a difference in photosynthesis response to elevated Co2 between short and long term exposure. Short term elevated CO_2 results in photosynthesis stimulation. It has been shown that, when plants are exposed to elevated CO_2 under controlled conditions, photosynthesis and activity of the CO_2 fixation primary enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), increases (Li P. et al. 2006a). However, the response to long term CO_2 enrichment is variable (Makino and Mae 1999). Down-regulation of photosynthesis capacity in response to long term elevated CO_2 has already been previously reported (Usuda 2006). A mechanism involved in this down-regulation is repression of photosynthesis related genes via sugar accumulation in source leaves (Long et al. 2004). Interestingly, this down-regulation is not observed in species with sink organs for carbohydrates accumulation (Makino and Mae 1999). It has been reported that photosynthesis down-regulation in response to elevated CO_2 is stronger under N limitation than adequate N supply (Isopp et al. 2000, Stitt and Krapp 1999). Under N limitation, development of new sinks is limited which results to source/sink imbalance of plants grown under elevated CO_2 (Hymus et al. 2001, Stitt and Krapp 1999). Another reason for stronger photosynthesis down-regulation in response to elevated CO_2 under low N condition is reduction in the amount of Rubisco (Stitt and Krapp 1999). However, FACE (Free Air CO_2 Enrichment) ring experiments revealed that Rubisco reduction is specific and it is not part of general protein reduction. Since Rubisco contains 25% of leaf N, reduction of its amount to 20% can results in 5% decrease in leaf N (Ainsworth and Long 2005, Spreitzer and Salvucci 2002).

In rice plants grown under elevated CO_2, genes encoding CO_2 fixation enzymes such as carbonic anhydrase, Rubisco, phosphoglycerate kinase and glyceraldehydes 3-phosphate dehydrogenase were down regulated while genes encoding Ribulose-1,5-bisphosphate (RuBP) regeneration enzymes including fructose bisphosphate phosphatase, fructose bisphosphate aldolase, sedoheptulose bisphosphate phosphatase, phosphoribulokinas and starch synthesis including ADP-glucose pyrophosphorylase and starch synthase were up regulated. Therefore, it has been suggested that elevated CO_2 leads to co-regulation of some of the genes involved in

chloroplast primary metabolism pathway (Fukayama et al. 2009). It is also reported that initiation of early senescence under elevated CO_2 is involved in photosynthesis repression (Ludewig and Sonnewald 2000).

Photosynthesis rate is dependent on leaf developmental stage and therefore response to elevated CO_2 is correlated with the developmental age of the leaf (Usuda 2006). For example, it has in poplar plants, gene expression changes in response to elevated CO_2 depend on the leaf developmental age (Taylor et al. 2005).

Due to the fact that human diet is largely based on crop plants and considering increasing amount of atmospheric CO_2 after industrial revolution, it is crucial to develop crop varieties which have higher ability to utilize CO_2 for photosynthesis and higher yield production (Kant et al. 2012). Abiotic stress plays an important role in decreasing the average yield of major crops and global agricultural sustainability. Plant carbon balance mainly determines the crop productivity. Improving Rubisco catalytic activity can result in improved plant productivity and yield (Parry et al. 2013). Analysis of data collected from the effect of CO_2 concentration on Rubisco activity revealed that high CO_2 results in lower Rubisco activity which is especially notable under 200% CO_2 (compared to control treatment) (Galmes et al. 2013). Long term exposure of plants to elevated CO_2, results in lower Rubisco content and down-regulation of genes involved in photosynthesis, such as Rubisco large subunit (RbcL), Rubisco small subunit (RbcS) and Rubisco activase (Rca) (Kant et al. 2012). Considering low NUE in oilseed rape and the cross talk between carbon and nitrogen, it is important to study the interaction between CO_2 enrichment and N supply in this crop. It has been reported that in oilseed rape, elevated CO_2 results in higher biomass acquisition and water use efficiency while it decreases yield and these effects are stronger under normal N compared to low N supply (Franzaring et al. 2011).

- Stomatal conductance

Stomatal pores are surrounded by a pair of special epidermal guard cells and are main gateways for CO_2 influx in the plants (Kim T. H. et al. 2010). Guard cells receive all of the environmental signals including biotic and abiotic stimuli and long distance signals from root and finally manage the suitable turgor pressure changes. In C3 and C4 plants stomata open in response to blue and red light, humidity and low CO_2. On the other hand, stomatal closure is induced by darkness. However, the latter is not the case in CAM plants which convert CO_2 into organic molecules such as malate during night time. Under elevated CO_2 less stomatal opening is

needed for efficient CO_2 influx. Reduction of stomatal conductance under elevated CO_2 leads to higher plant water-use efficiency (Hu H. et al. 2010, Kim T. H. et al. 2010). Drought and elevated ozone can also result in stomatal closure which protects the leaves from oxidative damage induced by ozone (Hirayama and Shinozaki 2007, Hu J. et al. 2007, Shimazaki et al. 2007). Under drought condition the hormone abscisic acid (ABA) is increased which can induce stomatal closure and consequently reduce water loss by transpiration. ABA effect on stomatal movement refers to more than 40 years ago. Due to the increasing level of atmospheric CO_2 concentration and climate changes causing drought stress in plants and because of limited water sources in many areas, ABA and CO_2 has got enormous attention in recent years. Other plant hormones such as auxin, cytokinin, ethylene, brassinosteroids, jasmonates and salicylic acid also play a role in stomatal closure under different environmental conditions (Acharya and Assmann 2009, Melotto et al. 2008).

Short term exposure to CO_2 results in decreasing stomatal conductance and long term exposure to elevated CO_2 can lead to lower stomatal density in the leaves (Gray et al. 2000, Lake et al. 2002). However, there is not much known about the mechanism of stomatal movement and development under elevated CO_2. Studies with *Vicia faba* has shown that anion channels and k^+_{out} efflux channels are activated under elevated CO_2 (Brearley et al. 1997, Gray et al. 2000), concomitantly chloride release from guard cells and finally depolarization happens (Hanstein and Felle 2002, Harada et al. 2003). Moreover, Ca^{2+} is another ion which is required for stomatal closure (Schwartz A. et al. 1988, Webb et al. 1996, Young et al. 2006). There is a big debate about this question that whether CO_2 signal is directly sensed by guard cells or by leaf mesophyll cells. Currently, none of mentioned hypothesis can be excluded (Kim T. H. et al. 2010). More studies are needed to unravel the exact mechanism involved in stomata closure under elevated CO_2. Unraveling the plant response to elevated CO_2 at the transcriptome level provides invaluable information about the genes playing role in this process. In the next section, an overview of several microarray studies focusing on plant transcriptome response to elevated CO_2 and the identified responsive genes is provided.

- Transcriptome response

Plant trascriptome response to elevated CO_2 has been reported in several microarray studies focusing on different species such as Arabidopsis (Li P. et al. 2006a), paper birch (*Betula papyrifera*) (Kontunen-Soppela et al. 2010), rice (Fukayama et al. 2009, Fukayama et al. 2011) and poplar (Gupta P. et al. 2005, Taylor et al. 2005). Some of these studies were performed in

Free Air CO_2 Enrichment (FACE) rings (Hendrey et al. 1993) in order to provide high CO_2 concentrations to plants growing in open area while keeping their intra and inter specific competitions intact and decrease the disturbance to the natural environment (di Toppi et al. 2002).

Gene Expression studies related to CO_2 enrichment showed diverse gene responses which suggest that response to elevated CO_2 differs strongly not only between plant species but also among different cultivars which makes it difficult to find common features (Fukayama et al. 2009). For example, one study with different *Arabidopsis thaliana* ecotypes including Col-0, Cvi and WS grown under elevated CO_2 in FACE ring conditions, aimed to find commonly regulated (Signature) genes in all three lines in response to elevated CO_2. The results of transcriptome analysis showed that there is functional plasticity between different ecotypes which results in only a small number of signature genes which are regulated in all three ecotypes in the same manner (Li P. et al. 2006a) . In the aforementioned study, analysis of gene expression changes in response to elevated CO_2 of three *A. thaliana* ecotypes (Col-0, Cvi-0, and WS grown in FACE ring) revealed that elevated CO_2 results in down regulation of genes involved in chloroplast functions in all lines; however the single genes of different multi-gene families showed different regulation between lines. Moreover, different lines showed a distinct response in case of carbohydrate biosynthesis and partitioning regulation, cell wall biosynthesis, N-allocation, amino acid metabolism and hormone responses independent of plant developmental stage. It seems that adaption of plants to their habitat during evolution and genetic plasticity is behind the nature of responses to elevated CO_2 (Li P. et al. 2006a). Functional annotation of elevated CO_2 responsive genes in soybean (Glycine max) revealed that respiratory break down of carbohydrates is induced which can meet the required energy and biochemical precursors for higher leaf expansion and growth under this condition (Ainsworth et al. 2006). Higher level of CO_2 (800 ppm) results in repression of genes involved in light harvesting complexes and Calvin cycle in *Populus deltoides.* On the other hand, genes involved in carbon distribution were shown to be up-regulated in response to elevated CO_2 which in turn changes the balance between hexose/sugars, organic acids and amino acids (Druart et al. 2006).

Analysis of gene expression changes in response to elevated CO_2 of two Arabidopsis ecotypes (Col-0 and Cvi-0) grown in FACE (Free Air CO_2 Enrichment) ring revealed that elevated CO_2 results in down regulation of genes involved in chloroplast functions in both lines; however the single genes of different multi-gene families showed different regulation between lines. Moreover, distinct responses in case of carbohydrate biosynthesis and partitioning regulation,

cell wall biosynthesis, N-allocation, amino acid metabolism and hormone responses independent of plant developmental stage was observed between two lines (Li P. et al. 2008).

There is not much known about signaling events involved in gene expression changes affecting plant development in response to elevated CO_2. Using deep-sequencing methods, (May et al. 2013) for the first time uncovered microRNAs that significantly alter the expression of genes under elevated CO_2. miR156/157 and miR172 and their target transcription factors including SPLs and AP2-like involved in juvenile to adult phase transition and flowering time of Arabidopsis, were shown to be affected by elevated CO_2 which suggests that they may have some correlations to earlier onset of flowering induced by elevated CO_2. At the same time, microRNAs involved in auxin-signaling, stress responses and potential cell wall carbohydrate synthesis were also observed to be regulated under CO_2 enrichment.

Transcriptome studies provide invaluable information about the regulatory gene networks involved in N deficiency and elevated CO_2 response. However, capacities of whole-genome approaches are particularly limited for species such as oilseed rape with recent polyploidy events in their ancestry, because of the difficulty in correctly assembling very closely related sequences (Bancroft et al. 2011). Therefore, the design of the microarray and subsequent data analysis is very crucial to gain correct information. In the next section, this issue is discussed in more details.

1.8 Aim of the thesis

Oilseed rape is an important crop plant which has a low nitrogen use efficiency (NUE). For improving the NUE of oilseed rape it is important to advance the understanding of N-remobilization and -allocation mechanisms to select genotypes with high NUE and to sensitively diagnose the N status of the plants. The aim of this study was the identification of gene expression biomarkers that indicate early stages of N deficiency and senescence before phenotypic symptoms are visible in order to manage fertilization application. Such early diagnostic tool can prevent subsequent environmental and economical negative effect of fertilizers. Moreover, it was of interest to unravel the genes involved in senescence associated network under both developmental and N-deficiency induced leaf senescence and beside that it was important to pin out the overlaps and discrepancies between leaf #4 as source and leaf #8 as

sink leaf in response to different N treatments. Such whole genome transcriptome response to senescence and N-deficiency has not yet been addressed in oilseed rape.

Nitrogen concentration and climate changes including elevated CO_2 affect yield quality and quantity of crop plants such as oilseed rape. Franzaring et al, (2011), reported the effect of different N supply and elevated CO_2 during development on physiological and phenotypical parameters of oilseed rape grown in growth chamber condition. Using the same plant material, it was aimed to unravel transcriptional changes in response to natural and N deficiency induced leaf senescence under normal and elevated CO_2 in source (leaf #4) and sink leaves (leaf #8) and more specifically to find those genes or biological pathways which are responsible for phenotypical and physiological observations. This study focused on leaf #4 as a source and leaf #8 as a sink leaf to explore if there are any similarities and discrepancies between these leaves under different nitrogen and carbon concentrations. Such information can finally be applicable for improvement of lines with better source to sink relationship. Taken together, the grand aim of this thesis was to unravel transcriptional changes in response to elevated CO_2 under normal and low N condition in source and sink leaves.

Previous oilseed rape microarray studies were all based on Arabidopsis cDNA. However, duplication events, rearrangements at chromosomal level and gene-level deletions makes difficulty for analysis of individual gene sequence in this plant. Moreover, detection of *B. napus* specific sequences which are not present in *A. thaliana* genome is not possible using Arabidopsis array. Therefore, one of the aims of this study was to develop a novel oilseed rape microarray resource using EST sequences belonging to *B. napus*, *B. rapa* and *B. olerace*

2 Materials and methods

2.1 Materials

2.1.1 Apparatus

SPAD fluorometer	SPAD-502PLUS, Konica Minolta
7500 Fast Real-Time PCR system	Applied Biosystems

2.1.2 Enzymes, proteins and kits

Ribolock	Thermo Scientific (#EO0381)
Superscript III	Invitrogen (Life Technologies) (#1808-044)
DNase I	Thermo Scientific (#EN0521)
RNA clean-up & concentrator	Zymoresearch (#R1015)
RNeasey mini kit	Qiagene (#74104)

2.1.3 DNA oligonucleotides

All oligonucleotides are designed based on *B. napus* EST assemblies used for the microarray design and are listed in in 5' → 3' orientation.

Table 1. Primer sequences of the two housekeeping genes (UBC9 and UP1), intron specific primer for gDNA control (SAG12) and primers designed close to 3' and 5' end of ACTIN2 gene for checking cDNA integrity.

Gene ID	Gene name	Forward primer	Reverse primer
At4g27960	UBC9	GCATCTGCCTCGACATCTTGA	GACAGCAGCACCTTGGAAATG

At4g33380	UP1	AGCCTGAGGAGATATTAGCAGGAA	ATCTCACTGCAGCTCCACCAT
At5g45890	SAG12	CGAGAATGCTCTAATGAAGGC	GATCAAGATACGTTGTGCACTC
AT3G18780	ACTIN 2_3’	GTTTCTGTGCACTTTGCGTGTAAC	AGCTGTTTCTTTCAAACGCAGACG
AT3G18780	ACTIN 2_5’	GTGTTGTTGGTAGGCCAAG	GCTTCGTCACCAACGTAG

Table 2. Primer sequences related to section 3.7. The first 34 genes belongs to marker genes identified in Mozart microarray study and the last 12 genes are from Apex and Capitol microarray study. All oligonucleotides are in 5' → 3' orientation.

Gene ID	Forward primer	Reverse primer
AT3G50060	TTTACCGGATCGGCCTGGAATC	AGAAGAAGACGAGCAGCTACGG
AT4G24280	AGCAGCTTCAGCAAGTAAAGGAG	TGTCTCACCCAAATCAAAGCCTAC
AT3G41768	AAGTCGTTTGGAGCTGTATTGAGG	GGCTGCTGGGAATGTCATACTG
AT1G15850	TTCGCCAGTGATGACGCCAATG	ACGCAGTTTCCTAGTCGAAGGTC
AT4G33980	TAACTCAAGGGCGTCCAGGAAAC	TTCACTTCCTATGCGCGGCTTC
AT1G71030	TCGGTCTCGTCCCGATAAAGAC	GCTAGGTTCTTGAAGGGAGCCAAC
AT1G75670	TGCAAAGCTTCTTCAGACTGCTG	ACTTCCGGCTAACACAGGATGC
AT5G02900	AACTGGCCTCTTATCGGGATGC	AATCATAGACCCGGTGGAGCAC
AT5G54490	GGCTTAGCCCTGAACTTGATGGAG	CTCTTCGATCGCTTCCGTGACAAC
AT5G61660	TGGTGGGTATGGTTATGGAAGCG	AGCGGCAACTTCACCATTAGAGC
AT5G54770	AAGACAGCAGGACGGTGGTATG	TCCATCAATAACATTGGGCTGTCC
AT4G27440	ACTCGTTCTCGAAAGACGCCGAAG	ACGCGTCCGCAAAGGATATGTCTC
AT5G16030	GAACGGTCGCTGAAACGATACG	CTTCCTTCTCATCCTCTGCCAAC
AT3G11964	TCAAACGTGTGGAGGCTTATGGC	ATCCAACCATGCCCGTTTGGTC
AT5G03545	GGATTGTGTGTGAATGGAGCGATG	AATCAAGCTGGTCTCCATCAACC
AT2G35930	ATCATGGAGCAGCCATAGCAGTG	ATCGCTAGCCGTCTGAGAAACC
AT5G61380	AGTGGCCTGTTCCTACTGGTTC	TTGACGTGGCTGTGCCATTAGC
AT4G04940	TGATGTAGGCCCGGAACAAACC	TATCTCCCGACAGCGATGGAATC
AT5G05600	TTACCGGACGATCAAGTCTTCGG	ACGCATGAGGATGAGGCTTGAC
AT5G42330	TGTCAAGATCAGAGCTGCTACGG	TCCTCCTGGCTGAATACACGTC
AT1G19300	TTCTGGTCTAACCCGACTCTCTCC	TCACTCCGGTGTTGAAGTAGCAG
AT1G12520	GCTGCTGTTGTTGCTAGAAGCG	TCCCAAACGGTGGTTCCATCAC
AT1G20220	TCAACCTCACCACGAAGCTCAG	ACTGTTGAGGAGCATTGTGTCC
AT4G15440	AATGGTCCACAAACCGGTACGC	CCATGTCTTTGGCTGCACACTG
AT1G17870	AAAGACCAGCGAAAGCGAGAGG	TTGCCAAATGCGGTCGAAGGAG
AT1G25275	CGCAAGTGCTCTATTCGATGTGG	AGTTCAAGCACCAGTCTTTCCTG
AT5G24660	TGAAGCCGAATCGTTGGACGAG	AGAGACACGAGAGAGTTCGTTCAC
AT5G08690	TGCATTGCGATTCCCGTCATTTG	AGTGAGTGAGTATGTCCGTTCAGC
AT5G42900	ATGTGAGGCAGCCTGAGTATCG	AGAAACTCGTGAGAGCGACGAC
AT4G13850	GGATTCTCCTCTGCCTCTTTCCTC	CGGTCTGAGAAACCACACCTTGTC
AT5G67300	TACGGACAACGCGGTGAAGAAC	GGCGTAGAAACCACCGCATTTC
AT4G39200	ATGAGGAAAGTGCCTCGAAAGGG	TCAGCACATCTTCCGTCTCAGC
AT3G22840	TTAGCGGTCCAGCACCAGAAAG	TGGAAAGTTCAACCGCCAAGG
AT3G15590	ATGAAGAGGGCAGGGTATGCAG	TCACATACGCCAACAGCACAGTC

AT1G69490	ACGAGAGCTTTCTTGACGCCTAC	TTGGAAAGCGACCGGTACAGAC
AT4G22920	CTTCACTTGATGGTCACAGGACTC	ACCGGTGGGAAACAACAACTAC
AT5G20280	TCTGGACAATGGTCTCCTTGTGG	ACAAGCTTGAGAAGAGCTTCGG
AT1G26440	TCTGGTGTCGCATGCTTCTTGG	AGCAGAGTTTGAAGAGTGGACAGC
AT4G35420	TCCACACGTTACCCATCAGTCC	AGTCGTAATGCAGCCTGTTAAGC
AT4G22880	TGAGCCACCCAAGGATAAGATCG	GCACCAACTCCTCTTGTTCGTTTC

2.1.4 Plant material and growth conditions for qPCR analysis and extension of candidate marker genes

2.1.4.1 *Brassica napus,* spring cultivar (cv. Mozart) plant material and growth conditions for microarray experiment

Nursing, growth and physiological parameters of the oilseed rape cultivar cv. Mozart are described in Franzaring et al., 2011. In brief, plants were grown in the growth chambers. The maximum light intensity was set to 1000 µmol m^{-2} s^{-1}. Climatic conditions and CO_2 concentrations which simulated ambient profiles of climatic conditions (temperature, relative humidity and irradiation) in terms of daily and seasonal variation. CO_2 concentrations were set to a constant value of 380ppm in the ambient and to 550ppm in the elevated treatment and were measured with Vaisala CO_2 sensors which were cross-checked with an external CO_2 monitor (Sick-Maihak, Hamburg, Germany) from time to time.

The supply of nutrients was based on (Egle et al. 2008). Nitrogen was added to each of the pots in three equal gifts as NH_4NO_3. Leaf samples were collected at multiple time points and from different whorls to identify genes that are differentially regulated during developmental and N deficiency-induced senescence in early and late developing leaves including leaf #4 and leaf #8, respectively. Leaf discs from representative areas of the leaf #4 and leaf #8 were collected at 78, 85, 92, 99 and 106 days after sowing (DAS) from plants grown at normal N supply ("NN": 150 kg N ha^{-1}) or under N deficiency ("LN": 75 kg N ha^{-1}). For each sample three biological replicates from different plants were collected, with the exception of leaf #4 at 78 and 99 DAS (H5) where only two replicates could be analyzed. Due to the limitation in the available number of microarrays, it was not possible to analyze more leaf samples.

For studying the transcriptome response to elevated CO_2, leaf #8 and leaf #4 from 92 DAS (H4) plants grown under both normal and N deficiency conditions were chosen for expression profile study.

2.1.4.2 Greenhouse grown plants (winter cultivars)

Growth and nutrient treatments of 4 different winter cultivars including NPZ2, NPZ5, NPZ1005 and MSL101B was conducted in collaboration with group of prof. Walter Horst, Leibniz University, in Hannover. After vernalization and before transplanting initial fertilization based was added to the plants (Table 3). To provide enough nutrients during plant development, macro and micro nutrient was added to the plants (Table 4). Time table of N fertilization and other nutrients is provided in table 5.

Table 3. Initial fertilization including basic substrates (before transplanting).

Nutrient	Form	Amount (mg)
N	Ca(NO3)2·4H2O	250
K	K2SO4	100
Mg	MgSO4·7H2O	50
Micronutrients	Flory 10	100
P	KH2PO4	50

*Ferty10: Commercially available fertilizer containing micronutrient

Table 4. Amount of the nutrients added to the plants during development (after transplanting).

Nutrient	Concentration	Stock solution
	In the final solution [mg/100 ml]	[g/60 L]
P	2	5.27
K	20	26.75
Mg	2	12.17
*Ferty 10	10	6

Table 5. Time table of N fertilization and other nutrients applied during plant development (after transplanting). X represents fertilization (for N fertilization each time 100 ml N solution containing 250 mg N was given), DAT: days after transplanting. The oldest leaves of the plants at 25 DAT were analyzed for the experiment represented in section 3.7.1.

Date (DAT)	N fertilization		
0	X	X	X
7		X	X
14			X
21			X
Total N	250 mg	500 mg	1000 mg

Date (DAT)	other nutrients than N (see table 5)
7	X
11	X
18	X

2.1.4.3 Field grown plants (cv. Major)

In collaboration with group of Prof. Nicolaus von Wirén, IPK, Gatersleben, oilseed rape winter cultivar, Major, grown in the field under normal and low N conditions were harvested. At the time of harvest plants were in the early flowering stage. Plants were previously vernalized 8-12 weeks at 4 °C under short day conditions (8 h day, 16 h night). NH_4NO_3 fertilizer was applied in the granulate form (Yara company). Plants were fertilized with 20 kg N/ ha in autumn. During spring 40 kg N/ ha and 60 kg N/ ha fertilizer was respectively applied before any side branch was available and at the emergence of the single closed flowers on the main inflorescence. Only normal N treated plants received fertilizer and the low N plants did not receive any fertilization at these two time points.

2.2 Methods

2.2.1 Bioinformatics tools

For analyzing the large data set of microarray, different bioinformatics tools were used. Table 6 represents the main software and tools which were used in the current research.

Table 6. Computer programs, databases, online tools and softwares used for data anaylsis

Program	Application	Reference
MapMan	Displaying gene expression data onto diagrams of metabolic pathways or other processes	(Usadel et al. 2005)
SuperViewer	Identification of significantly overrepresented biological pathways relevant to the specified list of genes	(Provart and Zhu 2003)
DataAssist	qPCR data analysis	Applied biosystems
BioEdit	Sequence alignment editor and local blast	(Hall 1999)
LIMMA	Statisitcal analysis of microarray	(Smyth 2004)
QuantPrime	qPCR primer design	(Arvidsson et al. 2008)
TAIR	Containing *A. thaliana* genetic and molecular data	(Lamesch et al. 2012)
PlantGDB	Containing tools and resources for plant genomics (e.g. *B. rapa* and *B. oleracea* sequences)	(Dong et al. 2004)
NCBI	Publicly available genome sequence data: representation and analysis	(Tatusova et al. 1999)
MultiExperiment Viewer (MeV)	Microarray data analysis tool (clustering, visualization, classification, statistical analysis)	http://www.tm4.org

2.2.2 *Brassica napus* microarray design and annotation

With the aim to represent a maximum number of *Brassica napus* transcripts expressed in leaves a novel *B. napus* microarray was designed for the Agilent platform by applying a probe-preselection strategy established by ImaGenes GmbH, Berlin. All ~10^6 publicly available *B. napus* EST assemblies from the *Brassica napus* Gene Index (BNGI version 031910; http://compbio.dfci.harvard.edu/tgi/plant.html), the *B. napus* 95k microarray assembly (Trick et al. 2009), and the Agilent 4x44k *B. napus* microarray (G2519F-022520) were collected and clustered by BLAST-matching all-versus-all followed by accurate assembly, resulting in a total of 78,986 non-redundant clusters ('unigenes'). For each cluster ~10 different 60mer oligoprobes were calculated and printed on two microarrays. To select the best-performing probes for expressed genes, one array was hybridized with labeled cRNA that was synthesized from a mixture of total RNA from various *B. napus* cv. 'Mozart' shoot tissues including differently aged leaves and labeled using the Quick-Amp One-Color Labeling Kit (Agilent Technologies, CA). The second array was hybridized with labeled genomic DNA to select the best-performing probes for non-expressed genes. Eventually, 59,577 probes were selected for the production of microarrays in the Agilent 8x60k format.

To annotate the microarray, the 59,577 'unigenes' were BLASTed against the TAIR10 *Arabidopsis thaliana* cDNA set, the *Brassica rapa* complete transcript sequences (PlantGDB, http://www.plantgdb.org/) and all ~15,000 *B. napus* mRNA sequences in the NCBI GenBank. Local Blast search was performed using bioEdit software (Hall, 1999).

2.2.3 Microarray analysis and data processing

Microarrays were scanned on an Agilent SureScan Microarray Scanner and the images were read and processed with the Agilent Feature Extraction software using default settings.

Data processing, normalization and data analysis was performed using the Bioconductor package Limma (www.bioconductor.org/packages/release/bioc/html/limma.html; Smyth, 2004).

2.2.3.1 Statistical analysis using LIMMA (Linear Model for Microarray Analysis)

LIMMA library was downloaded from Bioconductor website (http://www.bioconductor.org/ packages/release/ bioc/html/limma.html; Smyth, 2004), then installed and loaded in R program. Tab-delimited targets file, which contains both the names of Agilent Feature Extraction raw data text files and the corresponding sample information were loaded. Read.maimages function was used to load the data into an RGList object. Within array (Background subtraction) and between arrays normalization (quantile normalization) was performed (Figure 6). The average of replicated spots was calculated using avereps function. A design matrix was build for the linear modeling function and the intensity values were applied as lmFit function. Each contrast matrix of interest indicating different comparisons between N treatment, CO_2 and leaf number was created and applied to modeled data for computing the statistics of the data. FDR level <0.05 was applied for multiple testing corrections and the fold change was set to ≥ 3.

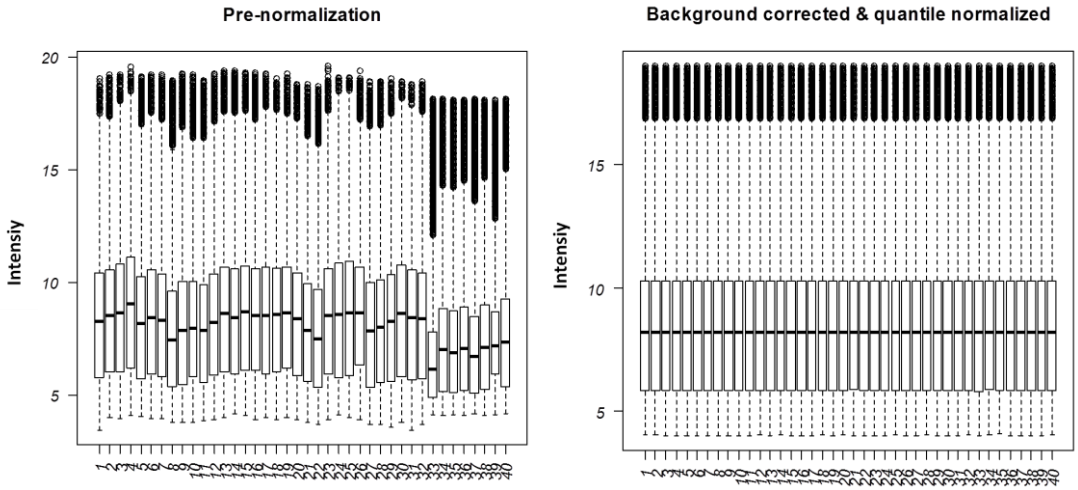


Figure 6. Box plots before and after data normalization. Box plots of the arrays representing the median and scales of intensities of arrays before (left panel) and after normalization (right panel).

2.2.3.2 Principle component analysis (PCA)

The normalized and log_2 transformed data were loaded in TIGR Multiple Experiment Viewer (MEV) software (v4.9) (source: http://www.tm4.org/index.html). PCA was conducted on all samples using median as centering mode. The first and the second components representing highest eigenvalues were considered as the main factors causing variances.

2.2.4 qPCR primer design for *B. napus* 'unigenes'

qPCR primer pairs were calculated by QuantPrime (www.quantprime.de; Arvidsson et al., 2008) after importing all *B. napus* EST assemblies used for the microarray design (collaboration with Samuel Arvidsson, MPI-MP Golm).

2.2.5 RNA extraction, cDNA synthesis and qPCR assay

Total RNAs were isolated from the leaves using hot phenol method. Concentrations and purities of RNAs were measured by Nanodrop ND-1000 spectrophotometer. 1µg RNA was used for DNA digestion using DNase I (Fermentase). Absence of gDNA was verified using *SAG12* intron specific qRT primers (Table 2). cDNA was synthesized using superscript III enzyme (Life Technologies) following manufactor's instructions. cDNA integrity was checked using primers designed close to 3' and 5' end of ACTIN2. qPCR reactions were performed in 5µl total volumes including 2.5 ml SYBR Green master mix, 2µl forward and reverse primers (0.5 µM final concentration, each) and 0.5µl cDNA. UP1 and UBC9 were used as reference genes. The following standard thermal profile was used for all PCRs: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analyzed using the DataAssist v3.01 software (Life Technologies).

3 Results

3.1 *Brassica napus* microarray and experimental set-up

- *Brassica napus* microarray

With the aim to represent a maximum number of *B. napus* transcripts expressed in leaves a novel *Brassica napus* microarray was designed on the Agilent platform by applying a probe-preselection strategy established by ImaGenes GmbH, Berlin (now SourceBiosciences, http://www.sourcebioscience.com). All ~10^6 publicly available *B. napus* EST sequences from the *B. napus* Gene Index (BNGI version 031910), the *B. napus* 95k microarray assembly and the Agilent 44k *B. napus* microarray were collected and clustered by BLAST-matching all-versus-all followed by accurate assembly, resulting in a total of 78,986 non-redundant clusters ('unigenes'). For each cluster ~10 different 60mer oligo probes were calculated and printed on two microarrays. To select the best-performing probes for expressed genes, one array was hybridized with labeled cRNA that was synthesized from a mixture of total RNA from various *B. napus cv*. 'Mozart' shoot tissues including differently aged leaves. The second array was hybridized with labeled genomic DNA to select the best-performing probes for non-expressed genes. Eventually, 59,577 probes were selected for the production of microarrays in the Agilent 8x60k format.

To annotate the microarray, the 59,577 'unigenes' were BLASTed against the TAIR10 *Arabidopsis thaliana* cDNA set, the *Brassica rapa* complete transcript sequences (PlantGDB, http://www.plantgdb.org/) and all ~15,000 *B. napus* mRNA sequences in the NCBI GenBank. When applying a threshold of E-value <10^{-6}, for 54,676 (92%), 55,887 (94%) and 14,794 (25%) of the *B. napus* 'unigenes' on the microarray homologs were identified in *Arabidopsis thaliana*, *B. rapa* and *B. napus*, respectively.

For only 5,522 of the 19,195 Arabidopsis genes with homology to *B. napus* 'unigenes', a single *B.napus* 'unigene' is represented on the microarray, whereas for 71% more than one *B.napus* 'unigene' with a BLAST E-value <10^{-6} is found on the microarray (Table 7).

Due to genome triplication, genome fusion and gene loss events in the evolution of *B. napus* since the divergence of the Arabidopsis and Brassica clades from an ancestral Brassicaceae progenitor with a duplicated genome (Rana et al. 2004), from zero to six (or even more) orthologs of any Arabidopsis gene may occur in *B. napus*. To facilitate functional pathway analyses with tools developed for *Arabidopsis thaliana* (e.g. MapMan), all *B. napus* 'unigenes'

with homology to one Arabidopsis gene were hierarchically ranked according to their BLAST E-value and regulation in any plant samples. Among a group of *B. napus* homologs for the same Arabidopsis gene the significantly regulated one with the lowest E-value was selected as the most informative homolog of that *Arabidopsis thaliana* gene for further bioinformatical analysis. The number of transcripts belonging to each biological categoriy in MapMa (based on TAIR10), number of unigenes (one or multiple unigenes representing the same *A. thaliana* gene) and number of genes (based on lowest E-value for *A. thaliana* gene) belonging to each biological category is provided in table S1. Out of 66 MapMan biological categories (and subcategories) 41 showed more than 70% coverage in *B. napus* microarray indicating a high coverage of biological categories in the custom made microarray used in this study (Table S1). However, several categories such as DNA synthesis (10.05%), micro RNA and natural antisense (14.8%), cell cycle (23.6%) and biotic stress (25.5%) showed less than 30% coverage. This can be attributed to the source of ESTs which was used for the oilseed rape microarray. The present design of the Bn-microarray is based predominantly on three sets of ESTs from B. *napus*, *B. rapa* and *B. oleracea* that were available in September 2007 (Trick et al., 2009). Only a small fraction of these ESTs was generated from leaf material, most came from flowers and developing seeds. Therefore, it is probable that some of the genes with tissue specific expression (e.g. expressed only in the leaves) may not be available in the current version of Bn-microarray. This may explain the low coverage of the genes belonging to the above mentioned biological functions. The possible strategies for improving the available Bn-microarray are discussed in section 4.1. Moreover, it cannot be completely excluded that some of the Arabidopsis genes may have evolved differently in oilseed rape to gain a new function and therefore it is not possible to represent them as the Arabidopsis homologue genes, due to sequence dissimilarity.

Table 7. Number of *Brassica napus* unigenes representing *A. thaliana* homologue genes.

B.n. 'unigenes' / A.t. gene	A.t. genes	(Bn/At)*At
1	5522	5522
2	5015	10030
3	3336	10008
4	2178	8712
5	1342	6710
6	766	4596
7	461	3227
8	255	2040
9	132	1188
10	70	700
11	34	374
12	28	336
13	20	260
14	5	70
15	6	90
16	2	32
17	4	68
18	3	54
19	2	38
20	2	40
	19183	54095

- Experimental set-up

Old leaf (#4) and young leaf (#8) from oilseed rape plants grown in growth chamber under sufficient (150 kg N ha^{-1} NH_4NO_3) or limiting (75 kg N ha^{-1} NH_4NO_3) nitrogen were harvested at 78, 85, 92 and 99 DAS (leaf #4) and 99 and 106 DAS (leaf #8), for expression profile study. Figure 7 shows the plants during these time scales.

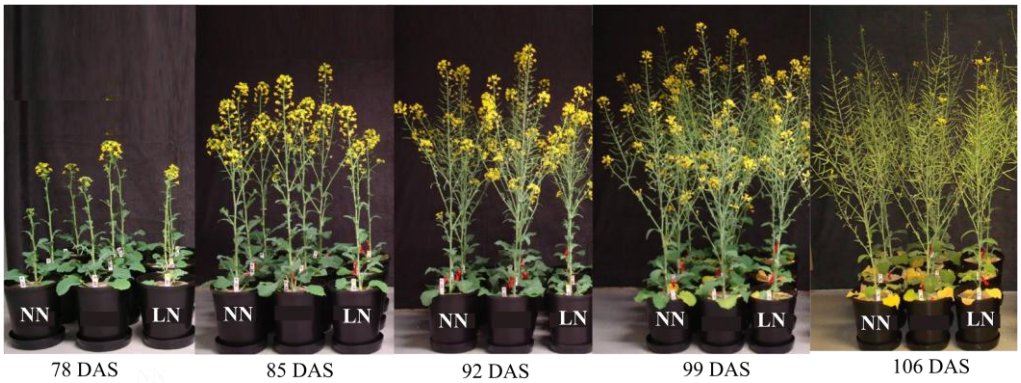


Figure 7. Oilseed rape plants (cv. Mozart) grown in growth chamber under normal (NN) and low (LN) N conditions during development from 78 until 106 DAS. DAS: days after sowing. (Collaboration with Prof. Fangmeier, university of Hohenheim).

Morphological and performance analysis of the plants used in the current study has been previously reported by Franzaring et al., 2011. Using an oilseed rape custom-made microarray it was aimed to unravel the transcriptome response of these plants to different N conditions during development to pin out the molecular networks playing role behind the morphological observations. N limitation caused lower chlorophyll content, reduced biomass and lower harvest index. General speaking, plants grown under N starvation showed earlier induction of leaf senescence compared to those with adequate N supply. This study was focused on leaf #4 and leaf #8 as early and late developing leaves respectively. It was of interest to compare the transcriptome response of old leaves (#4) with young leaves (#8). Old leaves act as a source and transport nutrients toward younger leaves and seeds while young leaves act as an active sink for nutrients transported from older leaves. Therefore, I compared the transcriptome response of source and sink leaves under different N supplies during development in oilseed rape. Moreover, this study was extended to elevated CO_2 samples to examine the effect of future level of CO_2 on the transcriptome response of oilseed rape as a main crop plant. Elevated CO_2 resulted in a strong branching out, especially at higher N level, which seems to be due to the lack of meristem switch off under this condition. At the same time plants produced less ripe pods resulting in the lower final yield. The exact molecular mechanism underlying the higher vegetative and lower reproductive growth under elevated CO_2, has not yet been elucidated. Moreover, plants grown under elevated CO_2 showed significantly higher water use efficiency, however this effect was also N dependent. Since the most drastic phenotypical changes in response to high level of CO_2 occurred at a later time point, 99 DAS (H5), it was expected that transcriptome changes can be observed at earlier harvest, 92 DAS (H4) and therefore this was the time point which was chosen for CO_2 transcriptome study. Transcriptome response of leaf #4 (source) and leaf #8 (sink) in response to elevated CO_2 were analyzed.

3.2 Comparison of senescence indicator genes between normal and low N conditions

Since N limitation causes an earlier senescence induction, it was of interest to compare the effect of normal and low N treatment over the time on the expression of well known senescence progression molecular marker, *SAG12*, in oilseed rape plants.

The *B. napus* microarray carries 30 probes with highest homology to the senescence marker gene AtSAG12, of which 3 are derived from BnSAG12-1 and 27 from BnSAG12-2 (but only 8 of the BnSAG12-2 probes show differential signals). All SAG12 probes exhibited only

background signals until 85 DAS (H3). In plants grown under optimal N supply, the SAG12-1 probes showed in leaf #4 a moderate increase (~27 fold) between 85 DAS (H3) and 92 DAS (H4), and a massive increase (~600 fold) between 92 DAS (H4) and 99 DAS (H5). In plants grown under N deficiency, SAG12-1 expression increased ~700 fold between 85 DAS (H3) and 92 DAS (H4), and another ~100 fold between 92 DAS (H4) and 99 DAS (H5) (Figure 8). Thus, senescence initiated one week earlier under N deficiency compared to optimal N supply, which is consistent with the earlier observations by Gombert et al. (2006). Interestingly, *SAG12-2* appears to follow the same trend like SAG12-1 (Figure 8). Furthermore, Arabidopsis genes that are known to be down regulated early or late in senescence, e.g. *Lhcb2* (At3g27690) or *Lhcb3* (At5g54270), were also down-regulated early or late in the oilseed rape leaves that were analyzed.

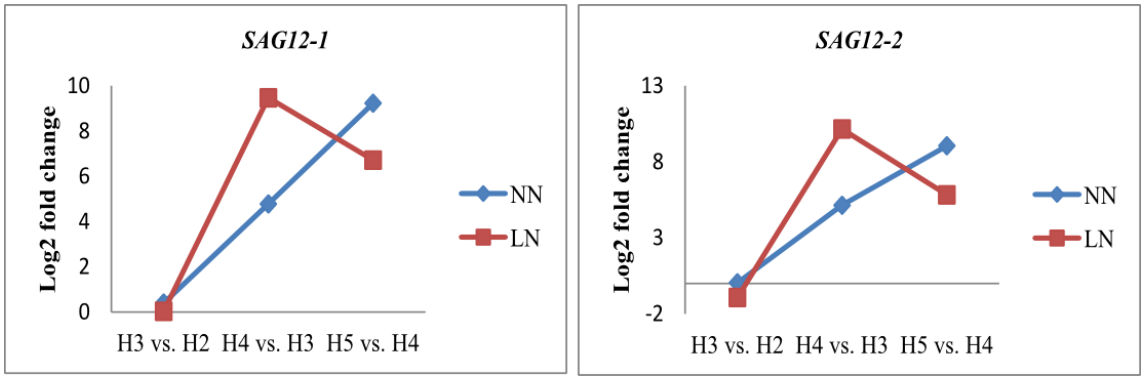


Figure 8. SAG12-1 fold changes during developmental and N-deficiency induced senescence. Log2 fold changes of senescence marker genes *SAG12-1* and *SAG12-2* in leaf #4 under normal (NN) and low (LN) N conditions. H stands for harvest. The values are the log2 ratio of expression level of each harvest divided by the previous one. H2: 78 DAS, H3: 85 DAS, H4: 92 DAS and H5: 99 DAS (DAS: Days After Sowing, H: Harvest).

Taken together, it can be concluded that at optimal N supply senescence is initiated in leaf #4 between 85 and 92 DAS (H4) and under N deficiency conditions a couple of days earlier. Chlorophyll fluorescence measurements of the same plants with a SPAD meter (Figure 9) that revealed a strong decline in chlorophyll content around 85 DAS (H3) in leaf #4 under low N supply. This indicates that senescence associated gene expression changes which results to this phenotypic observation likely have been occurred at the previous time points (Franzaring et al. 2011). Analysis of gene expression changes, provided in the following section, will give more insight in to the most crucial time points for N-deficiency induced leaf senescence in oilseed rape.

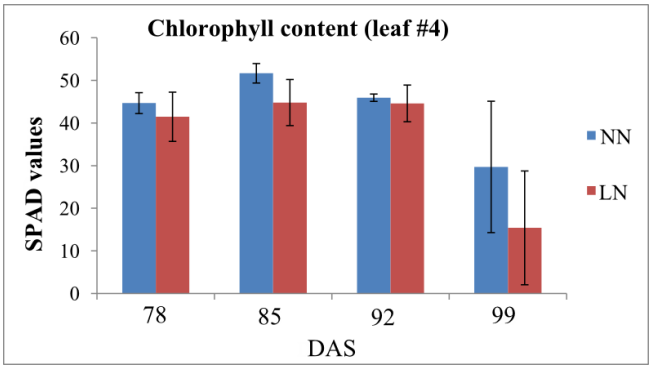


Figure 9. SPAD values of leaf #4 under normal (NN) and low (LN) N conditions during development. DAS: Days after Sowing. Each value is the mean of the three biological replicates (n=3) and the error bars correspond to the SD. SPAD values are retrieved from Franzaring et al., 2011.

3.3 Analysis of transcriptome response of oilseed rape to developmental and N-deficiency induced leaf senescence in early (#4) and late (#8) developing leaves

To identify the molecular network involved in response to normal and low N and also to unravel the overlaps and discrepancies between source leaf (#4) and sink leaf (#8), the transcriptome response of the relevant samples were studied. Differentially expressed genes were determined using LIMMA method. Functional categories of the identified genes are shown using MapMan software (based on TAIR10) and the overrepresented biological pathways are identified via SuperViewer tool.

3.3.1 Comparison of N effect on transcriptome response in leaf #4

It was of interest to identify differentially regulated genes in response to different N treatments in leaf #4 and to unravel the similarities and differences between normal and low N supply. To achieve this, two different comparison approaches were performed. In the first set of comparison, normal and low N samples were compared and the genes with differential regulation under low N relative to normal N were retrieved and were further analyzed to identify their relevant biological functions. In the second comparison approach, gene expression changes at each time point relative to the previous time point at each N treatment was calculated and differentially regulated genes were further analyzed to identify unique and shared pathways in response to different N treatments.

-N dependent transcriptome response of old leaf (#4) at different time points

To identify the genes with significant regulation changes between two different N treatments at each time point, gene expression fold changes under low N relative to normal N was calculated and significant differentially expressed genes were retrieved. The genes were further analyzed for identification of relevant biological functions using SuperViewer software. The results are provided in the following section.

Comparison of gene expression changes between normal and low N conditions at each time point showed that the differences are more pronounced at later time points because there are higher numbers of differentially expressed genes at 92 DAS (H4) and 99 DAS (H5) between normal and low N samples (Figure 10).

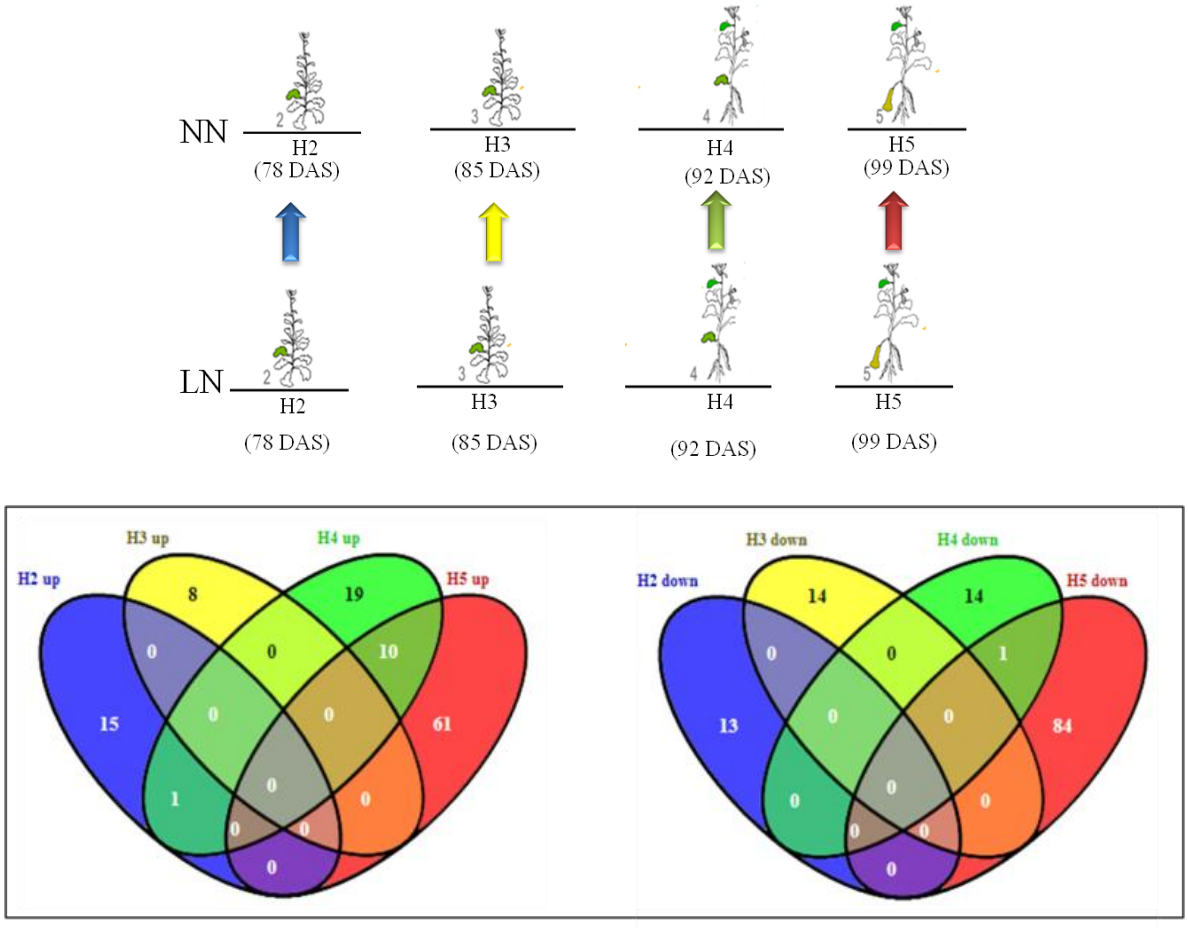


Figure 10. Venn **diagrams representing number of differentially regulated genes at each time point**. The number of unique and shared significantly deregulated genes in leaf #4 between normal (NN) and low N (LN) at each harvest time point (H2: 78 DAS, H3: 85 DAS, H4: 92 DAS, H5: 99 DAS) is represented. Venn diagrams are created using Venny online tool. All of the regulated genes are provided in table S2.

Data analysis using SuperViewer revealed that the only functional categories which showed up-regulation at 78 DAS (H2) was protein (2 synthesis gene out of 1452 and 3 degradation genes out of 2137 genes in TAIR10) while oxidative pentose phosphate (OPP) (1 out of 31 genes in TAIR10) and hormone metabolism (2 out of 543 genes in TAIR10) showed down-regulation. There was no significantly up-regulated pathway at 85 DAS (H3) while co-factor and vitamin metabolism (1 out of 81 genes in TAIR10) were down-regulated at this time point. At 92 DAS (H4), polyamine metabolism (1 out of 16 genes in TAIR10) was up-regulated but no pathway showed down-regulation. At 99 DAS (H5), polyamine metabolism (1 out of 16 genes in TAIR10) and secondary metabolites (3 out of 445 genes in TAIR10) showed up-regulation whereas gluconeogenesis/glyoxylate cycle (1 out of 13 genes in TAIR10) and tetrapyrrole synthesis (1 out of 48 genes in TAIR10) were down-regulated. Taken together, not so many genes were regulated at this set of comparisons. However, N-related pathways such as protein and polyamine metabolism showed up-regulation. Interestingly, between up-regulated genes were the protein degradation genes indicating higher protein catabolism under low N condition. All of the regulated genes are provided in table S2.

- Time dependent transcriptome response of leaf #4 to different N treatments

To explore the transcriptome response of old leaf (#4) during developmental and N-deficiency induced leaf senescence, I compared the gene expression changes at each time point relative to the previous time point under each N treatment. Such analysis provides a time scale snapshot representing the unique and overlapping genes involved in natural and/or N deficiency induced leaf senescence.

In order to have an overview about the factors which cause the most variances between the samples and also to see which sample are more close or deviating, principle component analysis (PCA) was performed using MEV software (Figure 11). Leaves belonging to the same time point but different N concentrations show a more similar gene expression pattern (represented by less distance in figure 11) than leaves belonging to different time points with the same N treatment (represented by higher distance in figure 11). At later time points (92 and 99 DAS for leaf #4 and 106 DAS for leaf #8) there is more variation in gene expression between leaves from plants grown under different N concentrations. These results indicate that 1) age has a stronger impact on gene expression compared to N. 2) The effect of N on gene expression is greater in older plants (92 and 99 DAS). 3) The above-mentioned results are true for both old (#4) and young (#8) leaves.

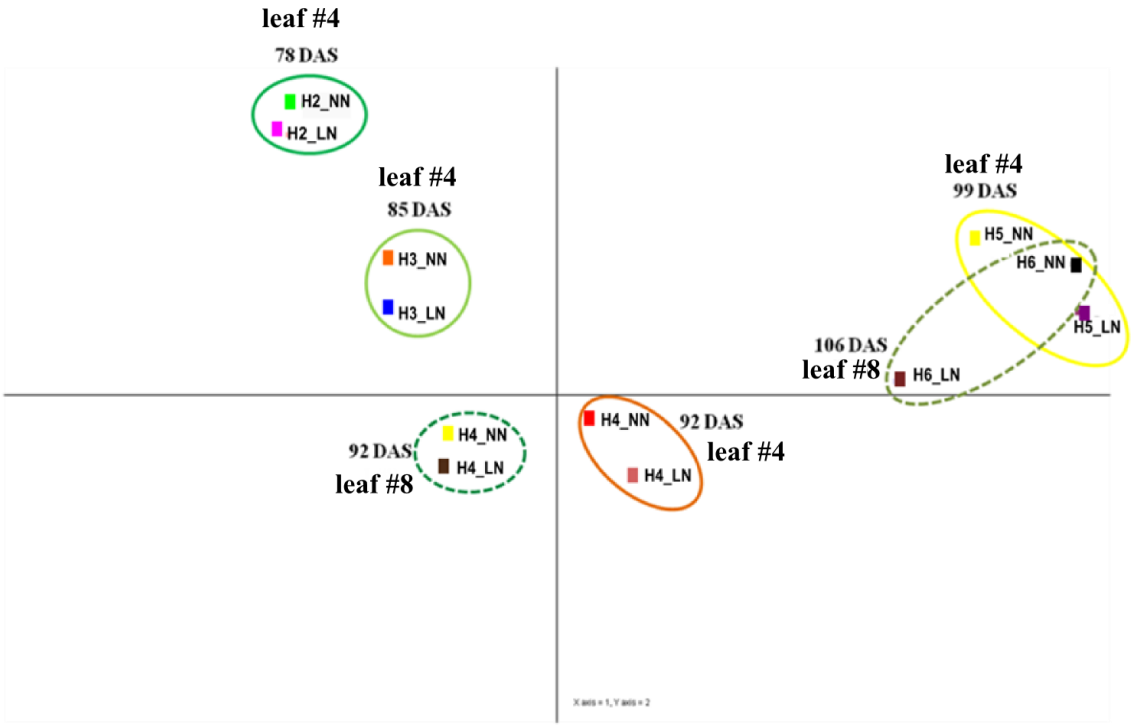


Figure 11. PCA representation of leaf transcriptome response from plants grown with normal (NN) and low (LN) N supply. The X axis represents the first component "time" and the Y axis represents the second component "N treatment". PCA was performed using MultiExperiment Viewer (MeV).

The comparison of the numbers of differentially expressed genes between different time points (from 78 until 99 DAS with weekly intervals) revealed (Figure 12) that at earlier time points (between 78 and 85 DAS) there are more gene expression changes under normal N while most of gene expression changes in response to N limitation occurs at later time points (between 85 and 99 DAS as well as between 92 and 99 DAS). Increasing number of the genes during time was observed for unique genes, overlapping genes and genes with opposite regulation in response to normal and low N.

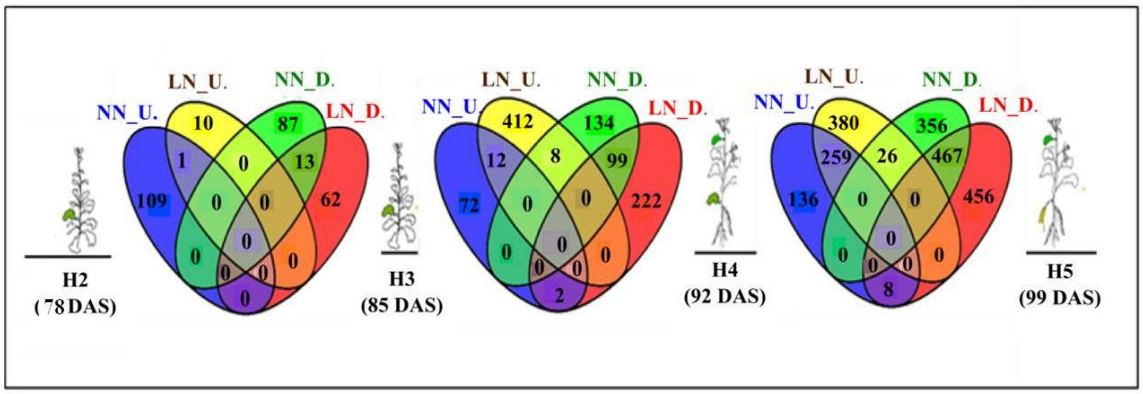


Figure 12. Venn diagrams representing number of differentially regulated genes. The number of unique and shared significantly regulated genes in leaf #4 under normal (NN) and low (LN) N between different harvest time points (78, 85, 92 and 99 DAS) are provided. U. and D. stand for up and down-regulation, respectively. Venn diagram was created using Venny online tool. All of the regulated genes are provided in table S3.

Differentially regulated genes in leaf #4 during development at each N treatment belonged to several biological categories which some were shared between normal and low N and some were uniquely regulated in response to one of N treatments. Figure 13 represents the biological categories related uniquely regulated pathways under each N treatment. It must be noted that a subset of genes in each biological category is regulated, not all the genes in that category. Total number of genes belonging to each biological category and the number of regulated genes in each category is provided in table S4**.** Genes with significant ($p<0.05$, using LIMMA method) and ≥3 fold expression changes between each consecutive time point were identified. The genes were sorted in different biological categories using SuperViewer software. Under normal N supply between 78 DAS (H2) and 85 DAS (H3) up-regulated genes involved in amino acid metabolism, stress response, protein (synthesis, degradation and posttranslational modification (kinases) are significantly overrepresented while there was no pathway with significant up-regulation under low N condition. Glycolysis, tetrapyrrole synthesis, hormone metabolism and stress response showed down-regulation only under normal N condition while major carbohydrate (CHO) metabolism, cell wall (synthesis, degradation and modification), RNA (regulation of transcription) and DNA (synthesis) categories showed unique down-regulation in response to low N condition. It was observed that between 85 DAS (H3) and 92 DAS (H4), polyamine metabolism and minor CHO metabolism were up-regulated only under normal N condition. More gene expression changes were observed between 85 DAS (H3) and 92 DAS (H4) under low N condition than normal N. These genes were involved in mitochondrial electron transport/ATP synthesis, amino acid metabolism, photosynthesis, RNA, development,

stress response, signaling, protein and DNA. The only pathway which was uniquely down-regulated between 85 DAS (H3) and 92 DAS (H4) under normal N was protein (synthesis, degradation, targeting, posttranslational modification) while more pathways such as nucleotide metabolism, cell (organization, division, vesicle transport, cell cycle and cell death), development and transport showed down-regulation only under low N condition. Compared to earlier time points, the two latest time points, 92 DAS (H4) and 99 DAS (H5) showed more pathways with regulation changes under both normal and low N conditions. Uniquely up-regulated pathways under normal N were photosynthesis and stress. Mitochondrial electron transport, TCA/org transformation and secondary metabolism and cell (organization, division, cell cycle and vesicle transport) were up-regulated only in response to limited N condition. Photosynthesis was the only pathway which showed unique down-regulation under normal N while more pathways such as N-metabolism, redox, cell, cell wall, stress and secondary metabolism showed unique down-regulation in response to N limitation. Taken together, pathway analysis showed that there are differences between normal and low N conditions in the context of uniquely regulated pathways. This difference is very clear between 85 DAS (H3) and 92 DAS (H4). In this developmental phase, the number of LN-specifically, mostly up-regulated pathways is higher than in plants grown with normal N supply. This suggests that N deficiency induced leaf senescence is initiated at this time point under low N condition. This is an earlier induction of leaf senescence compared to normal N condition.

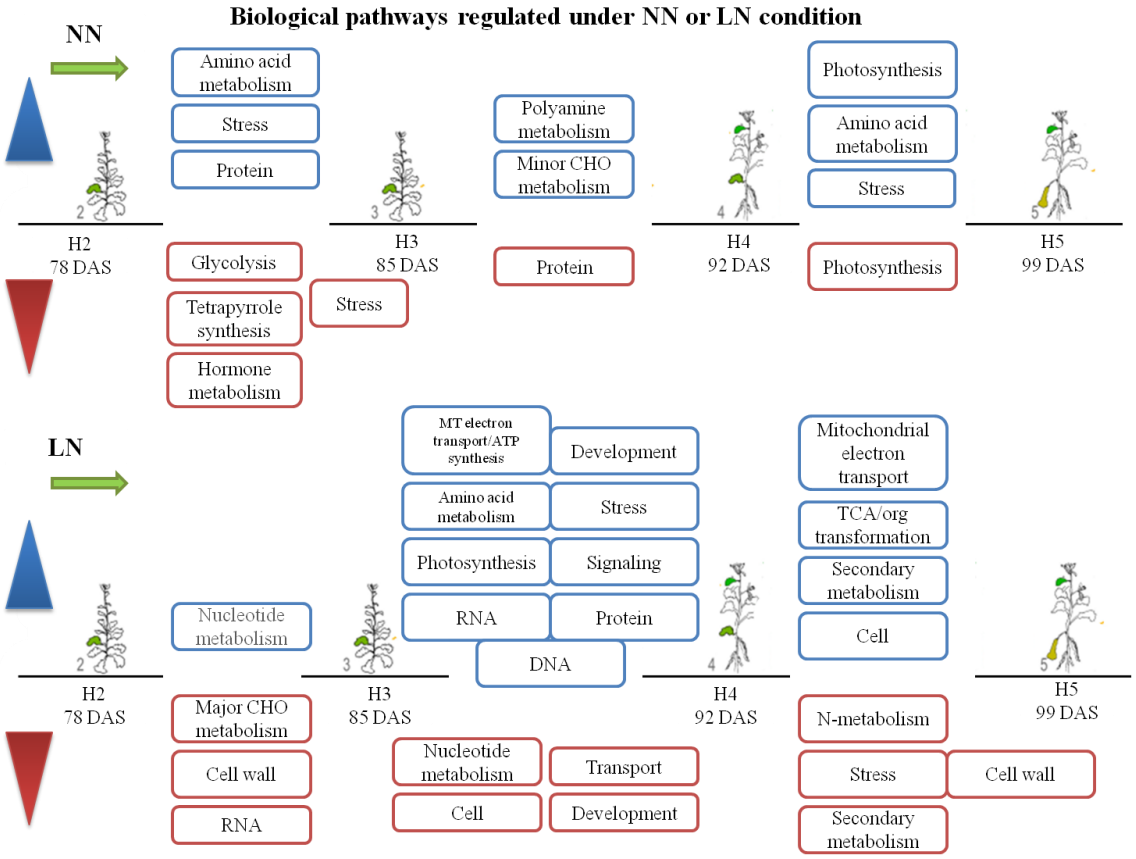


Figure 13. Biological categories regulated only under one of N conditions (uniquely regulated) in leaf #4 during development. Only significantly represented biological categories ($p<0.05$, based on hypergeometric distribution in SuperViewer tool) are shown in the figure above. P-value of the hypergeometric distribution is calculated as: $p = BC(M,x) * BC(N-M, n-x) / BC(N,n)$ with BC as the binomial coefficient calculated as follows: $BC(n,k) = n! / (k! * (n-k)!)$, x as the number of input genes with the selected classification, n as the total number of input genes, M as the number of genes with the selected classification in the database (MapMan, TAIR10) and N as the total number of genes in the database (TAIR10). (source: http://bar.utoronto.ca). The blue and red boxes indicate up- and down-regulation, respectively. Total number of genes belonging to each biological category and the number of regulated genes in each category under normal and low N is provided in supplemental table S1 and S4, respectively.

Figure 14 presents those pathways which were regulated under both N conditions. Total number of genes belonging to each biological category and the number of regulated genes in each category is provided in table S1 and S4, respectively. The only pathway which was up-regulated under both N conditions between 78 DAS (H2) and 85 DAS (H3) was related to not assigned genes. The category “not assigned”, which is attributed to those genes which are not related to any known functional categories, showed up-regulation between 78 DAS (H2) and 85 DAS

(H3) and also 85 DAS (H3) and 92 DAS (H4) (not shown). No pathway was significantly down-regulated between 78 DAS (H2) and 85 DAS (H3) while co-factor and vitamin metabolism, photosynthesis, cell wall and DNA were down-regulated under both N conditions between 85 DAS (H3) and 92 DAS (H4). Most of shared pathways under normal and low N condition were observed between 92 DAS (H4) and 99 DAS (H5). Glycolysis, major and minor CHO, development, protein, RNA , not assigned and DNA were up-regulated while tricarboxylic acid cycle/ organic acid (TCA/org) transformation, mitochondrial electron transport/ATP synthesis, nucleotide metabolism, lipid metabolism, amino acid metabolism, transport, RNA, development, protein, misc, not assigned, DNA and micro RNA/natural antisense showed down-regulation. Different pathways showed up-regulation under both normal and low N including major CHO metabolism, minor CHO metabolism, glycolysis, amino acid metabolism, photosynthesis, cell, secondary metabolism, development, signaling, RNA, miscellaneous, protein, hormone metabolism, stress, DNA and micro RNA/ natural antisense. Pathways such as TCA/ org transformation, redox, photosynthesis, mitochondrial electron transport/ ATP synthesis, lipid metabolism, cell, transport, glycolysis, cell wall, nucleotide metabolism, development, RNA, hormone metabolism, signaling, protein, miscellaneous, not assigned, stress, DNA and microRNA/ natural antisense showed down-regulation under both N conditions. Since different set of genes related to the same pathways are up- or down-regulated, some of pathways are observed as both up and down-regulated. Even though, higher number of genes showed regulation changes under low N condition (genes between 85 and 92 DAS and also between 92 and 99 DAS) compared to normal N (genes between 85 and 92 DAS and also between 92 and 99 DAS), the biological pathway belonging to these regulated genes between 85 and 92 DAS showed less overlap between 85 and 92 DAS while most of the biological categories associated with regulated genes at latest developmental phase (between 92 and 99 DAS) were observed under both N conditions. This can indicate an earlier senescence initiation under low N condition (between 85 and 92 DAS) followed by advanced senescence which occurs between 92 and 99 DAS under both N conditions. Therefore, it is possible that the regulated genes under low N, between 85 and 99 DAS, are involved in senescence initiation under low N condition.

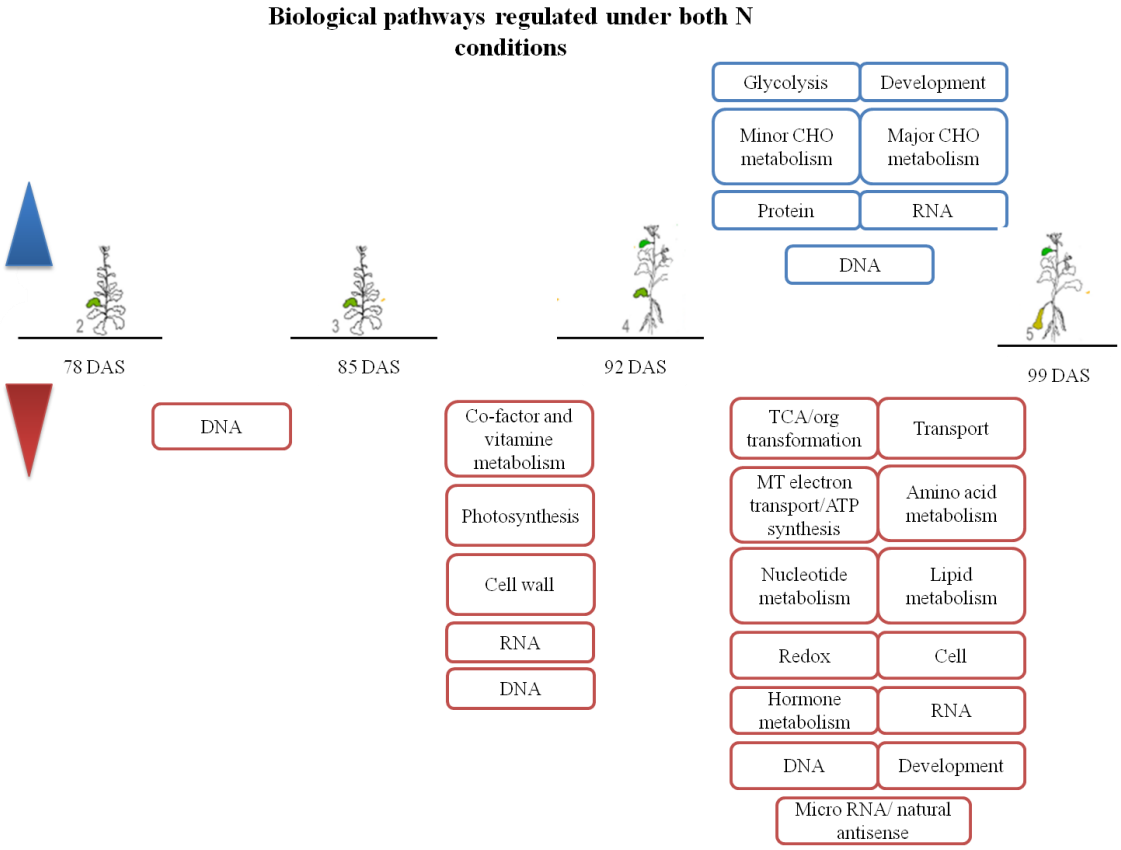


Figure 14. Overlapping biological pathways under both N conditions in leaf #4 between different time points. Only significantly represented biological categories ($p<0.05$, based on hypergeometric distribution in SuperViewer tool) are shown in the figure above. P-value of the hypergeometric distribution is calculated as: $p = BC(M,x) * BC(N-M, n-x) / BC(N,n)$ with BC as the binomial coefficient calculated as follows: $BC(n,k) = n! / (k! * (n-k)!)$, x as the number of input genes with the selected classification, n as the total number of input genes, M as the number of genes with the selected classification in the database (MapMan, TAIR10) and N as the total number of genes in the database (TAIR10) (source: http://bar.utoronto.ca).The Blue and red boxes indicate up- and down-regulation, respectively. Total number of genes belonging to each biological category and the number of regulated genes in each category is provided in supplemental table S1 and S5, respectively.

The time span between 85 and 92 DAS represents higher gene expression changes which are correlated with different biological categories under low N condition compared to normal N (Figure 13). Moreover, *SAG12-1* expression (Figure 8) showed higher up-regulation at the same time span under low N and one week later (between 92 and 99 DAS) under normal N condition. These data indicates an earlier senescence initiation under low N condition (between 85 and 92 DAS). Since senescence initiation is correlated with nutrient remobilization, identification of the underlying genes involved in precocious senescence under low N condition is of great importance. Particularly interesting are the genes that encode master regulators such as

transcription factors and the genes involved in signaling process, because they control many downstream biological processes which lead to the final physiological and phenotipic response of the plant to N deficiency condition. Identification of the signaling or transcription factor genes with differential regulation changes in response to N deficiency will provide key genes which regulate down-stream biological processes for plant survival under this condition.

Phytohormones are considered as regulatory substances which coordinate long distance signaling pathways in plants in response to nutritional changes (Kiba et al. 2011). Phytohormone related genes which show expression changes in response to N-deficiency, especially at earlier stages, between 85 and 92 DAS, may indicate that they play a role in precocious senescence initiation under low N condition.

Beside signaling genes, transcription factor encoding genes and the genes involved in hormone metabolism, it has been shown that under low N condition protein degradation plays an important role for plant survival under this condition (Peng et al. 2007). Thus, regulated genes involved in these biological categories have the potential for improving nitrogen use efficiency in crop plants such as oilseed rape. Therefore in the following sections the focus will be on the differentially regulated genes involved in these four biological categories which are possibly involved in an earlier senescence initiation under low N condition (between 85 and 92 DAS). An overview of the expression changes and the number of the regulated genes belonging to the above mentioned biological categories is provided in figure 15.

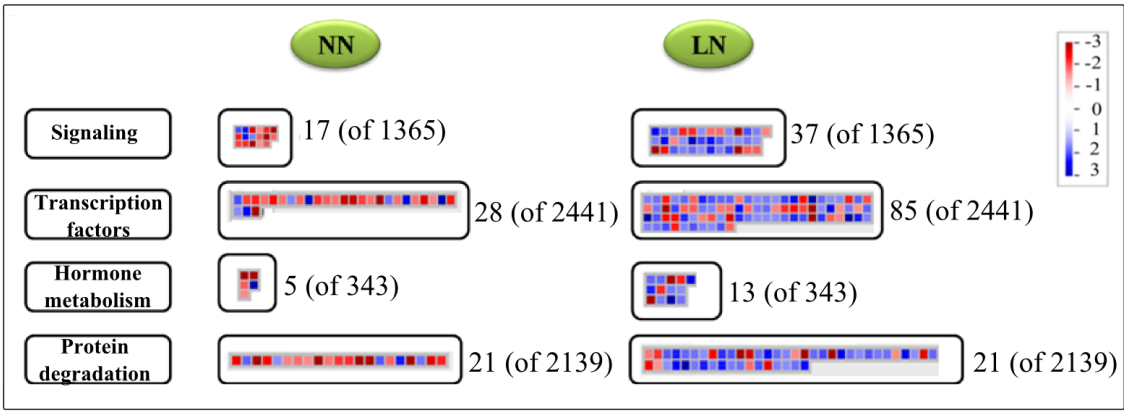


Figure 15. Expression changes and the number of the regulated genes involved in signaling, regulation of transcription (transcription factors), hormone metabolism and protein degradation in 92 DAS (H4) relative to 85 DAS (H3) in leaf #4 under normal (NN) and low N (LN) conditions. Blue and red squares represent up- and down-regulated genes, respectively. Image is modified from MapMan. The first and second numbers in parentheses indicate the number of regulated genes and the total number of the genes belonging to that category based on MapMan (TAIR10), respectively. The number of the respective unigenes on the microarray used in this study is provided in supplemental table S1.

3.3.2 Genes belonging to different signaling categories showed distinct regulation changes in response to different N condition

G-proteins are multi-subunit, integral membrane signal-transduction complexes that mediate intracellular responses to external stimuli in diverse eukaryotic organisms (New and Wong 1998). In a recent study it has been shown that plant G protein complex regulates nitrogen signaling. A major rice nitrogen-use efficiency quantitative trait locus (qNGR9) is synonymous with the previously identified gene DEP1 (DENSE AND ERECT PANICLES 1). Different DEP1 alleles underlie different nitrogen responses. Nitrogen-insensitive vegetative growth with increased nitrogen uptake and assimilation was observed in plants carrying dominant dep1-1 allele which results in improved harvest index and grain yield at moderate levels of nitrogen fertilization. The DEP1 protein interacts in vivo with both the Gα (RGA1) and Gβ (RGB1) subunits, and reduced RGA1 or enhanced RGB1 activity inhibits nitrogen responses (Sun H. et al. 2014). Authors suggested that G protein signaling unravel a missing link in the nutrient regulation of plant growth and development, and the modulation of heterotrimeric G protein activity also provides a potential new strategy for environmentally sustainable improvement of grain yield in rice (and possibly other crops) by increasing nitrogen-use efficiency.

Among different signaling categories shown in table 8, G-protein related genes showed the most distinct response in gene regulation changes under different N conditions, so that the numbers of up-regulated G-protein encoding genes were 7 and 1 under low and normal N condition, respectively while the numbers of down-regulated ones were 0 and 5, respectively. This shows that higher number of G-protein encoding genes are up-regulated under low N while opposite is true under normal N.

The role of G-proteins in N-deficiency signaling in oilseed rape has not yet been unraveled. Our results suggest a possible correlation between G-protein signaling and oilseed rape response to low N condition. This indicates that, similar to rice, G-proteins may have important role determining nitrogen use efficiency in oilseed rape. More studies are required to discover the exact mechanism by which G-proteins can affect oilseed rape response to nitrogen limitation. Beside G-protein encoding genes, other signaling related genes such as sugar and nutrient signaling genes, 14-3-3 protein, calcium dependent signaling, light associated signaling, phosphoinositides and different kinases (MAP kinases and receptor kinases) showed regulation changes under different N treatments. However, as it is shown in table 8, more up-regulation was observed under low N condition in each category, which may indicate that these signaling

genes are involved in N-deficiency induced leaf senescence and possibly are associated with N-remobilization during senescence in oilseed rape source leaves.

Table 8. Comparison of

involved in signaling in response to normal (NN) and low (LN) conditions in leaf #4 between 85 DAS (H3) and 92 DAS (H4). Numbers represent log2 fold changes of differentially expressed genes under NN and LN in leaf #4 between 92 DAS (H4) and 85 DAS (H3). Not significant genes are shown with (-) sign.

Gene	Function	NN_L#4	LN_L#4
at3g01090	sugar and nutrient physiology, AKIN10	-	2.50
at3g02970	sugar and nutrient physiology, EXL6	2.03	-
at1g22300	14-3-3 proteins, GRF10	-	2.00
at1g35160	14-3-3 proteins, GRF4	-	1.75
at3g57330	calcium, ACA11	1.73	-
at3g20410	calcium, CPK9	-3.17	-
at3g13460	calcium, ECT2	-1.61	-
at4g14750	calcium, IQD19	-	1.61
at2g26180	calcium, IQD6	-	2.83
at5g28850	calcium, calcium-binding EF hand family protein	-2.74	-2.21
at4g38810	calcium, calcium-binding EF hand family protein	-	1.83
at5g27540	G-proteins, MIRO1, emb2473	-1.58	-1.84
at3g59920	G-proteins, ATGDI2	-1.92	-
at5g55190	G-proteins, RAN3, ATRAN3	-1.99	-
at5g39960	G-proteins, GTP-binding family protein	-2.26	-
at4g01860	G-proteins, transducin family protein	-4.95	-
at3g55020	G-proteins, RabGAP	-	1.60
at3g54190	G-proteins, unknown	-	1.63
at4g35860	G-proteins, ATRABB1B, ATGB2, ATRAB2C	-	1.71
at5g17790	G-proteins, VAR3	-	1.95
at3g55660	G-proteins, ATROPGEF6, ROPGEF6	-	2.11
at5g47200	G-proteins, ATRABD2B, ATRAB1A	-	2.25
at2g03150	G-proteins, emb1579	-	2.34
at3g22170	light, FHY3	-	1.59
at5g13600	light, phototropic-responsive NPH3 family protein	-1.88	-1.91
at3g08570	light, protein binding	-	1.71
at1g55080	light, unknown	-	-1.91
at5g41790	light.COP9 signalosome, CIP1	-	-1.68
at2g43790	MAP kinases, ATMPK6, MPK6, MAPK6	-	1.61
at5g66850	MAP kinases, MAPKKK5	-	-3.35
at5g19010	MAP kinases, MPK16	-3.52	-4.81
at4g20260	phosphinositides, ATPCAP1, PCAP1	-	1.62

at1g22620	phosphinositides, ATSAC1	-	2.07
at4g33240	phosphinositides.phosphatidylinositol-4-phosphate 5-kinase	-	-1.84
at1g28390	receptor kinases.crinkly like, protein kinase family protein	-	-2.25
at5g16590	receptor kinases.leucine rich repeat III, LRR1	-2.27	-3.37
at3g02880	receptor kinases.leucine rich repeat III, putative	-	1.72
at2g45340	receptor kinases.leucine rich repeat IV, putative	-2.10	-
at1g56130	receptor kinases.leucine rich repeat VIII-2	2.35	2.37
at1g29720	receptor kinases.leucine rich repeat VIII-2	-	1.94
at1g09970	receptor kinases.leucine rich repeat XI, LRR XI-23	-	-2.36
at5g25930	receptor kinases.leucine rich repeat XI	2.49	-
at5g61480	receptor kinases.leucine rich repeat XI, putative	-	1.80
at2g16250	receptor kinases.leucine rich repeat XIV, l putative	-	-1.67
at1g70690	receptor kinases.misc, HWI1, PDLP5	-	1.68
at3g02130	receptor kinases.misc, RPK2, TOAD2	-	1.61
at5g20050	receptor kinases.misc	-2.20	-
at3g13690	receptor kinases.proline extensin like	-	1.95

3.3.3 Up-regulation of senescence associated transcription factor encoding genes under N-deficiency might be correlated with an earlier onset of leaf senescence

It has been shown that up-regulation of senescence-associated transcription factors are among the earliest events occurring during leaf senescence (Breeze et al. 2011). Transcription factors play major role in expression changes of their target genes and therefore are considered as master-control proteins. transcriptomic analyses have identified NAC, WRKY, AP2/EREBP, MYB, C2H2 zinc-finger and bZIP as being the largest families of transcription factors that are up-regulated during leaf senescence among which NAC and WRKY genes have been widely investigated for their regulatory roles in leaf senescence (Balazadeh et al. 2008, Breeze et al. 2011).

In the current study, members of several senescence associated transcription factor families such as AP2/EREBP, bHLH, bZIP, C2H2, MYB, NAC and WRKY showed more regulation changes under low N (LN) condition. It was noticeable that members of AP2/EREBP (e.g. *TOE2*, *RAP2.2* and *DDF1*), bHLH (e.g. *ICE2*, *bHLH071*and *BIM2*) and bZIP (e.g. *HYH*) were mainly up-regulated under LN compared to NN while C2H2 members (e.g. *ZAT10*, *ZFP7* and *ZAT11*) were mainly repressed under LN. Up-regulation of AP2/EREBP transcription factors which are ethylene-responsive element binding proteins (Riechmann and Meyerowitz 1998) is in accordance with up-regulation of ethylene related genes under LN. It has been shown that

AP2/EREBP members TOE1 and TOE2 are involved in the transitions between developmental stages of the shoot apical meristem as the *toe1-1:toe2-1* double mutant flowers much earlier than the wild type, whereas overexpression of *TOE1* leads to delayed flowering (Aukerman and Sakai 2003).

Two NAC transcription factors including an unknown member (At4g10480) and *NAC017* showed up-regulation under LN. Even though, the direct role of NAC017 during senescence has not yet been studied in detail, but BLAST search results revealed that its closest homologue (E=10e-158) is NAC016 which is a known senescence associated TF and has been shown to be a positive regulator of leaf senescence under developmental and abiotic stress induced leaf senescence (Kim Y. S. et al. 2013). Considering up-regulation of *NAC017* under LN in this study, it is likely that *NAC017*, like its homologue (*NAC016*) is involved in stress promoted senescence, especially under N-deficiency. The complete list of the differentially regulated transcription factors under different N conditions is provided in table 8. To have an overview about the extent of the regulation changes in each transcription factor family, the relative percentage of the number of the regulated genes were calculated based on the total number of genes belonging to each family available in MapMan (Table 9). AP2/EREBP, bHLH and bZIP are the families with the largest fraction of regulated genes under low N condition. Interestingly all of them show more up-regulation under low N condition than normal N (Table 10).

Table 9. Percentage of differentially regulated genes in each transcription factor family under normal (NN) and low N (LN). Calculation was conducted based on the complete number of genes in each family available in MapMan.

TF family	NN (%)	LN (%)
AP2/EREBP	0.87	7
bHLH	0.68	4.1
bZIP		4.1
HB	1.14	3.4
NAC	0.79	3.17
WRKY		2.74
C2H2		2.7
MYB	0.93	1.4

Table 10. Comparison of senescence associated transcription factors belonging to different families in response to normal (NN) and low (LN) conditions in leaf #4 between 85 DAS (H3) and 92 DAS (H4). Numbers represent log2 fold changes of differentially expressed genes under NN and LN in leaf #4 between 92 DAS (H4) and 85 DAS (H3). Not significant genes are shown with (-) sign.

Gene ID	Family, symbol	NN	LN
		Leaf #4	Leaf #4
AT1G53170	AP2/EREBP, ATERF-8, ATERF8, ERF8	-	-1.9
AT1G63040	AP2/EREBP, a pseudogene member of the DREB subfamily A-4 of ERF	-	-1.74
AT5G60120	AP2/EREBP, TOE2	-	1.68
AT2G44940	AP2/EREBP, AP2 domain-containing transcription factor TINY, putative	-	1.87
AT3G25890	AP2/EREBP, AP2 domain-containing transcription factor, putative	-	1.88
AT3G14230	AP2/EREBP, RAP2.2	-	1.89
AT1G12610	AP2/EREBP, DDF1	-	1.98
AT1G25560	AP2/EREBP, TEM1	2.01	2.25
AT2G31280	bHLH, CPUORF7	-	-2.37
AT1G12860	bHLH, SCRM2, ICE2	-	1.61
AT5G46690	bHLH, bHLH071	-	1.73
AT1G69010	bHLH, BIM2	-	2.09
AT1G06150	bHLH, transcription factor	-	2.35
AT1G10120	bHLH, DNA binding	-	5.63
AT2G18300	bHLH, basic helix-loop-helix (bHLH) family protein	-2.08	-
AT3G17609	bZIP , HYH	-	1.97
AT2G21230	bZIP , bZIP family transcription factor	-	1.88
AT3G14880	bZIP , unknown	-	3.17
AT1G27730	C2H2 zinc finger family, STZ, ZAT10	-	-2.26
AT2G37430	C2H2 zinc finger family, zinc finger (C2H2 type) family protein (ZAT11)	-	-2
AT1G24625	C2H2 zinc finger family, ZFP7	-	-1.82
AT5G22480	C2H2 zinc finger family, zinc finger (ZPR1-type) family protein	-	1.91
AT1G19700	HB, BEL10, BLH10	-	-1.83
AT1G23380	HB, KNAT6, KNAT6L, KNAT6S	-1.89	-1.78
AT5G47370	HB, HAT2	-	2.38
AT3G24310	MYB domain , MYB305, ATMYB71	-	-1.69
AT1G22640	MYB domain , ATMYB3, MYB3	-	1.74
AT4G32730	MYB domain , PC-MYB1, MYB3R-1, ATMYB3R-1, ATMYB3R1	2.8	1.83
AT1G18710	MYB domain , AtMYB47	-2.19	-
AT1G17880	NAC, BASIC TRANSCRIPTION FACTOR 3 (BTF3)	-2.78	-2.66
AT5G14000	NAC DOMAIN CONTAINING PROTEIN 84 (NAC084)	-	-2.58
AT4G10480	NAC, unknown protein	-	1.7

AT1G34190	NAC DOMAIN CONTAINING PROTEIN 17 (NAC017)	-	1.9
AT3G01970	WRKY domain , WRKY45, ATWRKY45	-	-1.59
AT3G56400	WRKY domain , WRKY70, ATWRKY70	-	1.78

Among different senescence associated transcription factor families, members of AP2/EREBP showed highest number of up-regulation under N-deficiency (6 genes) while only 1 gene was up-regulated under normal N. this propose that AP2/EREBP transcription factors may have important role in initiation of senescence under N-deficiency.

Two uncharacterized transcription factor encoding genes, AT1G10120 belonging to bHLH family and AT3G14880 belonging to bZIP family showed highest regulation changes (up-regulation), 25 and 9 fold, respectively, in response to low N condition. These genes are interesting candidates for further investigation in order to identify their role and their target

Taken together, higher number of up-regulated senescence associated transcription factors under low N compared to normal N, between 85 and 92 DAS, indicates that these transcription factors have possibly role as master regulators the complex regulatory network involved in precocious leaf senescence under N-deficiency in oilseed rape.

3.3.4 More up-regulated genes belonging to senescence associated hormones (ABA and ethylene) was observed under N starvation

It has been shown that nitrogen deficiency accelerates leaf senescence (Schildhauer et al. 2008). Plant hormones have important role in responses to senescence. Hormones such as ethylene, abscisic acid (ABA) and jasmonic acid (JA) that mediate plant responses to biotic and abiotic stresses, have been shown to accelerate senescence (Kim J. I. et al. 2011). Therefore, it was important to see which members of senescence related hormones are involved in senescence initiation (between 92 and 99 DAS) in response to N starvation in oilseed rape. Afore-mentioned genes may have crucial roles in induction of the down-stream senescence associated genes under low N condition.

Comparison of expression changes in hormone metabolism-associated genes revealed more up-regulation of genes belonging to two senescence associated hormones under N starvation compared to normal N condition: ABA (7.7%, low N and 1.5%, normal N) and ethylene (5% low N, 1% normal N). Different ethylene responsive factors including EFR12, ERF6 and ERF11 showed unique up-regulation under LN (Table 11). ERF6 has been shown to act as a positive antioxidant regulator during plant growth and in response to biotic and abiotic stresses

(Sewelam et al. 2013). All of differentially expressed genes at this time points belonging to ethylene and ABA is provided in table 11. Up-regulation of the genes belonging to senescence associated hormones (ABA and ethylene) in response to N starvation, between 92 and 99 DAS, can be correlated with senescence initiation at this time span.

Table 11. Comparison of hormonal related gene expression changes between leaf #4 under different N treatment. Log2 expression changes of hormonal regulated genes with classed with maximum distinct response to NN and LN in leaf #4 between 92 DAS (H4) and 85 DAS (H3). Not significant gene expression changes are shown with dash sign (-).

Gene ID	Function	Name	NN leaf #4	LN leaf #4
at2g44840	ethylene.signal transduction	ERF13	-1.81	-2.25
at1g28360	ethylene.signal transduction	ERF12	-	1.61
at1g04350	ethylene.synthesis-degradation	2-oxoglutarate-dependent dioxygenase, putative	-	1.74
at1g15360	ethylene.signal transduction	SHN1, WIN1 | SHN1 (SHINE 1)	-	1.80
at4g17490	ethylene.signal transduction	ATERF6	-	6.00
at1g28370	ethylene.signal transduction	ERF11	-3.74	-
at4g32810	abscisic acid.synthesis-degradation	CCD8, MAX4	-	-4.33
at2g40170	abscisic acid.induced-regulated-responsive-activated	ATEM6, GEA6	-	-2.21
at1g74520	abscisic acid.induced-regulated-responsive-activated	ATHVA22A	-	1.84
at1g04580	abscisic acid.synthesis-degradation	AAO4, ATAO-4	-	1.87
at1g19950	abscisic acid.synthesis-degradation	HVA22H | HVA22H	-	2.25
at2g22475	abscisic acid.induced-regulated-responsive-activated	GEM (GL2-EXPRESSION MODULATOR)	3.21	-

3.3.5 Protein degradation (ubiquitin dependent) genes showed stronger up-regulation under N-deficiency condition

.

Proteins are an important source of nitrogen and under N-limitation condition plants degrade proteins in source tissues in order to translocate nitrogenous compounds to sink tissues such as young leaves, flowers and seeds (Peng et al. 2007).

In this study, higher number of protein degradation genes (35 out of 51 regulated genes) showed up-regulation under low N condition compared to normal N (5 out of 21 regulated genes). Figure 16 represents an overview of all the regulated genes involved in both ubiqutin-

proteasome and ubiquitin independent protein degradation under low and normal N conditions (between 85 and 92 DAS).

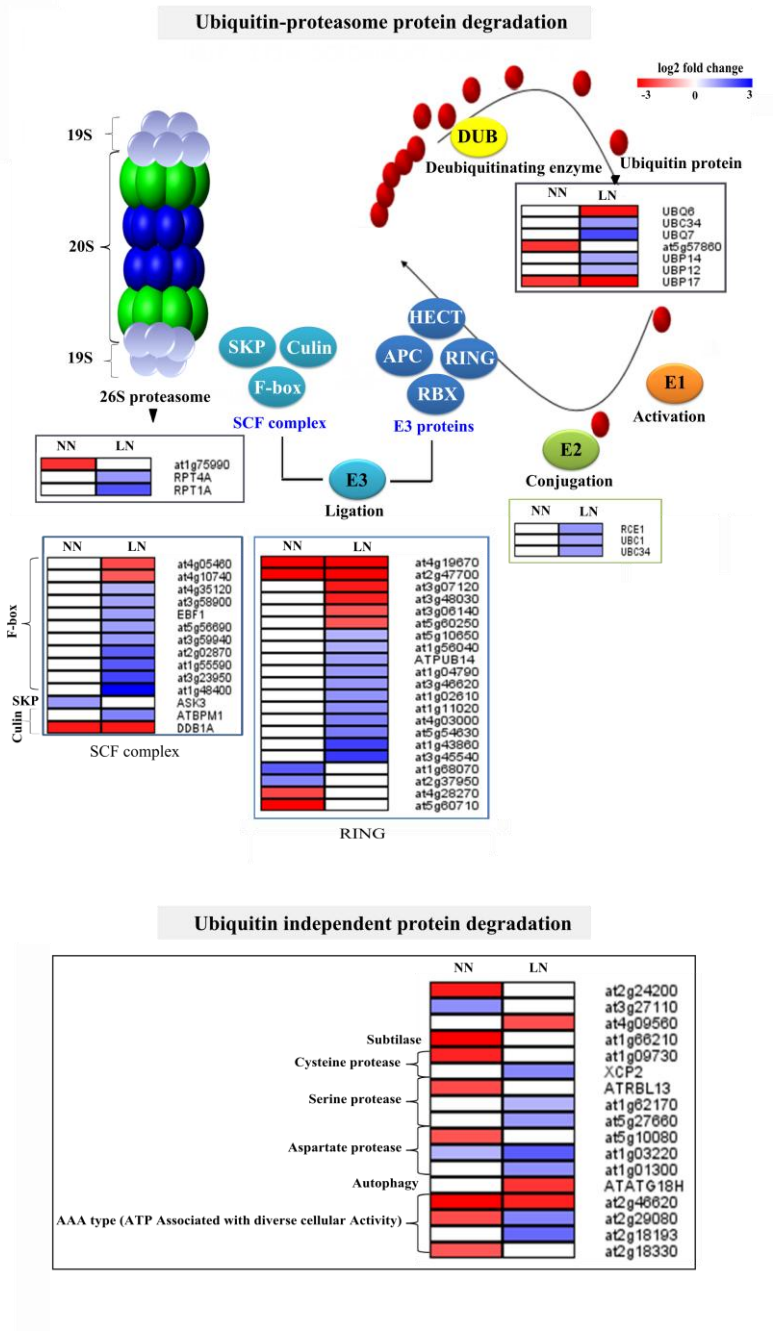


Figure 16. Heat map representation of the genes involved in the protein degradation (ubiquitin-proteasome and ubiquitin independent) depicting differentially regulated genes in leaf #4 (between 85 and 92 DAS) under normal (NN) and low (LN) N conditions. Ubiquitin activation (E1), ubiquitin conjugation (E2) and ligation of ubiquitin to proteins that are targeted for degradation (E3) are shown by orange, green and blue circles, respectively. SKP, Cullin and FBOX together constitute the SCF complex. RBX (for ring box protein), APC (for anaphase-promoting complex), HECT (for homology to E6-AP C terminus) and RING (for really interesting new gene) are different protein complexes in the E3 ubiquitin ligase family. DUB, Deubiquitinating enzyme. Red ovals represent individual ubiquitin proteins (Etalo et al. 2013). The image is modified from MapMan software.

Different amino acid proteases (three aspartate protease genes, one cystein protease genes and three serine protease genes) showed up-regulation under N limitation while they showed either down-regulation or no regulation changes under normal N condition. It was observed that the genes involved in ubiquitin dependent protein degradation, especially members of F-box family and zinc finger (C3HC4-type RING finger) proteins, showed more up-regulation under low N condition. The ubiquitin-proteasome system (UPS) has been shown to be an integral player in plant response and adaptation to environmental stresses such as drought, salinity, cold and nutrient deprivation. Moreover, it is involved in production and signal transduction of stress-related hormones (Stone 2014). In the current study, it was observed that a gene encoding a F-Box Protein (EBF1) showed >3 fold up-regulation under low N (between 85 and 92 DAS) but not under normal N condition. EBF1 binds to EIN3, which the latter is a known transcription factor involved in Ethylene signaling. EBF1 functions in ethylene perception by regulating EIN3/EIL turnover. In the absence of ethylene, EIN3 and possibly other EIN3-like (EIL) proteins are targeted for ubiquitination and subsequent degradation by E3 complexes of UPS system containing EBF1. Ethylene appears to block this ubiquitination, allowing EIN3/EIL levels to rise and mediate ethylene signaling. Up-regulation of EBF1 and its role in ethylene signaling is consistent with up-regulation of ethylene related genes (for example ERF12, SHINE1 and ERF6, table 11) at the same time point under low N condition in the current study. Moreover, up-regulation of several ethylene responsive transcription factors (members of AP2/EREBP family such as TOE2, RAP2.2 and DDF1, table 10) under low N condition and between 85 and 92 DAS, suggests that there is an orchestrated ethylene related gene regulatory network which is involved in the initiation of N deficiency induced leaf senescence of oilseed rape. Moreover, up-regulation of higher number of protein degradation genes under low N condition between 92 and 99 DAS, compared to normal N, is correlated with an earlier initiation of leaf senescence at this time span which can be associated with N remobilization from source leaves (leaf #4) towards upper part of the plant under N-limitation.

3.4 The effect of N treatment on gene expression changes in young leaf (#8)

Comparison of old and young leaves in response to different N treatment provide valuable insights regarding molecular players with different or similar functions or regulation changes in source and sink leaves. To identify N responsive genes in young sink leaf (#8) and to be able to

compare them with old source leaves, different comparison sets were considered and differentially expressed genes in response to normal and low N conditions were retrieved and further analyzed those genes to identify their biological function.

3.4.1 More gene regulation changes was observed at later time point in leaf #8 under low N relative to normal N

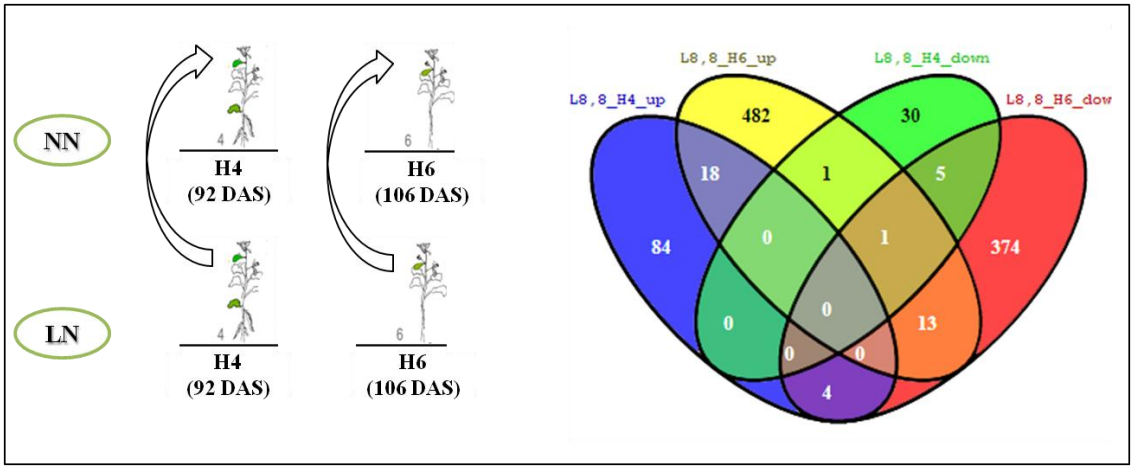


Figure 17. The number of unique and shared significantly regulated genes in leaf #8 between normal (NN) and low N (LN) at harvest time points 92 DAS (H4) and 106 DAS (H6). Arrows indicate that gene expression changes are calculated under low N (LN) relative to normal N (NN) condition.

Gene expression changes in leaf #8 and their relevant biological pathways were also examined at each time point so that normal N condition was considered as reference condition. Figure 17 represents the number of differentially expressed genes in leaf #8 between normal and limited N conditions at each time point. In total more genes showed up and down-regulation at later time point (106 DAS) than earlier time point (92 DAS). Regulated genes were incorporated in SuperViewer tool for functional categorization (Figure 18). It was noticeable that energy associated pathways such as photosynthesis (92 DAS), fermentation and mitochondrial electron transport/ATP synthesis (106 DAS) showed up-regulation under low N condition (Figure 18) which indicates that leaf #8 under low N is metabolically more active than leaf #8 under normal N condition. This is correlated with higher SPAD values and lower *SAG12-1* expression in leaf #8 under low N compared to normal N, at 106 DAS (Figure 21).

The data obtained from gene expression changes during development (between 92 and 106 DAS) in leaf #8 under different N conditions was strongly in agreement with the previous statement. More details are provided in the following section.

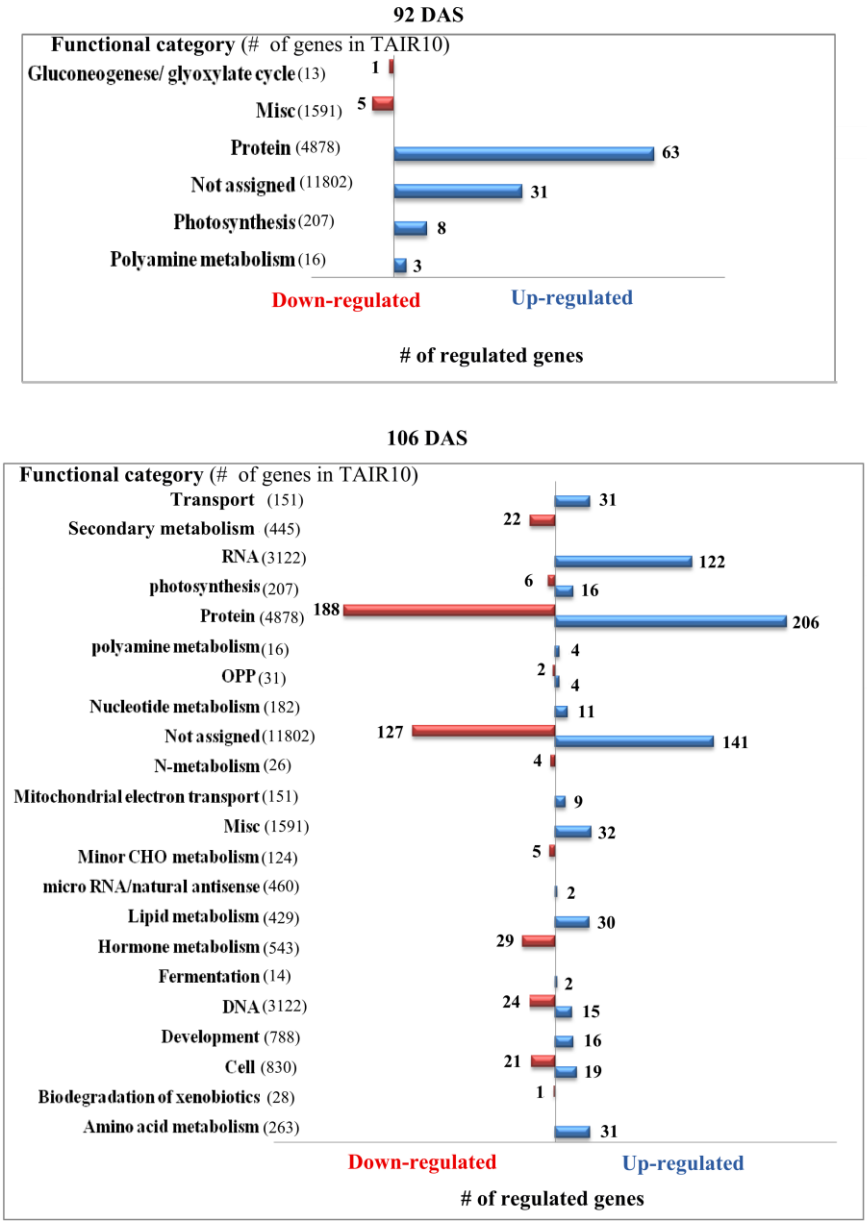


Figure 18. Functional categories (determined by SuperViewer tool) related to the regulated genes in leaf #8 under low N relative to normal N at earlier (92 DAS) and later (106 DAS) time points. Biological functions which are significantly represented ($p<0.05$, hypergeometric distribution). P-value of the hypergeometric distribution is calculated as: $p = BC(M,x) * BC(N-M, n-x) / BC(N,n)$ with BC as the binomial coefficient calculated as follows: $BC(n,k) = n! (k! * (n-k)!)$, x as the number of input genes with the selected classification, n as the total number of input genes, M as the number of genes with the selected classification in the database (MapMan, TAIR10) and N as the total number of genes in the database (TAIR10) (source: http://bar.utoronto.ca). All of the regulated genes belonging to each biological category are presented in table S6.

3.4.2 Senescence is delayed in leaf #8 under low N condition, possibly due to higher nutrient remobilization from old leaf #4

To identify gene expression changes in leaf #8 under normal and low N conditions, during development, transcriptome response of leaf #8 was compared between two time points including 92 DAS (H4), when leaf #4 is still attached to the plant and 106 DAS (H6), when leaf #4 has fallen down. This comparison was performed under both N conditions. The relevant biological pathways of these genes were further studied and similarities and discrepancies between normal and low N condition in leaf #8 were unraveled, as it is mentioned in the following section.

Figure 19 shows the number of differentially expressed genes in leaf #8 between 92 DAS (H4) and 106 DAS (H6). More expression changes were observed under normal N condition. Normal N samples showed more number of regulated genes in comparison with N limited samples.

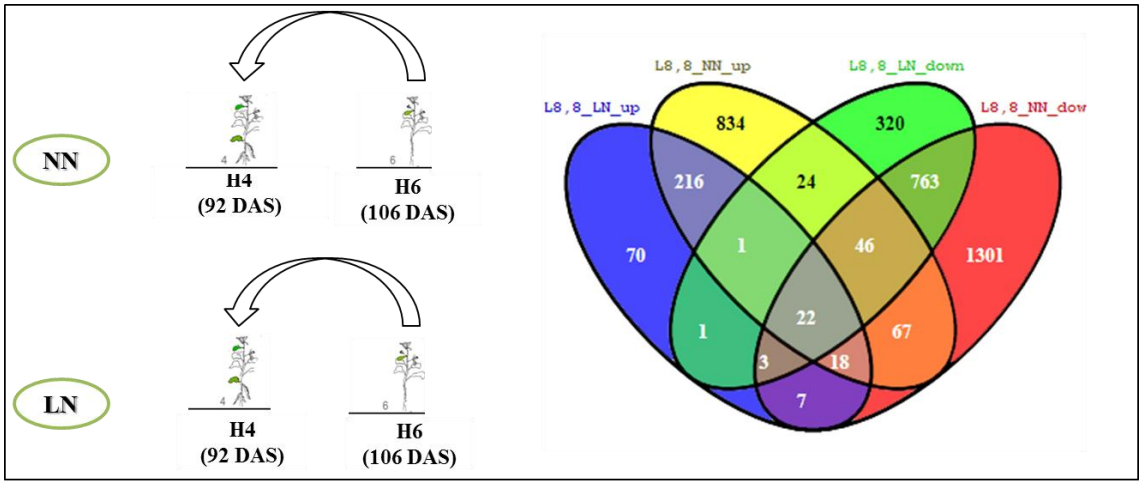


Figure 19. Number of unique and shared differentially regulated genes in leaf #8 between normal (NN) and low N (LN). Arrows show that comparison was conducted between H4 and H6. H4 was considered as reference.

Differentially regulated genes belonging to various functional categories are represented in figure 20, using MapMan software. As it is shown in figure 20, more genes belonging to C and N associated pathways such as photosynthesis (light reactions, Calvin cycle and tetrapyrrole

synthesis), cell wall metabolism, lipid metabolism, energy production (TCA cycle and mitochondrial electron transport) and amino acid synthesis showed down-regulation in leaf #8 under normal N compared to low N. Besides that, several secondary metabolites (S-misc "glucosinolates", phenylpropanoids, flavonoids and terpens) showed higher number of up-regulated genes (25 up-regulated genes of 445 secondary metabolites genes in TAIR10) under normal N compared to low N (7 up-regulated genes of 445 secondary metabolites genes in TAIR10). This indicate that there is a stronger senescence associated gene expression changes in leaf #8 under normal N compared to low N. Number of regulated genes and total number of genes in each category in MapMan (TAIR10) are represented in table S7 . The regulated genes belonging to the above-mentioned categories and their role during senescence are discussed in section 4. Moreover, the expression level of *SAG12-1* gene, a well known molecular marker of senescence progression is in agreement with the previous statement (Figure 21). It can be observed that *SAG12-1* expression is similar between two N conditions at 92 DAS, when leaf #4 is still available on the plant. However, its expression rises until 106 DAS, when leaf #4 has fallen down. At this time point, SAG12-1 expression is > 3 times higher under normal N than low N (Figure 21), indicating a more advanced senescence in leaf #8 under normal N supply compared to N-deficiency. This is in agreement with the senescence associated regulation changes which was mentioned before. This indicates that younger leaf (#8) under normal N has lower metabolic activity which is correlated with a more advance senescence. This is in accordance with lower SPAD values in this leaf at 106 DAS (Figure 21).

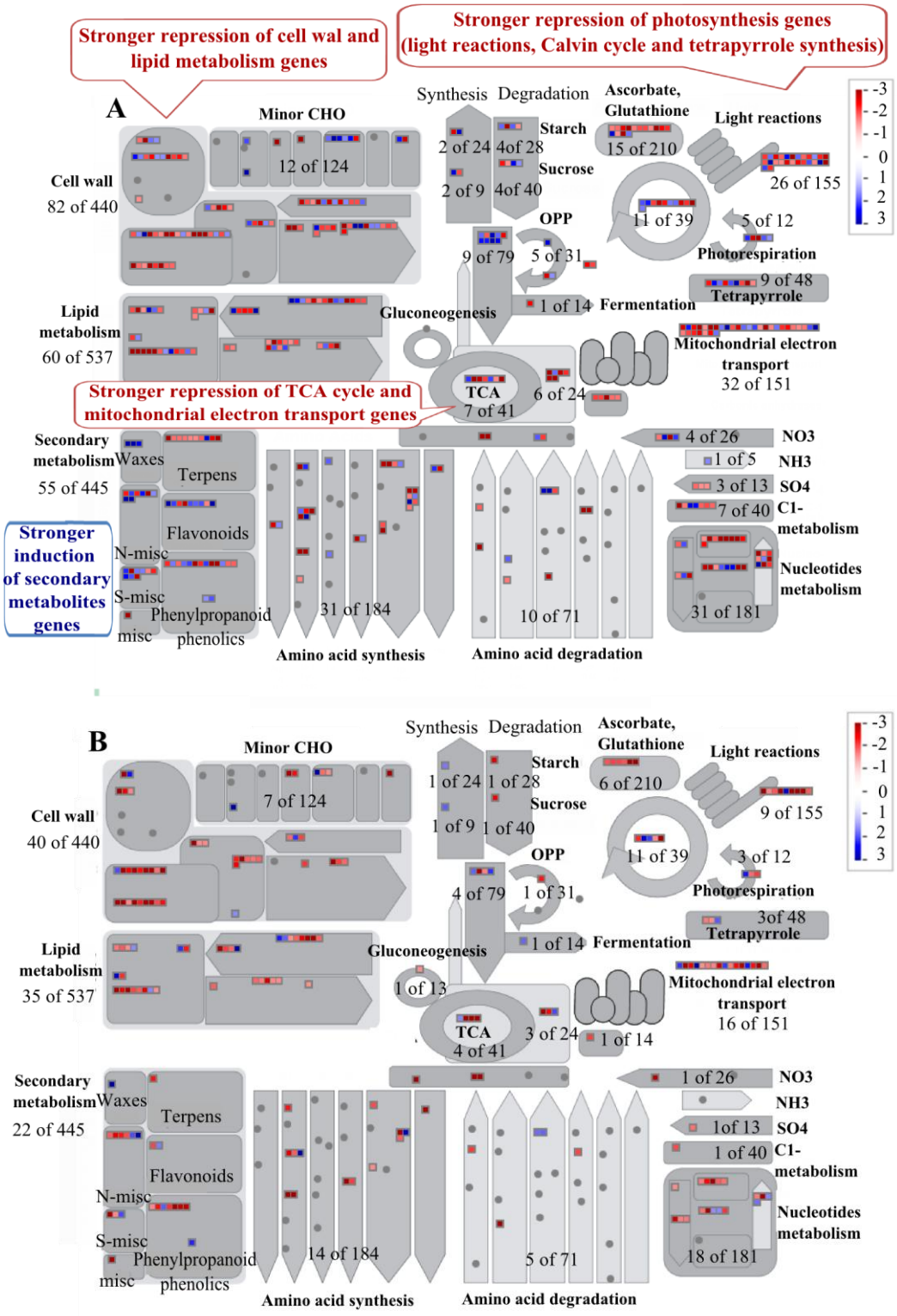


Figure 20. Overview of biological pathways and related regulated genes with differential expression changes in 106 DAS (H6) relative to 92 DAS (H4) in leaf #8 under (A) normal N (NN) and (B) low N (LN) conditions. Image is retrieved from MapMan software. Blue and red blocks denote up- and down-regulated genes, respectively. The first and the second numbers indicate the number of regulated genes and the total number of genes belonging to that category available in MapMan (TAIR10), respectively.

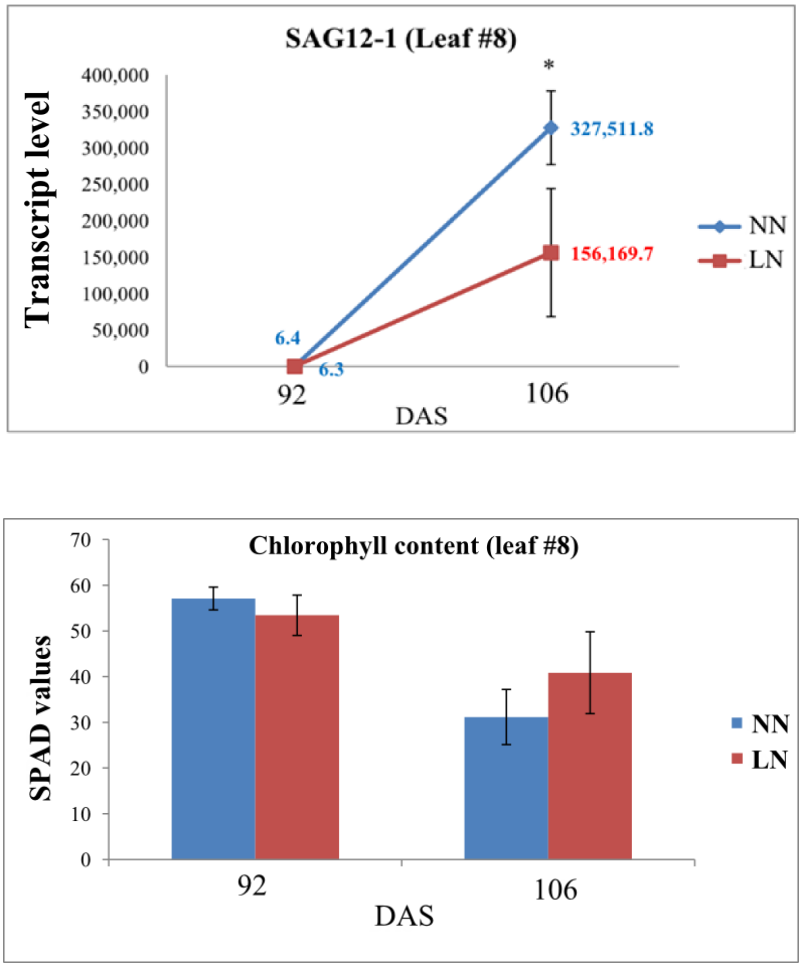


Figure 21. SAG12-1 transcript level (upper panel) and SPAD values (lower panel) in leaf #8 under normal (NN) and low (LN) N conditions at 92 DAS (harvest 4) and 106 DAS (harvest 6). DAS: Days after Sowing. Asterisk indicate statistically significant differences as determined by Student´s *t* test (*P < 0.05). Each value is the mean of the three biological replicates (n=3) and the error bars correspond to the SD. SPAD values are retrieved from Franzaring et al., 2011.

3.5 Identification of N sensitive potential biomarkers, as indicators of oilseed rape N status

Oilseed rape has a low nitrogen use efficiency (NUE) with only 50% of the N absorbed by the plant being present at harvest in the seeds (Schjoerring et al. 1995). Therefore, high N fertilizers are applied to the field to achieve optimum yield. Current requirement for N fertilization of oilseed rape is between 140 and 200kg N ha^{-1} $year^{-1}$ (Avice and Etienne 2014). Excessive fertilizer application has harmful effect on the environment. Moreover, it is one of the main costs of oilseed rape production (Avice and Etienne 2014).One of the aims of this study was the identification of gene expression biomarkers that indicate early stages of N deficiency and senescence before phenotypic symptoms are visible in order to manage fertilization application. Such early diagnostic tool can prevent subsequent environmental and economical negative effect of fertilizers. Figure 22 represent a summarized overview of the steps which were followed in the current study for marker gene identification and the details and the results of each step is provided in the following section.

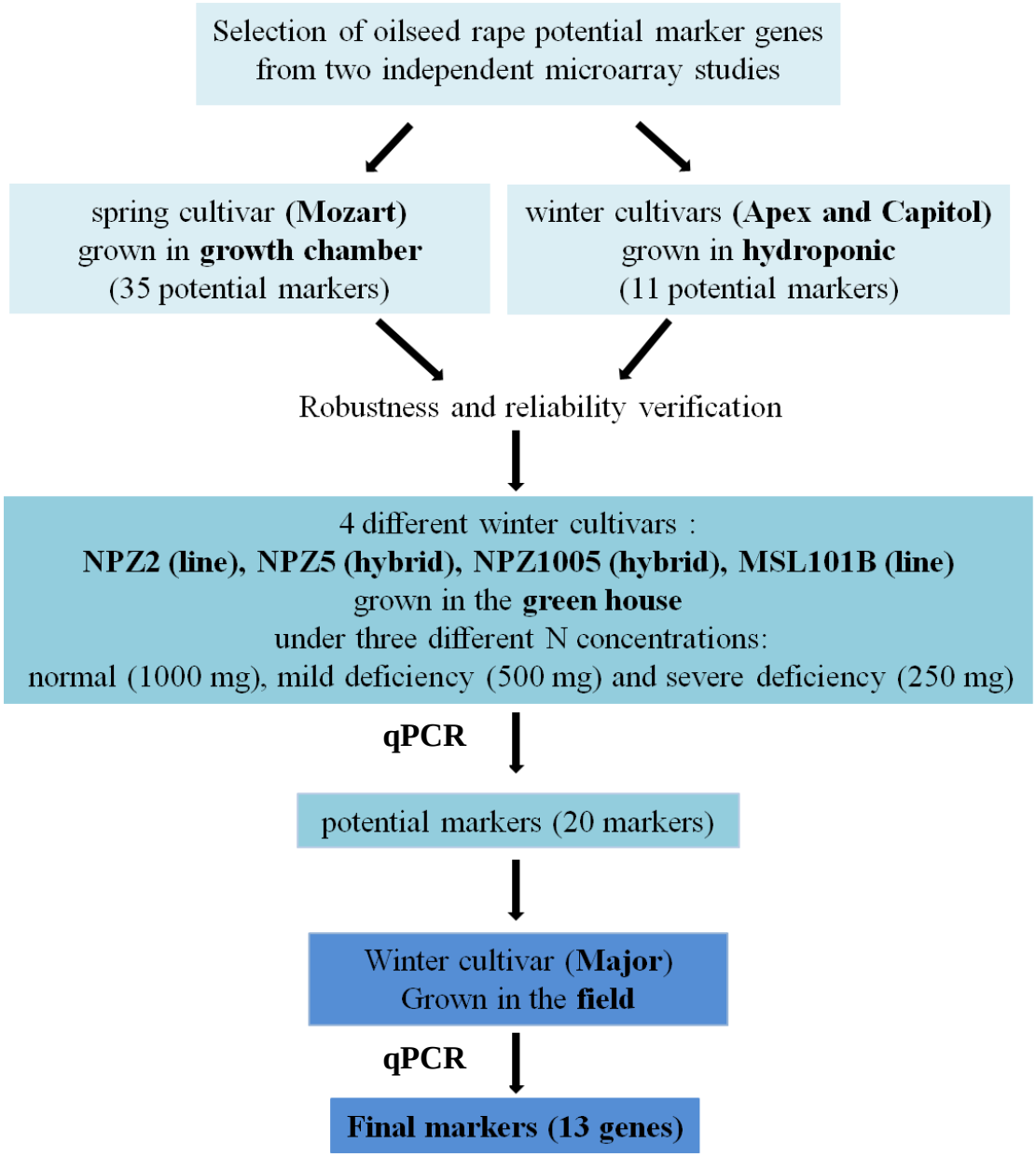


Figure 22. Schematic diagram indicating the overall view of different steps towards marker gene identification. HK stands for housekeeping genes.

Oilseed rape spring cultivar (Mozart) was grown at normal (150 kg N ha^{-1}) and low N supply (75 kg N ha^{-1}) and a custom made *B. napus* microarray (mentioned in section 3.1) was designed. Of interest were those probes on the Bn-microarray which can discriminate between plants grown at low or optimal N supply. Particularly interesting were probes that are early up-

regulated, i.e. between 78 DAS (H2) and 85 DAS (H3). A stringent regulation changes criterion (>10 fold change) was applied to identify marker genes. Moreover, since candidate marker needed to be further analyzed (by qPCR) under different growth conditions and cultivars, it was necessary to choose a criterion which results in the number of candidate marker genes which makes it possible to practically analyze them in various samples (avoiding too many genes) and also do not exclude many genes (avoiding very low number of genes and loosing potential candidates). After trying different thresholds a cut off value of >10 fold was chosen. This means those genes which showed >10 fold up- or down-regulation in each comparison (and less than 3 fold in the reference condition) were chosen. This resulted in identification of 130 genes as potential markers in different time points in leaves #4 and #8. Figure 23 represent all possible comparisons for marker gene selection. A senescence progression scale was defined based on *SAG12* expression (Figure 8) so that 78 until 85 DAS was considered as before senescence, 85 until 92 as early senescence, 92 until 99 mid-senescence and 99 until 106 as late senescence (Figure 23). In each time interval a different number of potential markers were observed (3, 68, 15 and 54, respectively). The complete list of the potential marker genes is provided in table S8.

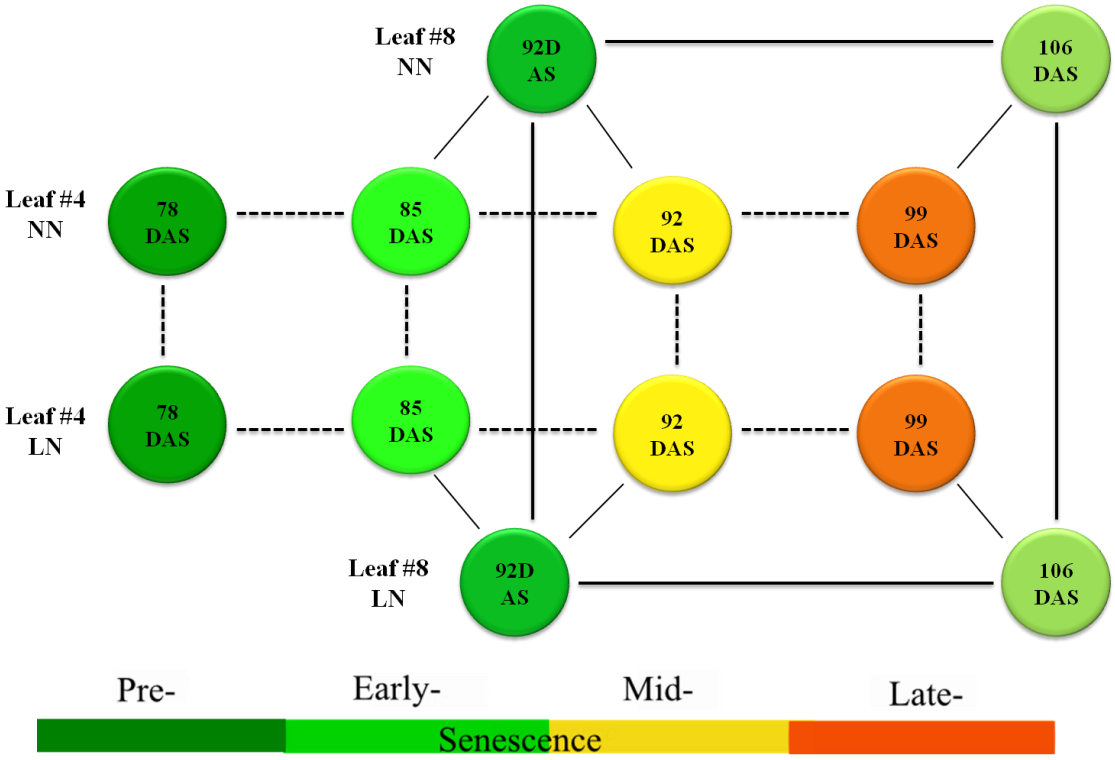


Figure 23. Schematic picture representing all possible comparisons of old (#4) and young (#8) leaves under different N treatment during development in Mozart study. NN and LN stand for normal N and low N, respectively. Comparisons were performed between leaf #4 vs. leaf #4 (dashed lines), leaf #8 vs. leaf #8 (bold solid lines) and leaf #8 vs. leaf #4 (thin solid lines) during development or at each time point. At 106 DAS there were no leaf #4 available and therefore the comparison of leaf #8 vs. leaf #4 was conducted using leaf #4 from 99 DAS. Markers were selected if they showed >10 fold

change between two N treatments at each time point or >10FC between two time points under one N treatment and <3FC under the reference condition. When comparing two N treatments, NN samples were considered as reference group. When comparing different leaves, leaf #4 was considered as reference.

In the next step, the expression profiles of all 130 selected potential maker genes were checked and those genes which showed an overall distinct expression profile between normal and low N during development (in all or almost all time points) were selected for further analysis (35 potential markers) which 4 of them are presented in . The expression profiles of the remaining 31 genes under normal and low N during development are available in Figure S2.

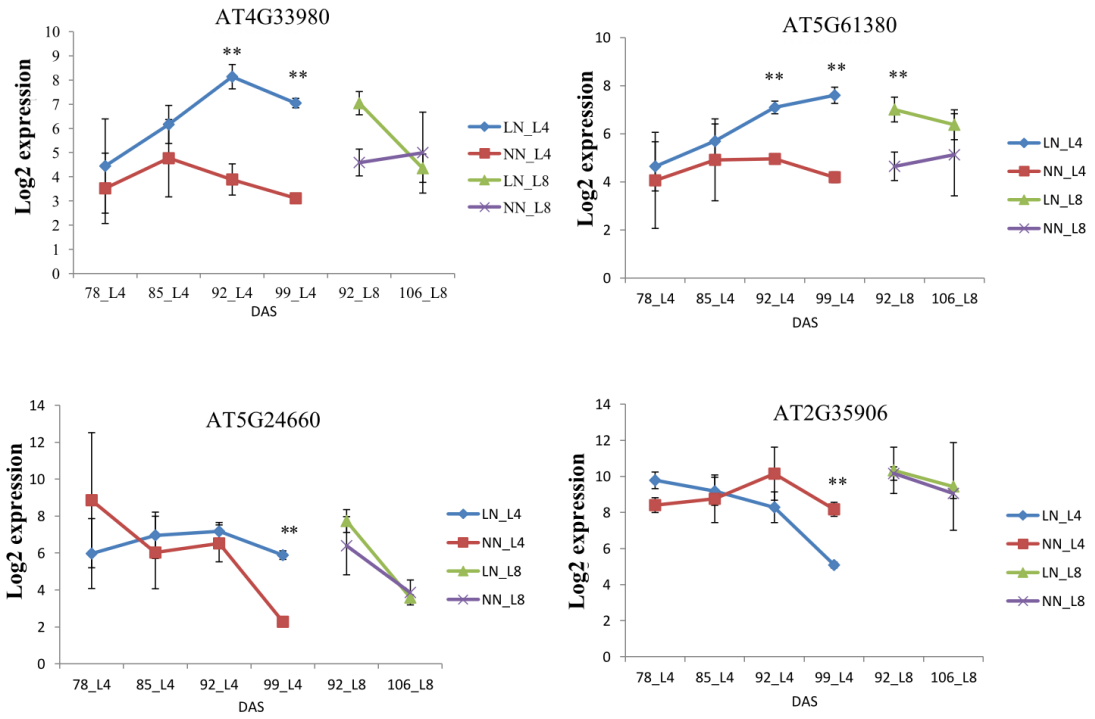


Figure 24. Log2 expression values of 4 potential marker genes from Mozart plants with distinct expression levels under normal (NN) and low N (LN) conditions at different time points in leaf #4 (L4) and leaf #8 (L8). Asterisks indicate statistically significant differences as determined by Student´s *t* test (*P < 0.05, **P <0.01).The error bars correspond to the SD (78, 85, 92 and 106 DAS: n=3, 99 DAS: n=2).

3.5.1 Expression validation of potential marker genes in 5 different oilseed rape winter cultivars using qPCR assay

In addition to these 34 genes, 12 genes were included which were previously identified as potential markers (in collaboration with Prof. Walter Horst, university of Hannover) in Apex

and Capitol cultivars which have been shown to be N sensitive and cultivar specific (Koeslin-Findeklee et al. 2015) , in press, a hard copy of the accepted manuscript which is available online (DOI: http://dx.doi.org/doi:10.1016/j.plantsci.2014.11.018) is provided with this thesis. Between these two sets of genes (one set from Mozart and another set from Apex and Capitol), one was shared. The expression of all 45 potential marker genes was further analyzed in 5 different winter cultivars (NPZ2, NPZ5, NPZ1005 and MSL101B, grown under greenhouse condition and Major, grown under field condition) by qPCR to identify the most reliable and robust markers. Using qPCR, I tested the reliability of these 46 marker genes in the oldest leaf of each four different oilseed rape winter cultivars including (NPZ2, NPZ5, NPZ1005 and MSL101B) which were grown in greenhouse under three different N conditions including normal N (1000 mg N), mild N deficiency (500 mg N) and severe N deficiency (250 mg N) (Figure 25).

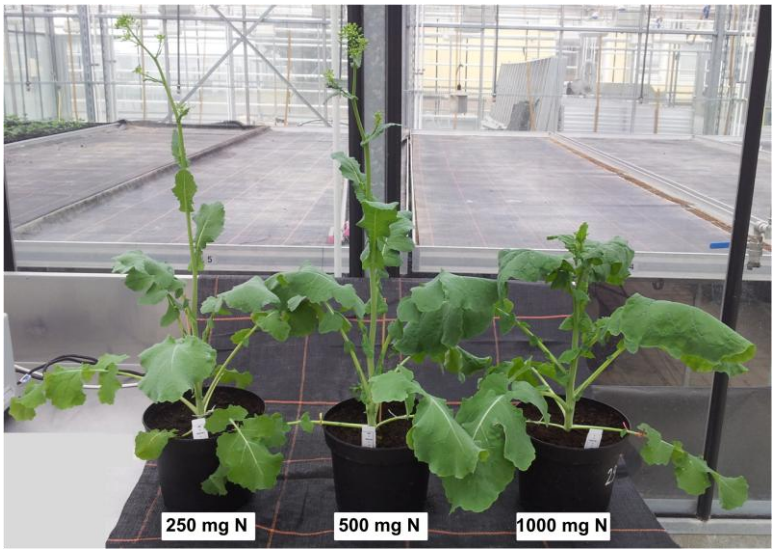


Figure 25. Greenhouse grown oilseed rape plants (collaboration with Professor Walter Horst, Hannover University)**.** Plants were treated with three different N concentrations: 1000 mg N (normal N), 500 mg N (mild N deficiency) and 250 mg N (severe N deficiency).

As it is shown in figure 26, from 46 marker potential genes, 20 genes showed distinct expression levels under different N conditions in all cultivars. Interestingly these genes showed the same trend under both mild (500 mg) and severe (250 mg) N deficiency.

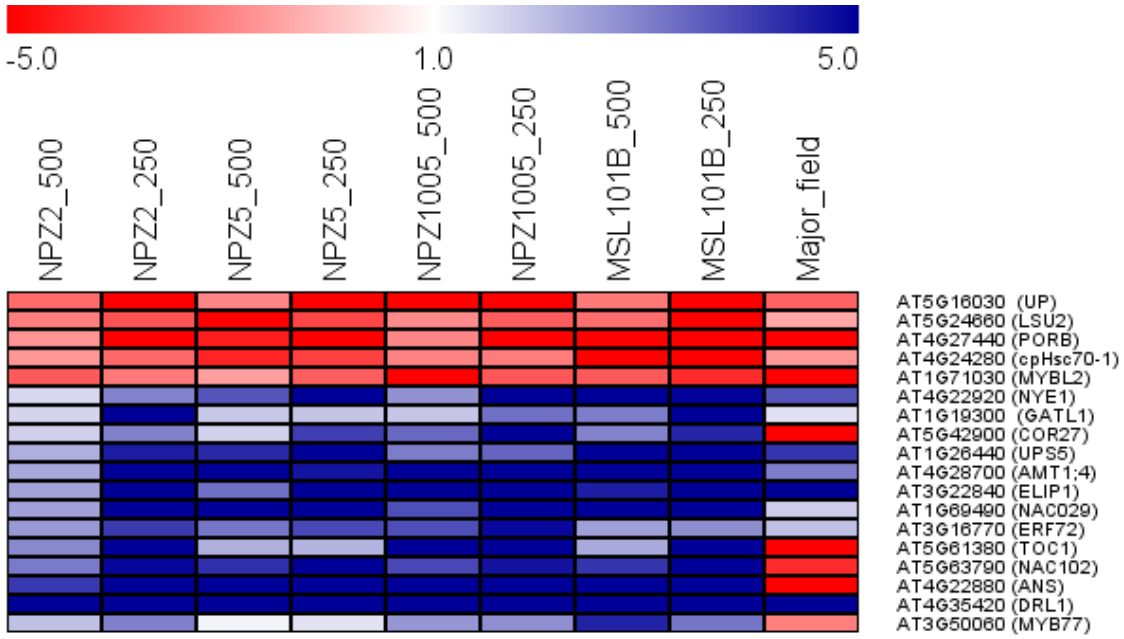


Figure 26. Heatmap of marker genes expression fold changes in response to N-deficiency in different cultivars grown in greenhouse (NPZ2, NPZ5, NPZ1005 and MSL101B) or filed (major). Column heads represent the name of the cultivar followed by the N concentration. Numbers represent expression fold changes of 20 marker genes with robust expression response in plants grown in greenhouse (column 3-10) under low N (500 and 250 mg N) relative to normal N (1000 mg N) and in field grown plants (column11) under low N (20 kg N/ ha) relative to normal N (120 kg N/ ha). Down- and up-regulated markers are shown by red and blue colors, respectively. Heatmap is generated using MultiExperiment Viewer (MeV**)** (www.tm4.org/mev**).**

To test the performance of 20 potential markers in field grown plants, in oilseed rape plants (cv. Major) grown in the field under low N (20 kg N ha^{-1}) and normal N (120 kg N ha^{-1}) , in collaboration with group of Prof. Nicolaus von Wirén, IPK, Gatersleben, figure 27, were used.

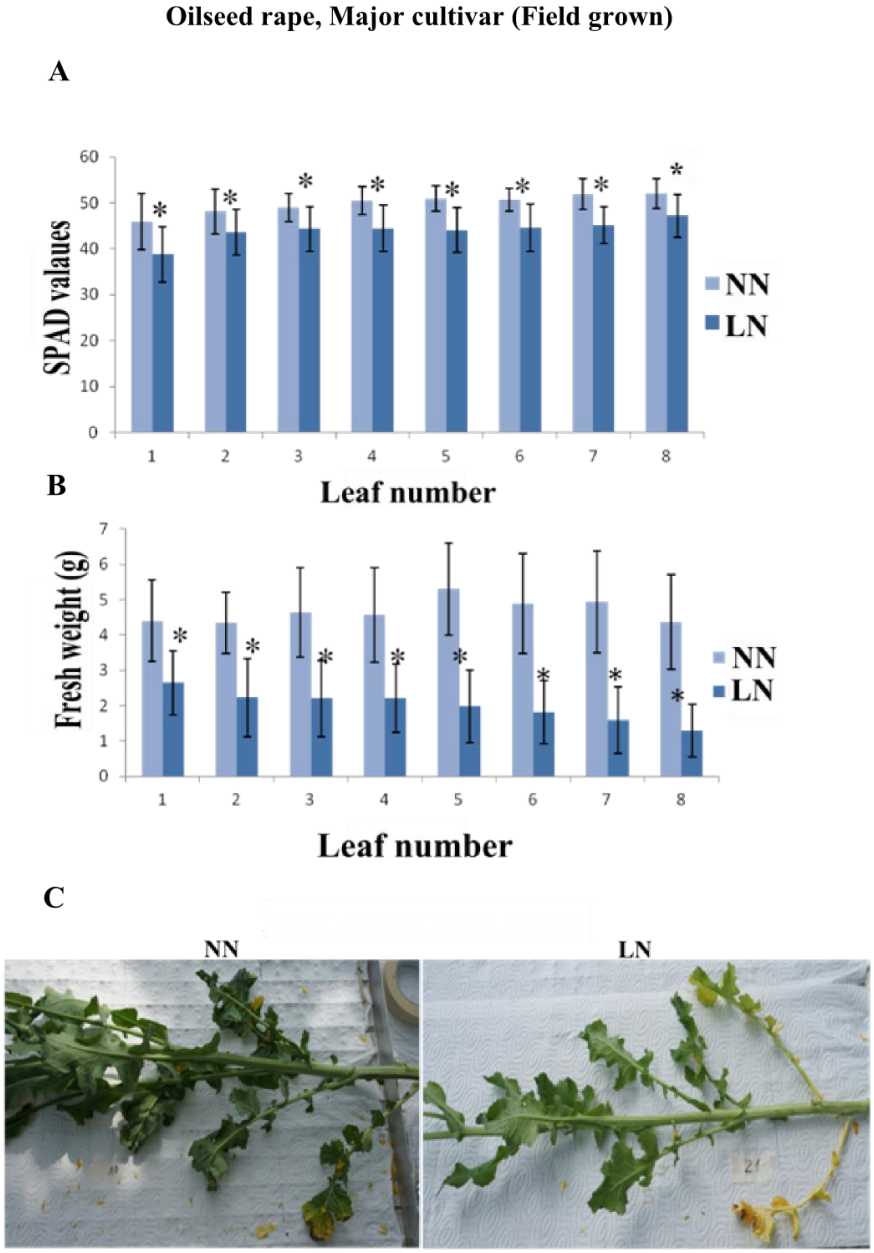


Figure 27. SPAD values (A), fresh weight (B) and phenotype (C) of oilseed rape, major cultivar, grown in the field under normal (120 kg N/ ha) and low (20 kg N/ ha) N supply (in collaboration with Prof. Nicolaus von Wirén , IPK, Gatersleben). Asterisks indicate statistically significant differences as determined by Student´s *t* test (*P < 0.05 and **P < 0.01). Each value is the mean of the three biological replicates (n=3) and the error bars correspond to the SD.

The expression changes of potential marker genes were further analyzed in the field grown samples. Eventually, 13 marker genes (*LSU2, PORB, UP, MYBL2, ELIP1, cpHsc70-1, GATL1, UPS5, NAC029, DFR-like1, ERF72, AMT1.4, NYE1*) were identified which showed distinct expression levels dependent on N condition but independent of cultivar and growth condition

Normalized CT values (obtained from qPCR assay) belonging to these 13 marker genes under normal and low N conditions are represented in figure 28.

Five of the remaining genes (*COR27*, *TOC1*, *NAC102*, *ANS* and *MYB77*) showed a distinct expression level under normal and low N conditions but showed opposite regulation changes in the field grown samples compared with those samples grown in controlled condition (e.g. up-regulation under low N in greenhouse plants but down-regulation in the field grown plants) (Figure 26). Therefore, they were not included in the final list of the marker genes. More cultivars and conditions must be tested to identify whether this is either an effect of the environment on gene expression and/or a cultivar specific response. It must also be noted that the difference between normal and low N concentration was strongly higher in the field compared to the controlled conditions. This can partly explain the above-mentioned observation.

Taken together, 13 marker genes were identified in this study which showed a clear distinct expression levels in response to different N conditions in several oilseed rape cultivars (spring and winter) grown under various conditions (growth chamber, hydroponic, greenhouse and field). Application of these identified marker genes, as early diagnostic tools in oilseed rape agriculture system, can potentially improve the N fertilizer management and minimize the cost and negative environmental effects of excessive amounts of fertilizers which is applied to the field. Statistical analysis data is provided in table S9.

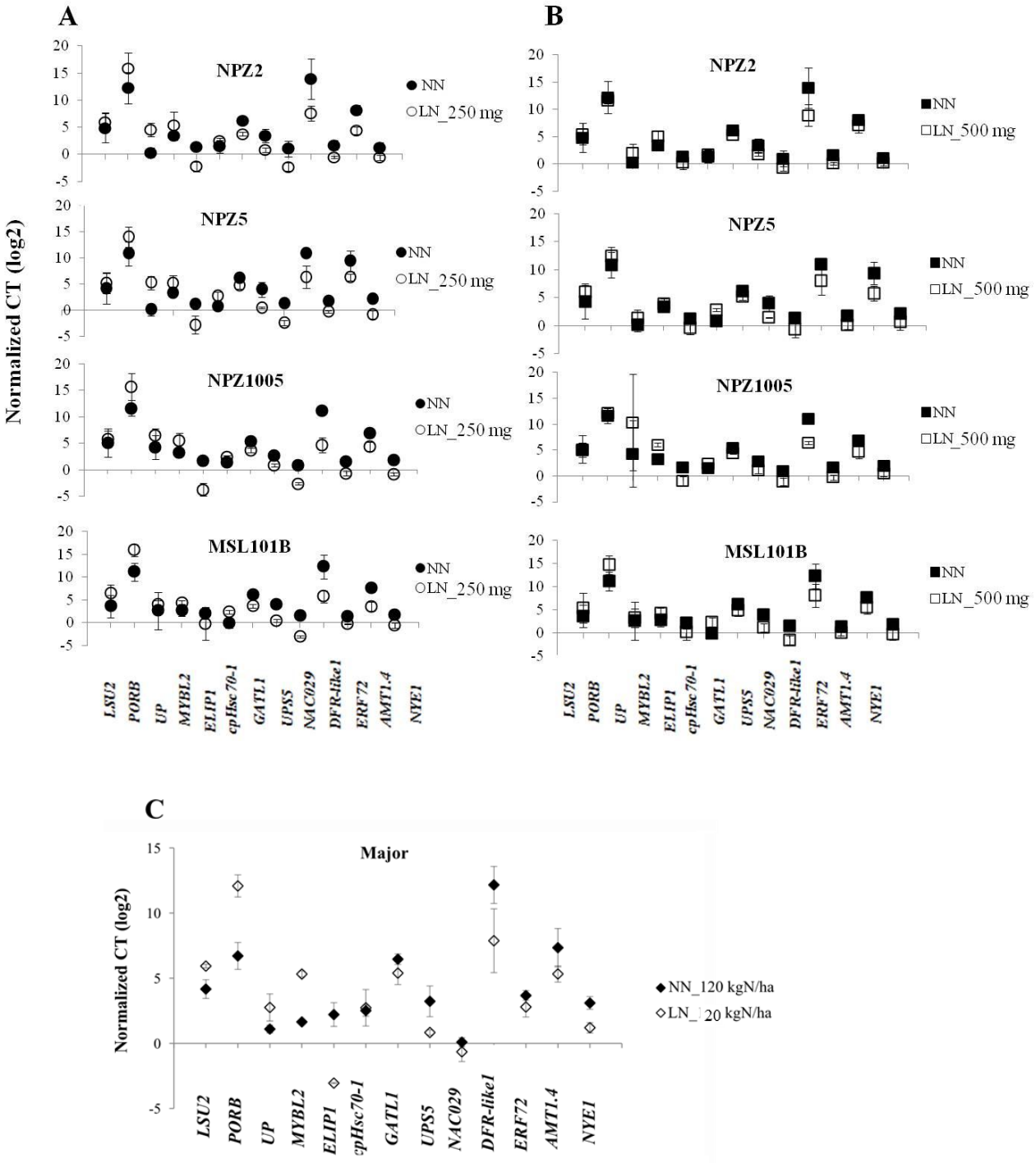


Figure 28. Normalized CT values (log2) belonging to the 13 marker genes with N-dependent expression levels in greenhouse grown plants under severe (A) and mild (B) N deficiency and in field grown plants (C). Normalized CT(gene) = CT(gene) – CT(housekeeping gene). UP1 and UBC9 were used as housekeeping genes. Error bars represent SD (n=4 for greenhouse samples and n=3 for field grown samples). NN: normal N (1000 mg), LN: low N.

Since N-deficiency induces senescence, it was of interest to monitor the expression of identified N status markers during leaf senescence. EFP browser data from *Arabidopsis thaliana* (Winter et al. 2007) showed that among 13 marker genes which are identified in this study, eight genes showed mild to strong expression in senescing leaves (Figure 29). Three genes, *MYBL2*, *UPS5*, and *NAC029*, showed strong expression in senescing leaves while *NYE1*, *LSU2*, cpHsc70-1, *ERF72* and *ELIP1* showed mild expression in senescing leaves (showed by the intensity of the red color in figure 29). The fact that *MYBL2*, cpHsc70-1 and *LSU2* which are expressed in senescing leaves are down-regulated under N-deficiency in this study indicates that these genes might be up-regulated by developmental leaf senescence but not with N-deficiency induced leaf senescence. *NYE1*, *UPS5*, *NAC029*, *ERF2* and *ELIP1* which shows mild to strong expression in senescing leaves and also up-regulation under N-deficiency in the current study, might be positively regulated by both developmental and N-deficiency induced leaf senescence. However, it must be noted that the data for developmental senescence which are retrieved from EFP browser are obtained from *Arabidopsis thaliana* while N-deficiency data from this study belongs to oilseed rape. Further investigations in oilseed rape are needed to confirm the above-mentioned statement.

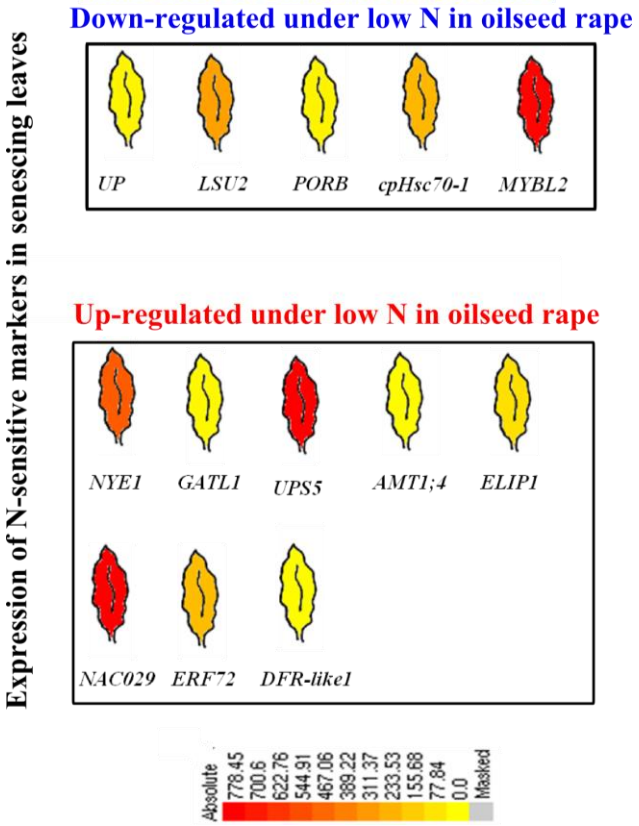


Figure 29. Expression of identified N-status marker genes in *A. thaliana* senescing leaves. Data are retrieved from EFP browser; http://bar.utoronto.ca (Winter et al. 2007).

3.5.2 Expression markers are earlier indicators of N deficiency than SPAD values

As it is shown in figure 30, SPAD values of 4 different winter cultivars grown in greenhouse did not show clear distinction between different N levels at earlier time points (15 until 26 DAT) and there were variations between different cultivars. For example, NPZ2 and NPZ1005 showed earlier significant differences between severe N deficiency and normal N at 26 DAT but not between moderate N deficiency and normal N treatments. Interestingly, NPZ5 did not show any significant differences in SPAD values between different N treatments during studied time points. Another observation was that even though there were significant differences in SPAD values of different N treatments this was not in all time points and not between all N concentrations. Moreover, in NPZ5 line, SPAD values of samples treated with sufficient N supply were even lower than mild N deficiency (though not significantly) and more close to the SPAD values related to the plants representing severe N deficiency. This is due to the more growth of the plants under sufficient N supply which their bigger leaves at the upper part of the canopy cause shadow on the lower leaves and therefore leads to lower chlorophyll content. This indicates that SPAD values are less reliable indicators of N status of the plant compared to expression markers which were able to distinguish between all different N treatments as early as 27 DAT in all cultivars.

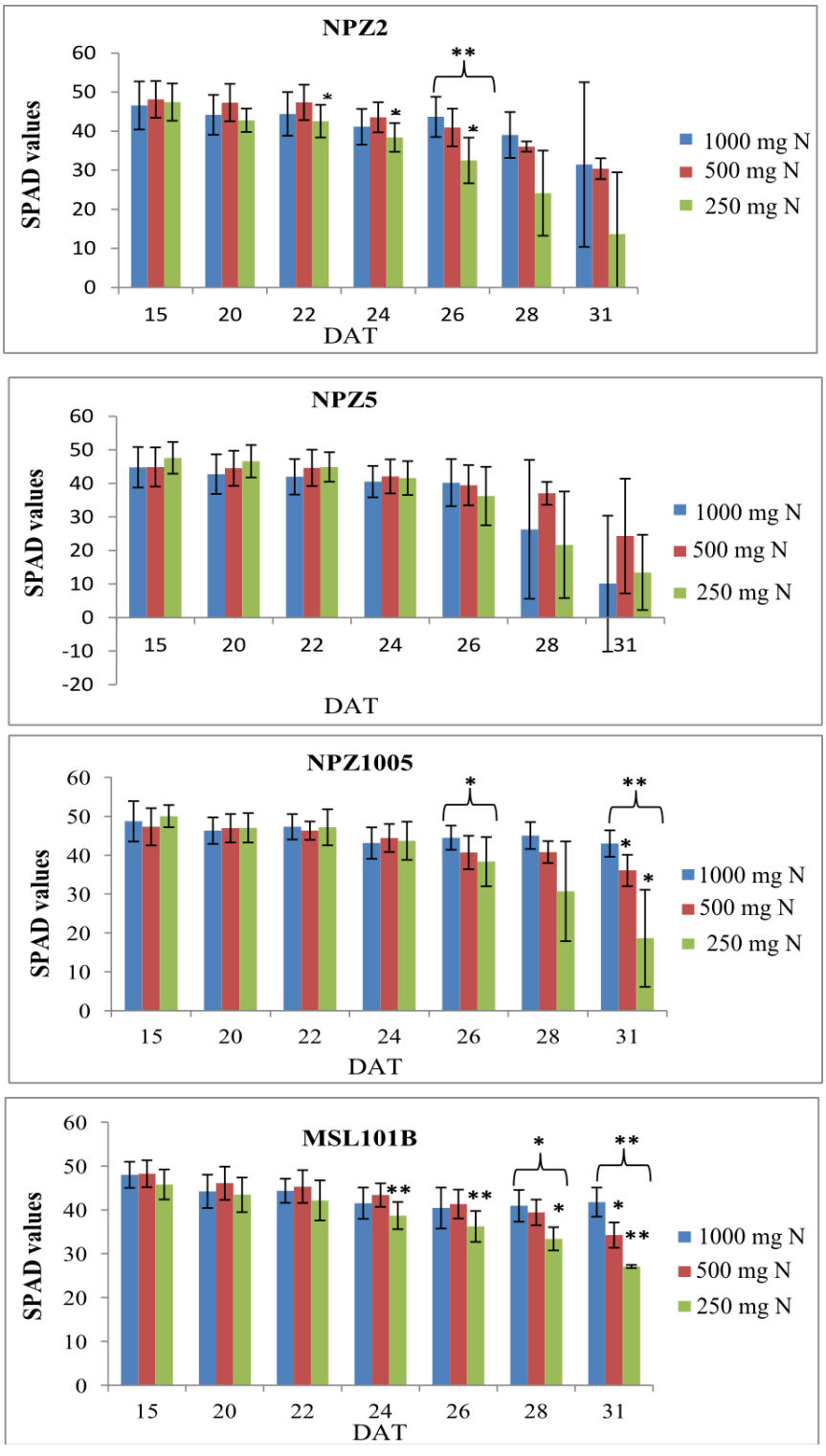


Figure 30. SPAD values of the oldest leaf belonging to four different oilseed rape winter cultivars (NPZ2, NPZ5, NPZ1005 and MSL101B) under three different N concentration. Normal N (1000mg N), mild deficiency (500 mg N) and severe N deficiency (250 mg N). Asterisks indicate statistically significant differences as determined by Student´s *t* test (*P < 0.05 and **P < 0.01). Each value is the mean of the 4 biological replicates (n=4) and the error bars correspond to the SD. DAT: days after transplanting.

3.6 Effect of elevated CO_2 on transcriptome response of oilseed rape under normal and low N conditions in old and young leaves

Since the Industrial revolution, CO_2 concentration has risen from 280 ppm in 1860 to 400 ppm in 2013, and if current trends in emissions continue, atmospheric CO_2 concentration will exceed 500 ppm by 2050 (Bishop et al. 2014). The rising requires higher crop plants production, makes it crucial to consider the potential benefits and risks of high CO_2 levels on the crop plants. Regarding the interaction of N and C metabolism and considering low NUE in oilseed rape it is necessary to study the response of this crop plant to elevated CO_2. Phenotypical analysis of the oilseed rape plants, cv. Mozart, under elevated CO_2 and different N concentrations has been previously reported (Franzaring et al. 2011). They observed that elevated CO_2 results in higher growth, increased water use efficiency and lower yield in oilseed rape plants under different N treatments. However, since transcriptome response of oilseed rape to elevated CO_2 has not yet been addressed, the molecular players underlying observed phenotypic responses are not known. Therefore, one part of this thesis was dedicated to transcriptome profiling of the same oilseed rape materials reported by Franzaring et al., 2011. In brief, plants were grown in growth chamber under ambient (380 ppm) and elevated (550 ppm) CO_2 in combination with low (75 kg N ha^{-1}) and normal (150 kg N ha^{-1}) N. The focus of the this section of my thesis is on the effect of elevated CO_2 on transcriptome response of young (#8) and old (#4) leaves of oilseed rape plants under different N conditions. Figure 31, represents oilseed rape plants which were used for transcriptome profiling (92 DAS) in the current study. Since most of phenotypical changes were observed at 99 DAS (personal communication with Dr. Juergen Franzaring, University of Hohenheim), it was postulated that underlying gene expression changes might have occurred at earlier time points. Therefore, oilseed rape plants harvested one week earlier than this time point, 92 DAS, were chosen for transcriptome profiling. The same *B. napus* custom made microarray which is described in the section 3.1, was used.

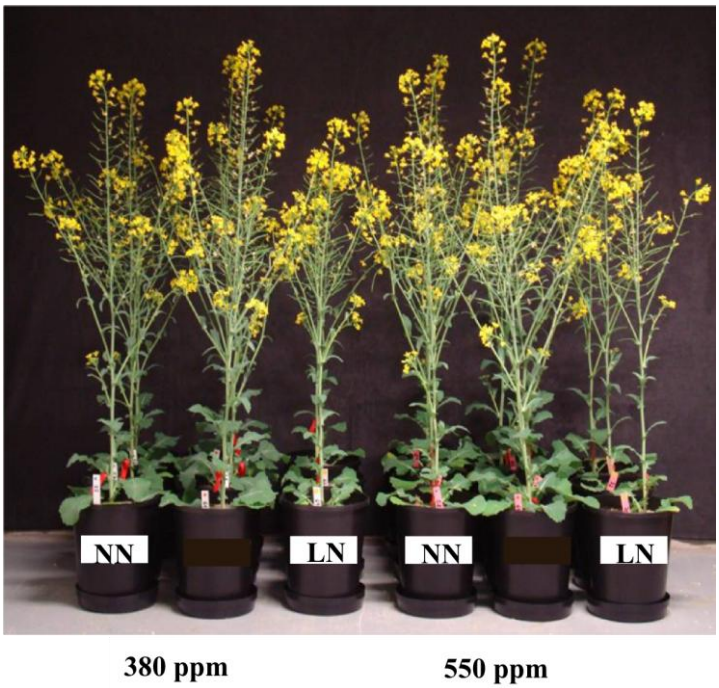


Figure 31. Plant materials used for studying the transcriptome response to elevated CO_2 (92 DAS). Plants were grown in growth chamber under ambient (380 ppm) and elevated (550 ppm) CO_2 in combination with normal (optN) and low (min N) (Franzaring et al. 2011).

In order to have an overview about the factors which cause the most variances between the samples and also to see which sample are more close or deviating in their gene expression, principle component analysis (PCA) was performed using MEV software (Figure 32). CO_2 was the first component ("X" axis in figure 32) which strongly affects transcriptome response of oilseed rape plants under both N conditions. Leaf position was the second component ("Y" axis in figure 32) affecting transcriptome response of different samples. Moreover, there was a higher difference in gene expression (represented by more distance in figure 32) between leaves from plants treated with different N concentrations under ambient CO_2 but these samples show more similarity under elevated CO_2. This can indicate that elevated CO_2 can overrun the effect of N deficiency and therefore samples with different N concentration show a more similar transcriptome response under elevated CO_2. These results indicate that elevated CO_2 have a clear impact on gene expression changes in oilseed rape and this impact is stronger compared to N treatment and leaf position.

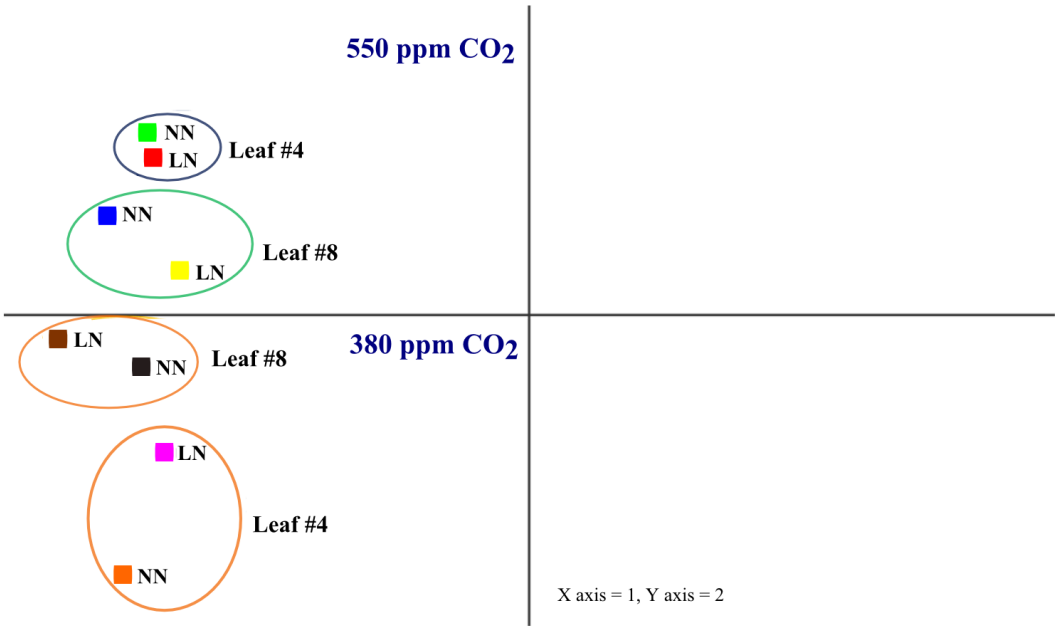


Figure 32. PCA representation of leaf transcriptome response to elevated CO_2 from plants grown under normal (NN) and low (LN) N supplies. The X axis represents the first component "CO_2" and the Y axis represents the second component "leaf". PCA was performed using MultiExperiment Viewer (MeV).

3.6.1 Analysis of transcritome response to elevated CO_2 under normal and low N in leaf #8

To analyze the transcriptome response of old (#4) and young (#8) leaves in response to elevated CO_2 and unravel the differences and similarities of this response between low and normal N treatment, gene expression changes of leaf #4 and leaf #8 under elevated CO_2 relative to normal CO_2 in the plants treated with two N supply was calculated. The genes with the cut off value of ≥ 3 fold change in the expression and differentially regulated (using LIMMA, R package) were identified. All of the differentially regulated genes under elevated CO_2 and their relevant functional categories are provided in table S10. Figure 33 shows the number of regulated genes. Even though the number of up-regulated genes in response to elevated CO_2 was higher under low N condition compared to normal N, a large fraction of regulated genes showed up- and down-regulation under both N conditions (Figure 33).

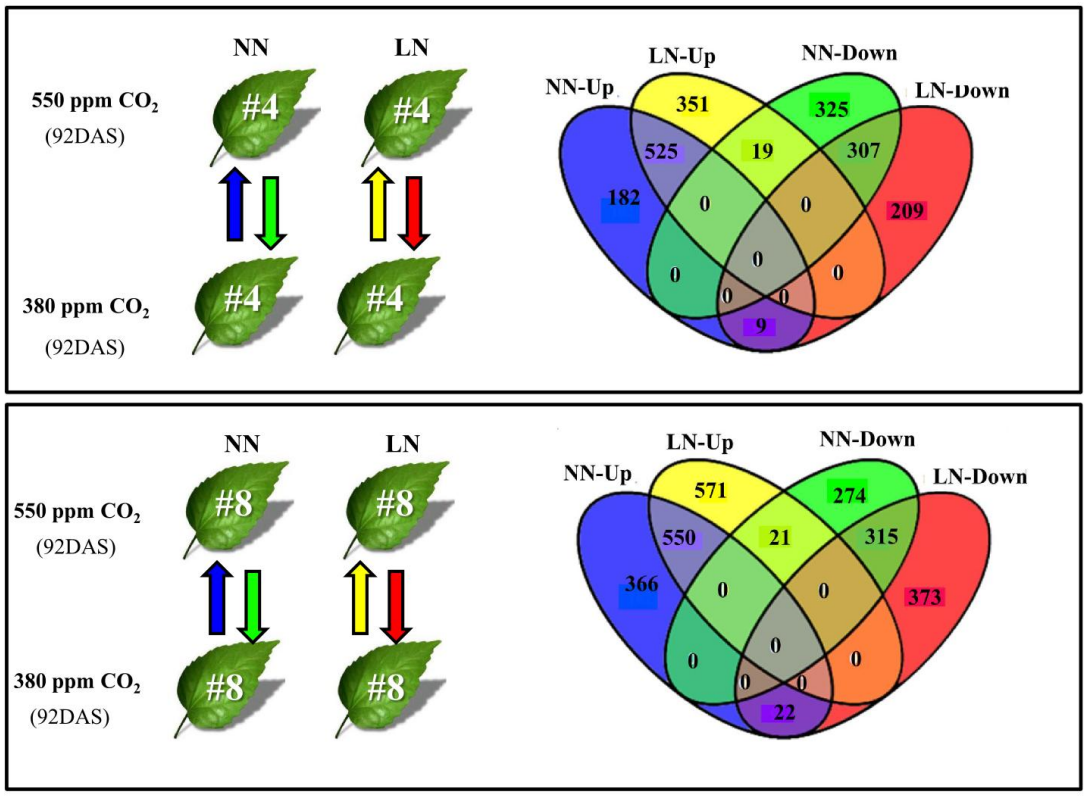


Figure 33. Number of regulated genes under elevated CO_2 (550 ppm) relative to ambient CO_2 (380 ppm) in leaf #4 (upper panel) and leaf #8 (lower panel). Comparison was conducted under normal (NN) and low (LN) N conditions. The color of the arrows is the same as the corresponding section in the Venn diagram.

3.6.2 Elevated CO_2 results in extensive gene expression changes in carbon associated pathways in young and old leaves

Higher C supply provided by elevated CO_2 stimulates gene expression changes related to biological processes involved in C assimilation and consumption such as photosynthesis (light reaction and Calvin cycle), cell wall (synthesis and degradation), mitochondrial TCA cycle and major carbohydrate (starch and sugar) metabolism which are presented in the following sections. This was observed in all of samples, regardless of N condition and leaf number. An overview of the different biological categories related to regulated genes in response to elevated CO_2 in old and young leaves under different N treatments is provided in figure S2.

Photosynthesis

Photosynthesis related genes belonging to Calvin cycle, light reactions and photorespiration showed regulation changes (more up-regulation) (Figure 35). As it is shown in figure 34, the number of regulated genes, especially up-regulated ones, was higher in young leaf (#8) under

both N conditions compared to old leaves (#4). This can be associated with higher metabolism of young leaves and the fact that young leaves are photosynthetically more active. It is possible that young expanding leaves can better benefit from the effect of elevated CO_2 on photosynthesis activity. The details of the regulated genes and their role are discussed in section 4.2.

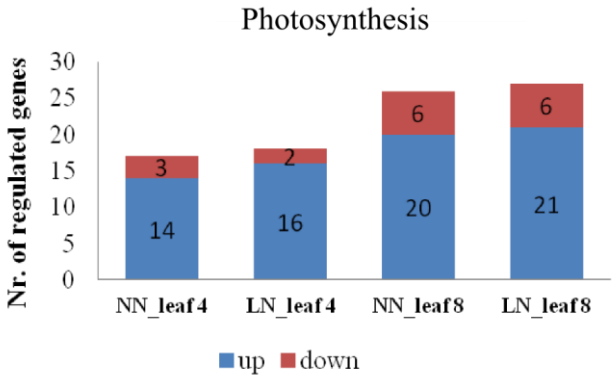


Figure 34. Number of photosynthesis regulated genes in response to elevated CO_2. NN: normal N, LN: low N.

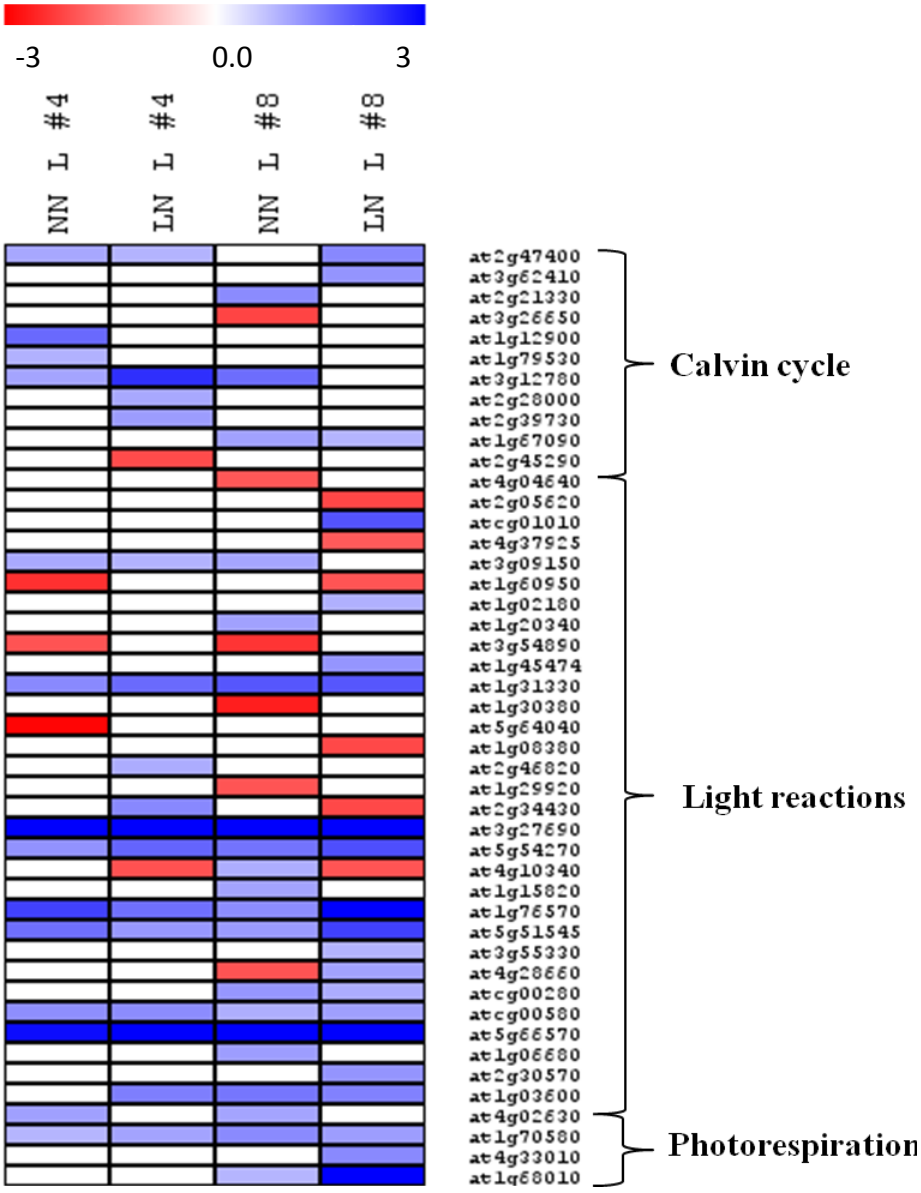


Figure 35. Expression pattern photosynthesis related genes in response to elevated CO_2. Data are log2 fold changes under elevated CO_2 relative to ambient CO_2. L #4: leaf #4, L #8: leaf #8, LN: low N, NN: normal N.

- TCA cycle and mitochondrial electron transport

Up-regulation of the genes involved in the breakdown of carbohydrates can provide energy which is required for higher growth at elevated CO_2. Leaf #4 under low N condition and leaf #8 under normal N showed higher number of the up-regulated genes (14 and 15 genes, respectively) (Figure 36) related to energy production. Figure 37 represent up- and down-regulated genes belonging to TCA cycle and mitochondrial electron transport, under normal and low N, in response to elevated CO_2. Since those leaves showed more senescence symptoms

under ambient CO_2 (e.g. lower SPAD values and higher up-regulation of the genes encoding senescence associated transcription factors) it seems that elevated CO_2 stimulates metabolism of these leaves which in turn leads to up-regulation of genes involved in TCA cycle and mitochondrial electron transport to provide the energy required for higher metabolism. Moreover, it is also consistent with up-regulation of photosynthesis genes which can results in higher C fixation and therefore providing more substrates for TCA cycle for energy production and producing precursors for biosynthesis of other macromolecules.

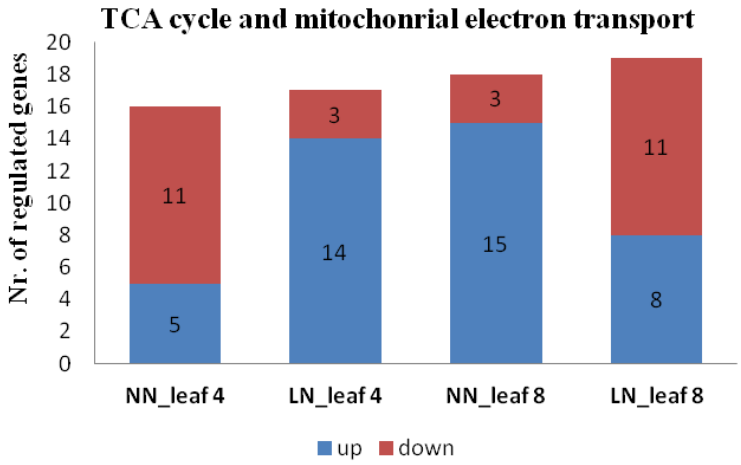


Figure 36. Number of regulated genes related to TCA and mitochondrial electron transport in response to elevated CO_2. NN: normal N, LN: low N.

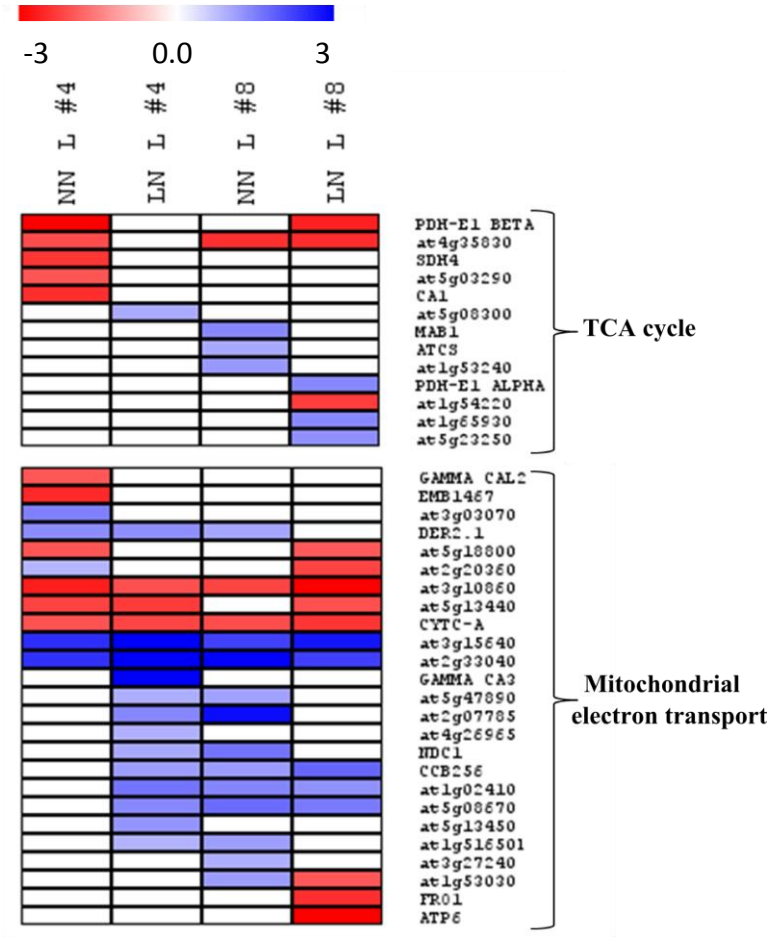


Figure 37. Expression pattern of genes related to TCA cycle and mitochondrial electron transport in response to elevated CO_2. Data are log2 fold changes under elevated CO_2 relative to ambient CO_2. L #4: leaf #4, L #8: leaf #8, LN: low N, NN: normal N.

- Cell wall

Cell wall is known as major sink for C (Gibeaut et al. 2001). Different genes involved in cell wall synthesis, degradation, cell wall proteins and cell wall modification showed expression changes under elevated CO_2 (Figure 39). Numbers of up- and down-regulated cell wall genes are shown in figure 38. Up-regulation of cell wall related genes, especially those involved in cell wall precursor synthesis and cell wall degradation implies their correlation with leaf expansion under elevated elevated CO_2 (Marga et al. 2005). More details about the cell wall related regulated genes are provided in discussion section 4.3.

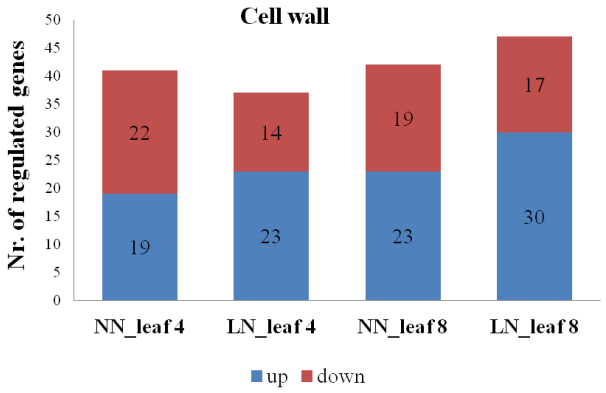


Figure 38. Number of cell wall regulated genes in response to elevated CO_2. NN: normal N, LN: low N. Blue: up-regulated, red: down-regulated.

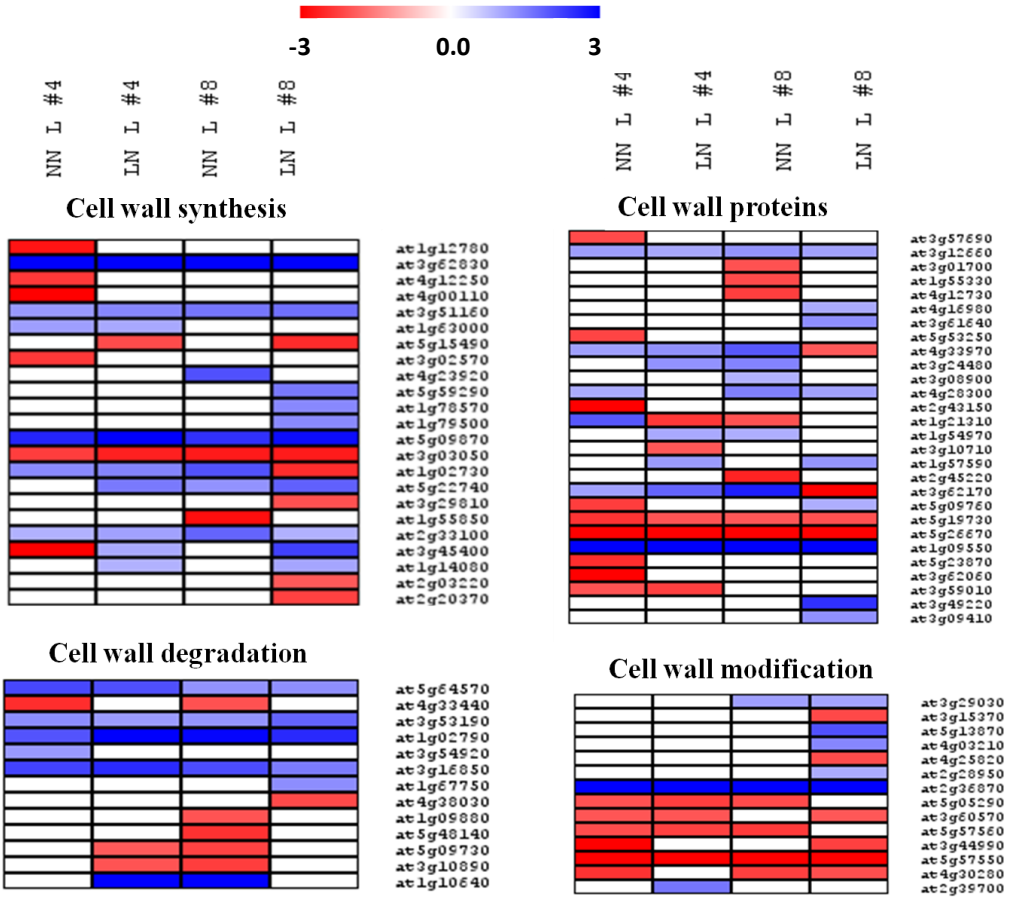


Figure 39. Expression pattern of genes related to starch and sucrose metabolism in response to elevated CO_2. Data are log2 fold changes under elevated CO_2 relative to ambient CO_2. L #4: leaf #4, L #8: leaf #8, LN: low N, NN: normal N

- Major carbohydrates (starch and sucrose) metabolism

Higher photosynthesis under elevated CO_2 results in accumulation of major carbohydrates (sucrose and starch). Consistent with the previous statement, in the present study, two starch synthesis genes encoding ADP glucose pyrophosphorylase (involved in the first and committed step of starch synthesis) and starch synthase 2 (SS2) showed up-regulation in all the samples under elevated CO_2 (Figure 41). A gene encoding a hexokinase like protein (HKL1), involved in sucrose degradation, showed up-regulation in all samples, except than leaf #4 under normal N (Figure 41). Number of up- and down-regulated genes involved in major carbohydrates synthesis and degradation is provided in figure 40.

Taken together, these data suggests that elevated CO_2 promotes synthesis of carbohydrates due to an increased level of C assimilation. However, it can also stimulate utilization of the accumulated carbohydrates which can provide more energy and biochemical precursors for leaf expansion and higher growth under elevated CO_2, as it was observed by Franzaring et al., 2011.

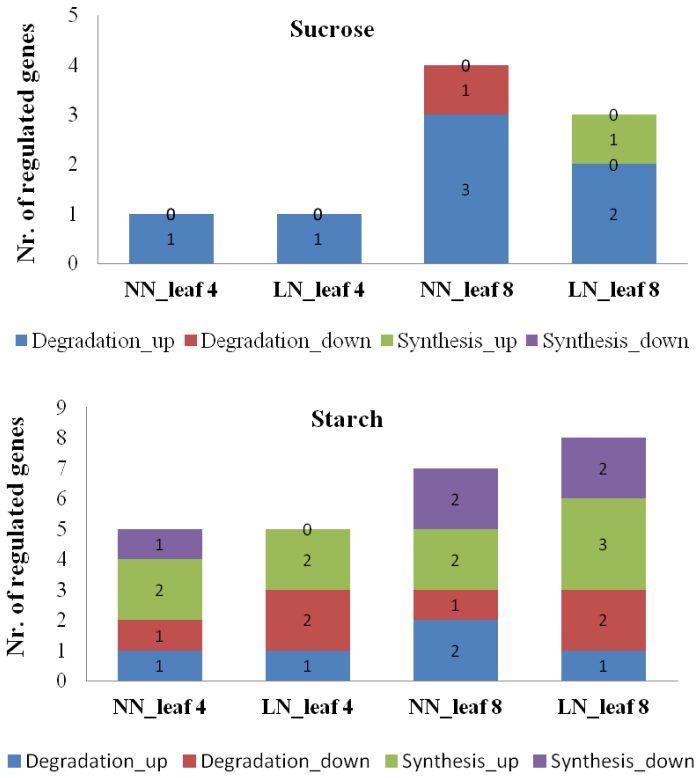


Figure 40. Number of regulated genes involved in major carbohydrates (sucrose and starch) metabolism in response to elevated CO_2. NN: normal N, LN: low N.

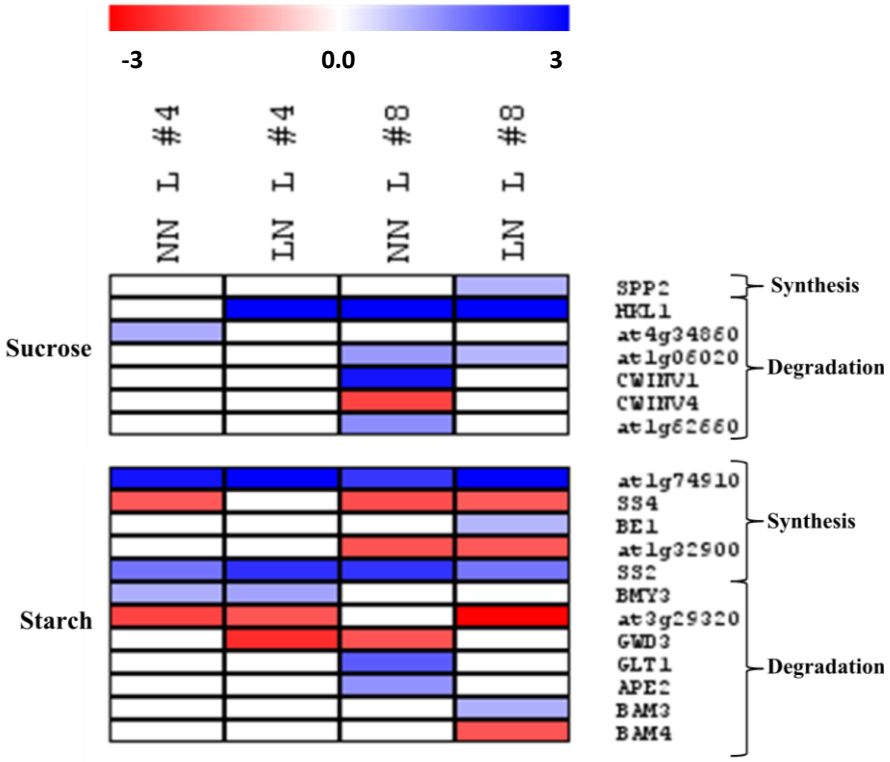


Figure 41. Expression pattern of genes related to starch and sucrose metabolism in response to elevated CO_2. Data are log2 fold changes under elevated CO_2 relative to ambient CO_2. L #4: leaf #4, L #8: leaf #8, LN: low N, NN: normal N.

Transcription factor and hormones orchestrate response to different environmental stimulus via transcriptional regulation of their target genes. To better understand gene expression changes of these master regulators in young and old leaves under high CO_2 level, transcriptome changes of leaf #4 and leaf #8 were compared. For visualization of the regulated genes involved in different biological pathways MapMan software was used.

3.6.3 Up-regulation of several brassinosteroid and ethylene related genes in response to elevated CO_2 can be correlated with higher growth and water use efficiency observed in oilseed rape

Different hormonal categories showed expression changes in response to elevated CO_2 under normal and low N conditions. Taken together hormone related gene expression changes in response to elevated CO_2 did not show a distinct picture between different N treatments except than IAA which showed higher up-regulation only in leaf #8 under low N condition while IAA

related genes were mainly down-regulated in the other samples. Individual genes belonging to each hormonal category which showed significant expression changes in response to elevated CO_2 under each condition are presented in table S10. As it is shown in figure 42 brassinosteroid (BA) and ethylene related genes showed highest percentage of up-regulated genes under elevated CO_2 regardless of N treatment and leaf number. Regulated brassinosteroid and ethylene related genes are involved in synthesis, signaling and also response to these hormones. In case of ethylene, two genes (*ERF13* and At5g25190) encoding ethylene responsive factors (ERFs) involved in ethylene signaling showed up-regulation in all samples in response to elevated CO_2. Even though, the biological function of above-mentioned ERF encoding genes is left to be discovered, it has been documented that ERF transcription factors are involved in various processes of plant development and stress responses (Wang F. et al. 2013).

It has been reported that elevated CO_2 stimulates biomass accumulation in Arabidopsis plants in a GA-independent manner by regulating the expression of growth-related genes such as BZR2 gene (involved in brassionsteroid signaling) (Ribeiro et al. 2013). In the current study, BZR2 showed more than 10 fold up-regulations in leaf #4 under normal N and more than 16 fold up-regulations in other samples in response to elevated CO_2. This implies that brassinosteroid may play a role in higher growth and biomass production under elevated CO_2 in oilseed rape. Up-regulation of those genes belonging to growth related hormones, ethylene and brassinosteroid (figure 43 and 44), is consistent with higher biomass accumulation which was observed in oilseed rape. This suggests that there is a coordinated gene regulation network likely via growth regulating hormones, such as brassinosteroids, which control plant growth and development under elevated CO_2.

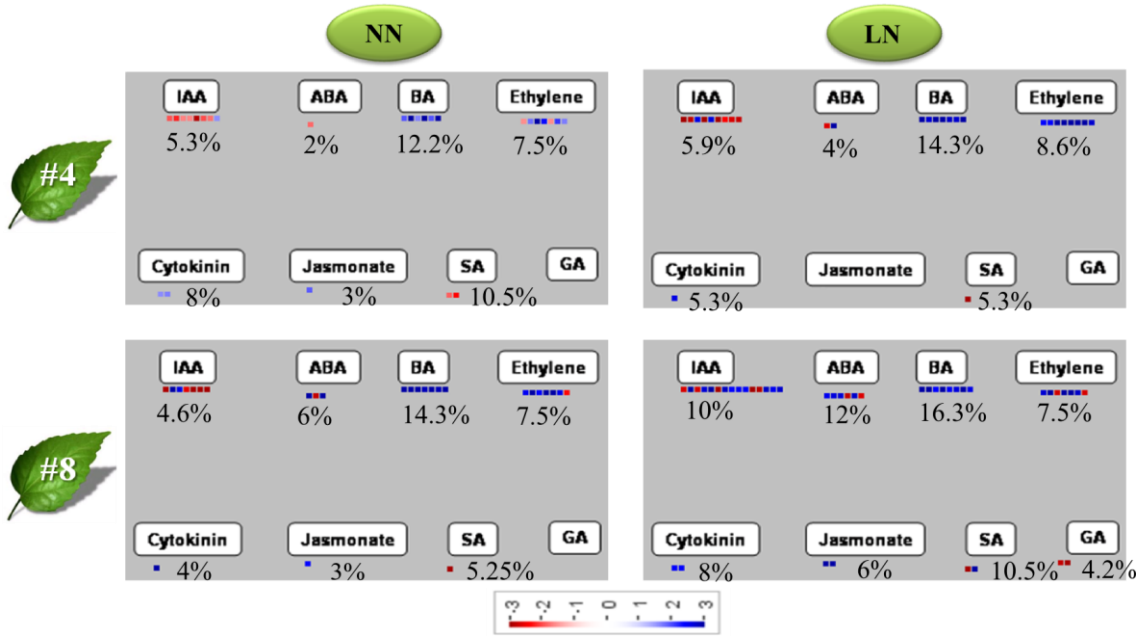


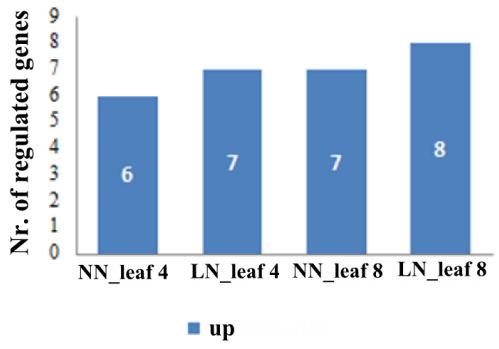
Figure 42. Overview of gene expression changes related to hormone metabolism in response to elevated CO_2 in leaf #4 and leaf #8 under normal (NN) and low N (LN) conditions. Blue and red squares represent up- and down-regulated genes, respectively. Numbers under each hormonal category represent the percentage of the regulated members of that family calculated based on the total number of the same family available in MapMan (TAIR10).

Brassinosteroid

MapMan (TAIR 10)	52
# of probes in *B. napus* microarray	158
… represented # of *A. thaliana* genes	46

Ethylene

MapMan (TAIR 10)	100
# of probes in *B. napus* microarray	187
… represented # of *A. thaliana* genes	68



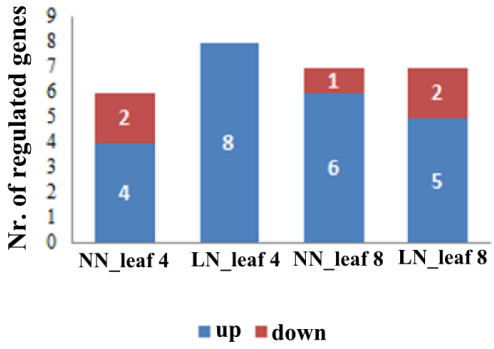


Figure 43. Total number of genes (upper panel) and number of regulated genes (lower panels, blue: up-regulated, red: down-regulated) belonging to brassinosteroid and ethylene hormones in response to elevated CO_2 in leaf #4 and leaf #8, respectively.

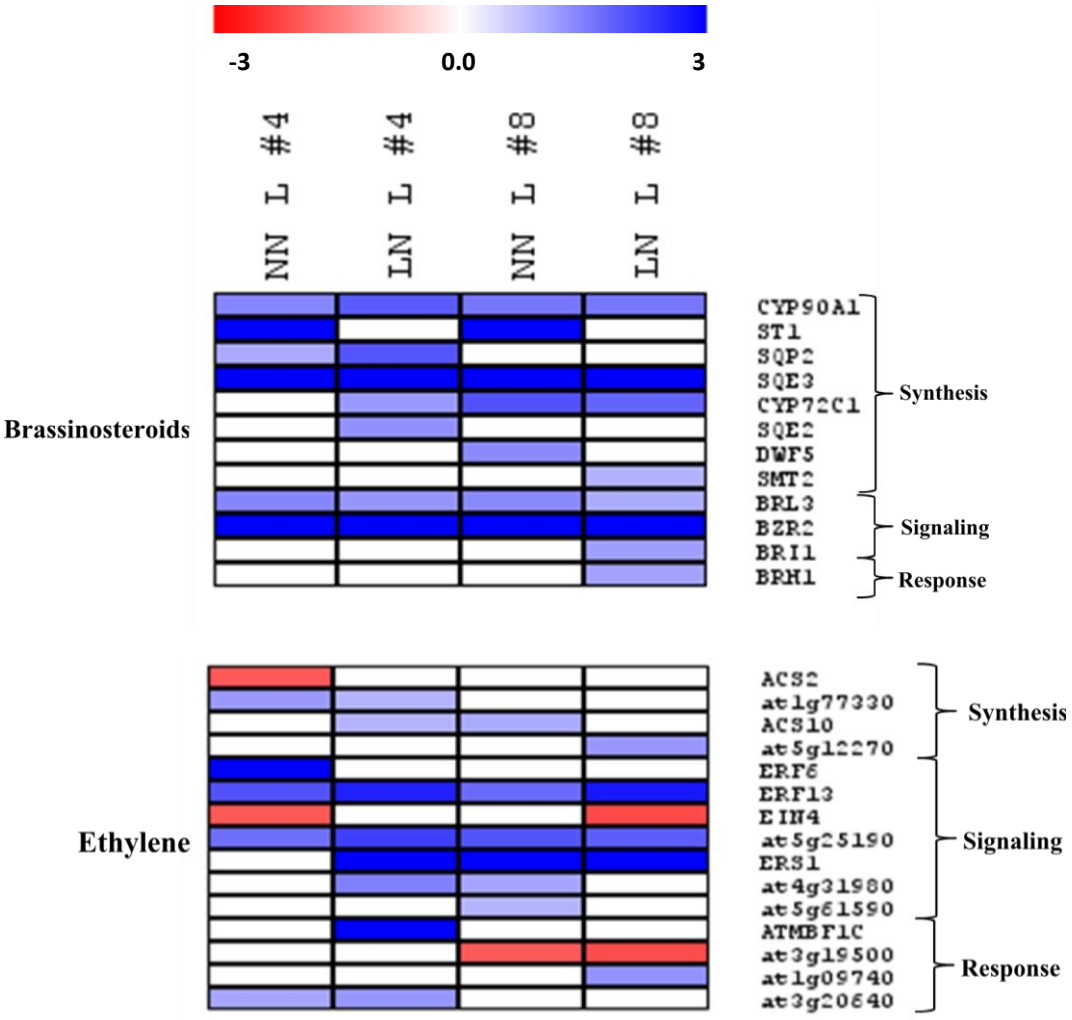


Figure 44. Expression pattern of brassinosteroid and ethylene related genes in response to elevated CO_2. Data are log2 fold changes under elevated CO_2 relative to ambient CO_2. L #4: leaf #4, L #8: leaf #8, LN: low N, NN: normal N.

3.6.4 Transcription factor families bHLH, bZIP and MYB showed high number of regulated genes in response to elevated CO_2

Members of different transcription factor families including AP2-EREBP, bHLH, bZIP, C2H2, G2-like, MYB, MYB-related, WRKY, chromatin remodeling, trihelix and SET-domain showed expression changes in response to elevated CO_2 (figure 45). Total number of genes related to different transcription factor families in TAIR10, number of *B. napus* probes and number of represented t *A. thaliana* transcription factors in *B. napus* microarray is provided in table 12.

Table 12. Total number of genes related to different transcription factor families in TAIR10, number of probes and number of represented *A. thaliana genes* in *B. napus* microarray.

Transcription factor family	TAIR 10	# of probes in *B. napus* micorarray	…represented # of *A. thaliana* genes
AP2/EREBP	115	180	81
bHLH	147	266	97
bZIP	73	157	55
C2H2	150	220	89
MYB	169	248	102
MYB-related	47	115	35
WRKY	73	134	51
Chromatin remodeling	37	121	33
Trihelix	30	76	25
Set-domain	40	73	32
G2-like	41	78	29

As it is shown in figure 45, MYB, bHLH and C2H2 families showed highest number of regulated genes in response to elevated CO_2 in all samples. Figure 46 shows the expression heat map of regulated genes belonging to these three families. MYB and bHLH members showed more up-regulation while C2H2 members were more down-regulated (Figure 45 and 46). The regulated genes and their role in response to elevated CO_2 is addressed in section 4.6. Even though, the importance of transcription factors (such as bHLH and MYB) in regulating of down-stream pathways has been documented (Feller et al. 2011), our knowledge of elevated CO_2 responsive transcription factors is very fragmental. The data from the current study provides the potential candidates for further investigations. Understanding the regulatory network involved in crop response to elevated CO_2 can help to improve crop sustainable agriculture in the context of climate change.

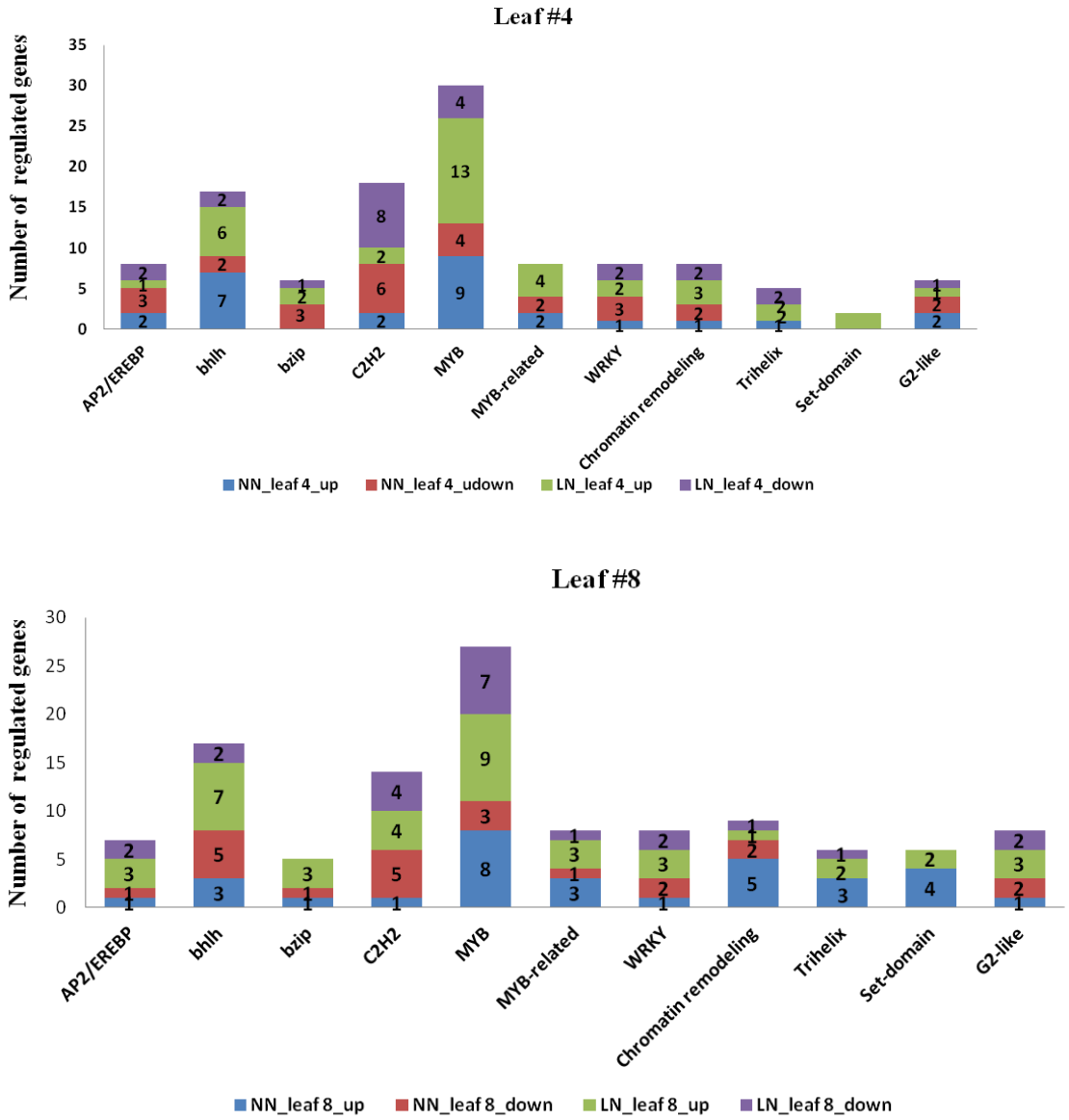


Figure 45. Number of regulated transcription factor encoding genes in response to elevated CO_2. Upper and lower panels represent number of transcription factor encoding genes which showed up regulation (up) or down-regulation (down) in leaf #4 and leaf #8, respectively.

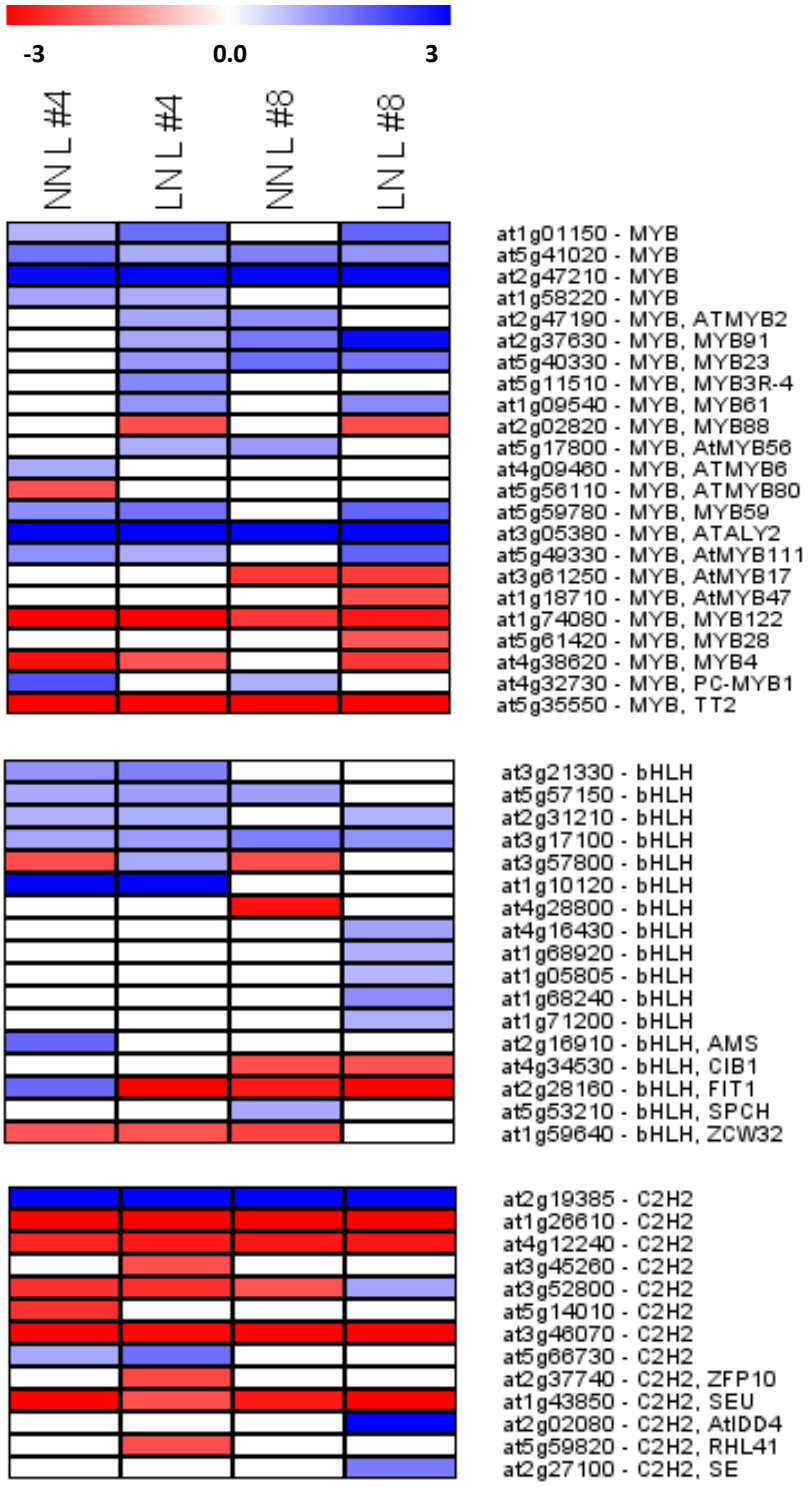


Figure 46. Gene expression heat map of regulated genes in response to elevated CO_2, belonging to MYB, bHLH and C2H2 family of transcription factors. Data represent means of two independent biological replicates. Heatmap is generated with the MultiExperiment Viewer (MeV) software (www.tm4.org/mev). L #4: leaf #4, L #8: leaf #8, LN: low N, NN: normal N.

In sum, elevated CO_2 showed a more universal effect on gene expression changes in all samples (old and young leaves under normal and low N conditions). This indicates that elevated CO_2 has a stronger impact on transcriptome response of oilseed rape than N treatment and leaf position. Moreover, regulated genes involved in master regulatory networks such as transcription factors together with hormone (brassinosteroid and ethylene) metabolism genes provide candidates which might play important role in the response of oilseed rape to elevated CO_2. Further investigations (e.g. combination of lab work and experiments in the field) are needed to unravel their possible role in improvement of oilseed rape production in the context of climate changes. Taken together, this study can provide the fundamental knowledge related to the transcriptome response of oilseed rape to N deficiency and elevated CO_2. It has the potential to be used as a genetic resource for relevant research community globally. However, further studies are needed to verify the functions of candidate genes for improving oilseed rape NUE (e.g. N-remobilization) and response to elevated CO_2 through genetic engineering.

4 Discussion

Using a microarray study in old and young leaves of oilseed rape (*Brassica napus*) it was possible to unravel transcriptome response of oil seed rape to developmental and N-deficiency induced leaf senescence and elevated CO_2. Oilseed rape is used worldwide as an important crop plant, however it has a low NUE. Moreover, C and N balance is important in determining biomass and seed yield. Therefore, understanding the molecular mechanisms and the genes involved in response to different N and C concentrations has the potential to improve oilseed rape NUE. In this PhD thesis, the transcriptome response of oilseed rape to developmental leaf senescence (under normal N) and N-deficiency induced leaf senescence is examined. The main transcriptome response differences and the key genes with differential expression at 92 DAS (H4) relative to 85 DAS (H3) between normal and low N condition is addressed and discussed in result sections 3.3.2, 3.3.3, 3.3.4 and 3.3.5. In the following section the main objective was to pin out the possible connections between gene expressions changes with phenotypical and physiological data of the same plants which have been reported by Franzaring et al., 2011. Hence, in the discussion section the focus is on the last two step including 92 DAS (H4) and 99 DAS (H5) for data analysis of leaf #4 because most of the phenotypical measurements were performed at the later time points when plants have already developed the reproductive organs. In case of elevated CO_2, plants were harvested at one time point, 92 DAS (H4) (92 DAS), and were compared with the samples grown under ambient CO_2 belonging to the same harvest. To facilitate the comparison of the ambient and elevated CO_2 effect on gene expression changes under normal and low N condition, the discussion section of ambient and elevated CO_2 is combined. As it was shown by PCA analysis (figure 32) the effect of N-deficiency on gene expression changes under ambient CO_2 was stronger while elevated CO_2 seems to overrun this effect. Therefore, to facilitate the comparison of the effect of N-deficiency (ambient CO_2) and C (elevated CO_2) on gene expression changes, the discussion of these two parts is combined.

4.1 *Brassica napus* microarray design and challenges for transcriptome analysis

Brassica napus is an allopolyploid (allotetrapolyploid), containing A and C genome originating from *Brassica rapa* and *Brassica oleracea*, respectively. Its chromosome number is n = 19 and the size of its genome is *ca.* 1200 Mb (Trick et al. 2009). The hybridization to form *B. napus* probably occurred during human cultivation, i.e. less than 10,000 years ago. Thus, *Brassica* species, in conjunction with Arabidopsis, provide an opportunity to study the evolution of genome structure over a wide range of time scales since polyploidization occurred (Trick et al. 2009).

B. napus and *Arabidopsis thaliana* are both belonging to Crucifereae family and there is 86% of protein sequence conservation between these two species (Li Fengling et al. 2005) and there are several successful microarray studies in oilseed rape has been performed using Arabidopsis cDNA array (Li Fengling et al. 2005, Shen et al. 2006, Yang Bo et al. 2007). However, duplication events, rearrangements at chromosomal level and gene-level deletions makes difficulty for analysis of individual gene sequence in this plant (Cheung et al. 2009). Moreover, detection of *B. napus* specific sequences which are not present in *A. thaliana* genome is not possible using Arabidopsis array. Therefore, developing a novel oilseed rape microarray resource using EST sequences belonging to *B. napus*, *B. rapa* and *B. oleracea* is important (Trick et al. 2009). The whole genome sequence of *B. napus* has been very recently released (Chalhoub et al. 2014). However, the functional annotation of its genes is still far behind *Arabidopsis thaliana*, for which the first complete plant genome sequence was obtained (Trick et al. 2009). The fact that *B. napus* and *A. thaliana* are closely related species and have high sequence homology provides the opportunity to extend Arabidopsis genome functional annotation to *B. napus* for transcriptome data analysis. Since ~9% of *Brassica* ESTs show no similarity with any Arabidopsis gene (Yang T. J. et al. 2006), it is estimated to assign for 90% of the *B. napus* unigenes the most similar Arabidopsis gene(s). To facilitate functional pathway analyses with tools developed for *Arabidopsis thaliana* (e.g. MapMan), all *B. napus* 'unigenes' with homology to one Arabidopsis gene were hierarchically ranked according to their BLAST E-value and regulation in any plant samples. Among a group of *B. napus* homologs for the same Arabidopsis gene which showed regulation changes, the one with the lowest E-value was selected as the most informative homolog of that *Arabidopsis thaliana* gene for further bioinformatical analysis.

The *B. napus* genome contains 101,040 gene models. Of these, 91,167 were confirmed by matches with *B. rapa* and/or *B. oleracea* predicted proteomes. Alternative splicing was shown in 48% of genes (Chalhoub et al. 2014). This new source of oilseed rape genome sequence will provide the opportunity to improve the gene coverage of the custom made microarray used in this study by designing new probes for the genes available in oilseed rape but absent in the array. Moreover, by designing the probes considering single nucleotide polymorphism it will be possible to distinguish the multiple copies of a gene (homologous alleles) originating from the two progenitors, *B. rapa* and *B. oleracea*.

The present design of the Bn-microarray is based predominantly on three sets of ESTs from B. *napus*, *B. rapa* and *B. oleracea* that were available in September 2007 (Trick et al., 2009). Only a small fraction of these ESTs was generated from leaf material, most came from flowers and developing seeds. Therefore, creating EST libraries from leaves (using the whole gneome sequence) will add novel sequences to the unigene set. This will update the Bn-microarray design by adding probes for new unigenes and removing probes from the present collection that are either redundant or did not yield a hybridization signal with any RNA.

4.2 Down-regulation of photosynthesis genes start earlier but proceeds slower under N starvation-induced leaf senescence while elevated CO_2 strongly enhances regulation of photosynthesis related genes

Nitrate assimilation and biosynthesis of nitrogen containing macromolecules require abundant energy, reducing equivalents (NADH and NADPH) and numerous organic carbon intermediates. Photosynthesis is one of the major metabolic pathways besides tricarboxylic acid (TCA) cycle and the pentose phosphate pathway to produce these compounds in plants. Under low N condition the genes involved in the synthesis of nitrogenous macromolecules are repressed which can lead to the reduction in the consumption of energy, reductants and organic carbon intermediates for nitrate assimilation and the anabolism of nitrogenous macromolecules. Therefore, repression of photosynthesis genes under low N is expected as it has been shown in Arabidopsis (Peng et al. 2007).

Down-regulation of photosynthesis in leaf #4 was observed between 85 DAS (H3) and 92 DAS (H4) under both N conditions. However, down-regulation of photosynthesis at later time points (between 92 and 99 DAS) was observed under normal N condition. This is consistent with the trend of chlorophyll and nitrogen content reduction. Although leaves 4 and 8 had lower initial SPAD values, respectively lower chlorophyll and nitrogen contents, the chlorophyll degradation

beginning at 85 DAS proceeded more slowly under low N condition than in plants grown under normal N (Franzaring et al. 2011). It is possible that leaves from plants with a low supply of N may keep their available resources for a longer time than leaves from well fertilized plants. Reasons for this could be that slow growing plants developing under a constantly low N supply produce more compact leaves with strong structural tissues, while leaves subjected to a sudden N starvation after luxurious supply will show a rapid degradation of chlorophyll and remobilization of resources to support the growth of other parts of the plant (Desclos et al. 2009, Franzaring et al. 2011). A very strong repression of photosynthetic genes related to both light reactions and Calvin cycle in leaf #8 under normal N condition in comparison to the same leaf under N limitation was observed. This is in agreement with SPAD values which were lower in leaf #8 under normal N at the latest time point (106 DAS) (Figure 21), indicating a delayed in senescence in young leaves under low N condition.

In leaf #8 the number of down-regulated photosynthesis and tetrapyrrole synthesis genes in leaf #8 under normal N (31genes out of 254) was higher than leaf #8 under low N condition (15 genes out of 254). Several photosystem II light harvesting complex (LHCBII) genes (*LHCB2.2*, *LHCB2.4* and *LHCB2.1*, *LHCB3.1*) were down-regulated only in leaf #8 under normal N conditions which among them LHCB3.1 was the only one which was down-regulated in leaf #8 under low N. Even though chlorophyll represents about 2% of the total cellular nitrogen content around 20% N are fixed in proteins associated with or directly binding chlorophyll and removal of chlorophyll seems to be a prerequisite for degradation of the corresponding apoproteins including LHCBII (Hörtensteiner 2006). It has been shown that the expression of genes such as those involved in photosynthesis and also in the structure of the chloroplast, decreases during leaf senescence (Gombert et al. 2006). It can be concluded that down-regulation of LHCBII genes is correlated with more advanced senescence progression in leaf #8 under normal N condition compared to low N which can be correlated with inefficient source to sink nutrient transport under normal N condition in oilseed rape.

Prior studies focusing on the effects of climate changes reported both positive and negative effects of CO_2 enrichment on photosynthesis in different species (Ainsworth et al. 2003, Makino and Mae 1999, Usuda and Shimogawara 1998). There are variations in physiological, biochemical and molecular responses to raising atmospheric CO_2 levels in different plant species including C3, C4 and crassulacean acid metabolic (CAM) pathways (Reddy et al. 2010). In C3 species, Rubisco enzyme is able to catalyze two competing reaction: carboxylation and oxygenation (Portis and Parry 2007). Oxygenation reaction leads to photorespiratory pathway which results in 20 to 30% loss of fixed C. Therefore, reduction of oxygenation reaction of

Rubisco can potentially increase C assimilation (Long et al. 2006). Down-regulation of Calvin cycle under elevated CO_2 has been attributed to starch degradation and related sugar signaling under elevated CO_2 which repress photosynthesis enzymes (Sharkey et al. 2004). Up-regulation of some of starch degradation enzymes under our condition is in accordance with the statement mentioned above. It was observed that under elevated CO_2, photosynthesis related genes including both light reactions and Calvin cycle related genes showed up-regulation in young and old leaves under both N conditions. This up-regulation can be correlated with a significantly higher shoot biomass due to prolonged vegetative growth under elevated CO_2 (Franzaring et al. 2011). More side branches in response to elevated CO_2 (under both N conditions, especially normal N) can act as strong sink for photoassimilates which results in a positive feedback to the leaves which eventually leads to up-regulation of photosynthesis related genes, e.g. the PSII light harvesting chlorophyll binding protein LHCB3 which showed up-regulation in all samples. It has been shown that LHCB encoding genes are involved in sensitivity to ABA and stomatal closure (Xu Y. H. et al. 2012). Interestingly, elevated CO_2 resulted in better water use efficiency in the samples used in this study (Franzaring et al. 2011) which is attributed to stomata closure. This can be positively correlated with up-regulation of LHCB genes in this condition. Moreover, photorespiration related genes were up-regulated in leaf #8 under both N conditions in response to elevated CO_2. In a study conducted on nine plant species from seven different families (oilseed rape was not included) under elevated CO_2, it was revealed that elevated CO_2 results in structural changes in major cellular organelles (mitochondria and chloroplast) (Griffin et al. 2001). In the afore-mentioned study, the amount of chloroplast stroma thylakoid membranes increased under elevated CO_2. The same observation was reported for Arabidopsis plants grown under elevated CO_2 (Teng et al. 2006). Distribution of PSI and PSII in grana and stroma thylakoids is not universal. PSII is mainly in grana and PSI in stroma thylakoids (Rojdestvenski et al. 2002). It has been reported that cyclic electron flow happens around PSI at high light to induce non photochemical quenching of chlorophyll fluorescence and is essential for photosynthesis (Miyake et al. 2004, Munekaga et al. 2004). It is likely that increasing cyclic electron flow in chloroplast of oilseed rape under elevated CO_2 is connected with changes in chloroplast fine structure including higher amount of stroma thylakoids. However, more structural studies are required to confirm such a connection between gene expression, biochemical, physiological and structural changes. It has been reported that C3 plants respond to elevated CO_2 with increased photosynthesis, growth, productivity and reduced stomata conductance which causes lower transpiration (Ainsworth and Rogers 2007). Oilseed rape plants studied here, showed higher phtotosynthesis and growth under elevated CO_2, however, the yield was negatively associated with elevated CO_2 (Franzaring et al. 2011) which can be

related to the higher investment of resources to vegetative growth compared to reproductive organs.

4.3 N-limitation results in up-regulation of cell wall degradation genes while elevated CO_2 results in up-regulation of lignin related genes

Besides the photosynthetic apparatus, the cell wall acts as N sink in plants (Onoda et al. 2004). It has been shown that primary cell wall N content is ranging from 0.4 to 2.2% in different species (Onoda et al. 2004).

Under both N conditions (92 until 99 DAS) expression changes were observed in the genes involved in the biosynthesis of important cell wall components including cellulose, hemicellulose and pectin plus many cell wall proteins such as expansins and hydroxyproline-rich glycoproteins. However, the intensity of the changes differed depending on the N treatment. Cell wall degradation genes showed more up-regulation under low N in leaf #4 which may indicate transporting of carbon and N containing compounds toward upper parts of the plant. N limitation in Arabidopsis results in up- or down-regulation of different cell wall related genes. Under low N condition, strong down-regulation of a cell wall gene *XTH18* was observed which encodes cell wall enzyme xyloglucan endotransglucosylase/hydrolase 18 involved in cell wall modification. Adding nitrogen to nitrogen deprived Arabidopsis seedlings resulted in transcriptional up-regulation of genes encoding cell wall modification enzymes such as xyloglucan endotransglycosylases, expansins, pectinesterases and polygalacturonases. These changes are considered as N associated growth related processes (Scheible et al. 2004).

Cell wall related gene expression changes under elevated CO_2 condition were also observed. Cell wall is also a major sink for carbohydrates which form 20 to 50% of plant dry weight and 70 to 80% of the carbohydrates (Gibeaut et al. 2001). Hence, changes in CO_2 affect cell wall as a major C sink. I observed that different cell wall related genes belonging to cellulose synthesis, precursor synthesis, pectin esterase, cell wall modifications and cell wall proteins showed expression changes in response to elevated CO_2. Also noticeable was up-regulation of lignin biosynthesis genes, such as *C4H* and *UGT72E1,* in all samples in response to elevated CO_2. Another lignin synthesis gene, *FAH1*, showed more distinct expression between old and young leaves so that, it was up-regulated in leaf #8 under both N conditions but not in leaf #4. A study with poplar trees grown under elevated CO_2 showed that even though some of enzymes and transcripts involved in lignin pathway showed reduction under elevated CO_2, lignin content was

increased under this condition due to higher C supply to the stem which supports higher lignin synthesis (Richet et al. 2012). Selective loosening and rearrangement of the cell wall stimulate a turgor-driven expansion which is correlated with cell growth (Marga et al. 2005). Two classes of proteins including expansins (Goh et al. 2012) and xyloglucan endotransglucosylase/endohydrolases (XTHs) (Van Sandt et al. 2007) are known to be involved in this process. It has been shown that elevated CO_2 increases cell wall extensibility and expansion in roots and leaves likely via XTHs activities (Taylor et al. 1994). It has been reported that expansins are involved in plant cell wall loosening and have been shown to be up-regulated under elevated CO_2 in aspen trees (Cosgrove 2000, Wei et al. 2013). Activity of UDP-Glc dehydrogenase which is a rate limiting enzyme in cell wall biosynthesis and is involved in biosynthesis of many cell wall polysaccharids increases under elevated CO_2 which is corresponding to higher growth rate under this condition (Gibeaut et al. 2001). Both up and down-regulation of expansin and XTH related genes was observed in this study. However, down-regulation of these genes was more remarkable in leaf #4, consistent with lower cell wall rearrangement and cell growth in older leaves indicating the effect of leaf age and growth rate on cell wall gene expression response to elevated CO_2.

4.4 Elevated CO_2 and senescence, but not N limitation alone, results in higher up-regulation of major CHO metabolism genes

Beside cell wall, carbon allocation is also connected to non structural carbohydrates such as starch and sucrose. It has been reported that low N supply increases starch synthesis in plants due to decreased consumption of organic carbon skeletons in nitrogen metabolism (Diaz et al. 2005, Wingler et al. 2006). Up-regulation of the gene encoding ADP-glucose pyrophosphorylase has been reported in Arabidopsis plants grown under constant N limitation (Peng et al. 2007). However, this gene did not show up-regulation in the present study. Taken together, starch synthesis genes showed up-regulation under both N conditions in leaf #4 between 92 DAS (H4) and 99 DAS (H5) and under normal N in leaf #8 in this study. However, higher up-regulation of starch genes under low N compared to normal N was not observed in this study, which is not in accordance with the previous report in Arabidopsis. This can be due to the different species specific C partitioning in response to N deficiency.

Under elevated CO_2 and especially in leaf #8, major CHO metabolism including sucrose and starch, showed overall up-regulation under both N conditions. Interestingly, ADP-glucose pyrophosphorylase encoding gene, a regulatory enzyme for plant starch synthesis which catalysis the first committed step of starch synthesis including production of ADP-glucose and

pyrophosphate (PPi) from glucose-1-phosphate (Ballicora et al. 2004) was up-regulated in all the samples except than leaf #4 under normal N which showed up-regulation of starch degradation genes such as genes encoding beta-amylase 1 (BAM1) in this leaf.

Sucrose metabolism related genes especially the genes involved in starch degradation toward sucrose production such as alpha amylase AMY2 and AMY3 showed up-regulation in response to CO_2 enrichment under normal and low N conditions in leaf #4 and #8. Degradation of starch under elevated CO_2 has already been shown to be involved in photosynthesis repression including down-regulation of photosynthesis enzymes through sugar signaling (Sharkey et al. 2004). However, repression of photosynthesis genes at 92 DAS was not observed in this study. Slightly, lower chlorophyll content (but not significantly) at 92 DAS under elevated CO_2 in both leaves #4 and #8 was observed (Figure S4).

4.5 Hormonal related transcriptome responses to different N treatments and elevated CO_2

Phytohormones are considered as key regulators of plant growth and developmental processes. Genetic and physiological evidences have shown that N status and availability promote morphological changes by modulating hormone homeostasis and/or signaling (Vidal and Gutierrez 2008). In the current study, gene expression changes in different hormonal categories including IAA, ABA, BA (BR), Ethylene, cytokinin, jasmonates, SA and GA was observed in response to developmental and N deficiency induced leaf senescence regardless of leaf number. The intensity of this response, however, was not similar in all of the conditions.

In leaf #4, under low N condition, up-regulation of two senescence associated hormones including ethylene and ABA was observed between 85 and 92 DAS. Up-regulation of ethylene related genes was observed at later time span (between 92 and 99 DAS) under normal N condition. In leaf #8, ethylene, ABA and another senescence associated hormone, jasmonate, showed up-regulation under normal N. Earlier up-regulation of senescence associated hormones in samples with lower SPAD values (leaf #4 under low N, figure 9, and leaf #8 under normal N, figure 21) can be correlated with a senescence induction.

Two lipoxygenase enzyme encoding genes involved in jasmonate biosynthesis including *LOX2* and *LOX3* were up-regulated under normal N in leaf #8 and the latter was also up-regulated in leaf #4 under low N condition. Interestingly, the jasmonate biosynthesis genes showed up-regulation in those samples which had lower SPAD values indicating it's correlation with senescence progression. Jamsonates has been reported to be associated with plant senescence. It

has been shown that endogenous levels of jasmonates increase during senescence (He et al. 2002, Seltmann et al. 2010). In oilseed rape application of methyl jasmonate results in the reduction of N uptake while it induces a strong remobilization of N from senescing leaves and a concomitant accumulation of the 23 kDa putative vacuolar storage protein (VSP). It has been reported that in oilseed rape, the 23 kDa protein acts as a storage buffer between N losses from senescing leaves promoted by methyl jasmonate and grain filling (Rossato et al. 2002).

Elevated CO_2 resulted in overall up-regulation of ethylene and brassinosteroid (BR) genes. 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase are key enzymes in ethylene biosynthesis which catalyzes the reaction from S-adenosylmethionine to ACC and from ACC to ethylene, respectively. The ethylene signal transduction genes including *ERF13*, *ERF6* and *ERS1* showed up-regulation in response to elevated CO_2. *ERF13* was up-regulated in all of the samples regardless of N treatment and leaf number while *ERF6* was only up-regulated in leaf #4 under normal N and *ERS1* was up-regulated in all the other samples except than leaf #4 under normal N. Ethylene synthesis related genes including *ACC oxidase* gene showed up-regulation in leaf #4 under normal N while a *PUTATIVE ACC OXIDASE* and *ACC SYNTHASE 10* (*ACS10*) genes showed up-regulation in leaf #4 under Low N condition while the latter gene was also up-regulated in leaf #4 under normal N condition. This data indicates that elevated CO_2 is associated with higher ethylene signal transduction and to some extent with ethylene synthesis. Ethylene has been shown to be involved in senescence induction in an age dependent manner (Jing et al. 2005). Ethylene is an effector of stomatal closure. However, its mechanism of action in stomatal closure is not as well known as ABA. Of the known ethylene receptors, only ethylene receptor 1 (ETR1), has been shown to be involved in ethylene-induced stomatal closure (Beguerisse-Diaz et al. 2012). However, it can be proposed that up-regulation of *ERF13* in response to elevated CO2 might be correlated with stomatal closure and therefore increased water use efficiency which was reported by Franzaring et al. 2011.

Brassinosteriod (BR) was another hormonal category which showed up-regulation in response to elevated CO_2. BRs are polyhydroxylated steroidal plant hormones that play pivotal role in the regulation of various plant growth and developmental processes (Fariduddin et al. 2014). BRs are required for normal growth and development including shoot and root growth (Nemhauser et al. 2004). BRs related genes showed up-regulation in response to elevated CO_2 under both N conditions regardless of leaf position. Genes involved in BRs synthesis and degradation including *CYP90A* and *SQE3* (*SQUALEN EPIOXIDASE 3*) showed up-regulation in all of the samples while *SQP2* was up-regulated only in leaf #4 under both N conditions. CYP90, a cytochrome P450, is involved in the biosynthesis of active brassinosteroid, which are essential for the regulation of cell elongation during plant development (Szekeres et al. 1996). Moreover,

two genes involved in BR signal transduction including a receptor kinase encoding gene (*BRL3*) and a transcription factor encoding gene, *BRASSINAZOLE RESISTANT 2* (*BZR2)* showed up-regulation in all of the samples under elevated CO_2. The effect of elevated CO_2 on plant biomass and metabolism in *Arabidopsis thaliana* in relation to the availability of gibberellins (GA) has been recently studied (Ribeiro et al. 2012). GA biosynthesis inhibitor paclobutrazol (PAC) inhibited the growth of plants under ambient CO_2 but not elevated CO_2. Moreover, under high CO_2, GA did not control the expression of the growth-related genes such as expansins (Goh et al. 2012) and xyloglucan endotransglucosylase/endohydrolases (XTHs) involved in cell wall loosening and rearrangement. Since, brassinosteroid (BR) are the main pathway to induce growth-related genes whereas GA quantitatively enhances BR potentiated growth (Bai et al. 2012). Authors suggested that the induction of growth related genes during elevated CO_2 might depend on BR and be uncoupled from GA. A couple of GA related genes showed down-regulation only in leaf #4 under low N condition. Considering low GA response and high up-regulation of some BR related genes under elevated CO_2 it is possible that BRs are involved in promoting the growth and higher biomass production of oilseed rape plants observed in the present study.

4.6 Transcription factor genes responding to senescence, N-deficiency and elevated CO_2

Transcription factors are key players that control the age-dependent expression of thousands of SAGs. Senescence associated transcription factors are also induced by various stress responses, indicating that senescence is an integrated response of plants to endogenous developmental signals and environmental cues (Woo et al. 2013). Various transcriptomic analyses have identified NAC, WRKY, AP2/EREBP, MYB, C2H2 zinc-finger, bZIP, and GRAS being the largest families of transcription factors that are up-regulated during leaf senescence (Breeze et al. 2011).

The response of senescence associated transcription factor genes in leaf #4 between 85 DAS (H3) and 92 DAS (H4) has been addressed and discussed in result section (3.3.3).Therefore, here the focus is more on the later time points, between 92 DAS (H4) and 99 DAS (H5), in leaf #4 and its comparison with leaf #8 transcription factor response, between 92 DAS (H4) and 106 DAS (H6). Since Different transcription factor families such as AP2/EREBP, bZIP, bHLH, C2H2, HB, MYB and WRKY showed gene expression changes in response to developmental

and N deficiency induced leaf senescence. Among them, AP2/EREBP and HB showed higher regulation changes in leaf #4 under low N while it was opposite in leaf #8. This indicates that the regulation changes of these two families are higher in samples with lower SPAD values (Figure 9 and 21). Interestingly, *RAP2.4* showed down-regulation under normal N while no expression changes observed under low N. RAP2.4 is a positive regulator of senescence and has been shown to promote developmental and dark induced leaf senescence in Arabidopsis (Xu H. et al. 2010). Moreover, a member of DREB subfamily, *DREB2B*, encoding dehydration responsive element binding protein 2B, which has shown up-regulation in response to other stresses such as dehydration and high salt stress in Arabidopsis (Nakashima et al. 2000) was up-regulated by N limitation in oilseed rape in the present study.

Three members of HB transcription factor family including *ML1*, *HB-7* (*HDG5*) and *BEL10* showed up-regulation in leaf #4 only under low N condition. ML1 is involved in epidermal cell differentiation (Mitsuda and Ohme-Takagi 2009) while HB7 has been shown to modulate abscisic acid signaling by regulating protein phosphatase 2C and abscisic acid receptor gene activities (Valdes et al. 2012). BEL10 function has not yet been discovered.

Taken together, more stress related transcription factors showed up-regulation in leaf #4 under low N condition. Comparison of transcription factor changes between leaf #8 belonging to normal and low N revealed that all of 7 senescence associated transcription factor families which were mentioned before, showed higher regulation changes under normal N. WRKY and HB families represented highest discrepancy for the number of regulated members between two N conditions in leaf #8. WRKY is a major family of plant transcription factors involved in the regulation of various processes, including pathogen defense, wound response, trichome development, and senescence (Turck et al. 2004). *WRKY6*, one of the senescence associated members of WRKY family, showed down-regulation only under low N condition in this study. One of the targets of WRKY6, *SIRK*, encodes a receptor-like protein kinase, which is strongly induced during leaf senescence and its expression is associated with *WRKY6*. *SIRK* transcript level is strongly reduced in senescing leaves of *wrky6* knockout mutants while it is elevated in green leaves of *WRKY6* overexpression lines (Robatzek and Somssich 2002). *WRKY6* has been reported to be associated with different nutrient deficiencies such as boron (B) and phosphate. It has been shown that *WRKY6* plays role in response to B deficiency in *Arabidopsis thaliana* and it is essential for normal root growth under low B conditions in this plant (Kasajima et al. 2010). Moreover, *WRKY6* plays an important role in modulation of plant responses to low Pi stress via regulation of *PHOSPHATE1* (*PHO1*) expression (Chen Y. F. et al. 2009). Other WRKY members such as *WRKY1*, *WRKY2* and *WRKY10* showed up-regulation only under normal N in leaf #8 in the current study. It has been reported that *WRKY1* is involved in drought and salt

stress tolerance in durum wheat (Mondini et al. 2012). Moreover, in tobacco plants, *WRKY1* is phosphorylated by the MAP kinase SIPK and is involved in HR-like cell death (Menke et al. 2005). *WRKY70* and *WRKY30* which has been shown to interact with each other and are negative regulators of leaf senescence showed down-regulation in leaf #4 under normal N condition. *WRKY70* and *WRKY30* interaction mediates the onset and progression of leaf senescence (Besseau et al. 2012). Down-regulation of senescence negative regulators is indeed consistent with the very low SPAD values of these leaves at latest time points in the current study (Figure 9). Moreover, *WRKY30* has been shown to be responsive to reactive oxygen species (ROS) such as hydrogen peroxide, a crucial player in leaf senescence (Besseau et al. 2012). *WRKY45* was another member of WRKY family which was repressed under normal N in leaf #4. WRKY45 is involved in Pi starvation response via direct up-regulation of *PHT1;1* which encodes an inorganic phosphate transporter (Wang H. et al. 2014). Direct correlation of *WRKY6* and *WRKY45*, which are involved in phosphate starvation response, with nitrogen deficiency, has not yet been shown but considering the cross talk between different nutrient and their homeostasis in the cell, it is not surprising that regulation changes of these two genes was observed.

Under elevated CO_2 different transcription factor families including AP2/EREBP, bHLHL, bZIP, C2H2, G2_like, MYB, MYB-related, WRKY, chromatin remodeling, trihelix and set-domain showed striking gene expression changes. Physiological analysis of oilseed rape plants revealed significantly higher water use efficiency in response to elevated CO_2 under both N conditions (Franzaring et al. 2011). The decreased stomata conductance observed in plants grown at elevated CO_2 can increase the water use efficiency (WUE) (Franzaring et al. 2011). *MYB61* was up-regulated in response to elevated CO_2 under low N condition in both leaves #4 and #8. MYB61 was the first transcription factor identified to be involved in stomata aperture (Liang et al. 2005). It is a member of R2R3MYB family, a subgroup of MYB transcription factors (Yanhui et al. 2006). *AtMYB61* is mainly expressed in guard cells in the darkness, when stomata are closed. Larger stomatal pores were observed in the *myb61* loss-of-function mutant, while the constitutive expression of *AtMYB61*, promoted stomata closure (Liang et al. 2005). *GTL1* is another transcription factor involved in water use efficiency belonging to trihelix family which showed down-regulation in both leaves #4 and #8 under low N condition and elevated CO_2. It has been shown that *GTL1* negatively regulates water use efficiency and drought tolerance by modulating stomata density via transcriptional repression of *SDD1* which the latter is a negative regulator of stomata development (Yoo et al. 2010). These data are consistent with the higher water use efficiency of oilseed rape samples used in this study (Franzaring et al. 2011). More detailed research on these genes will provide more insight about

drought tolerance in crops in response to climate changes (e.g. elevated CO_2) in the future and may have the potential to be considered by breeders for producing new lines having an improved drought tolerance.

SEUESS (*SEU*) gene, a member of C2H2 family, was down-regulated in all samples under elevated CO_2. It has been reported that SEU plays crucial role in the proper formation of ovules from the carpel margin meristem (CMM) in Arabidopsis. Ovule initiation is impaired in *seu* mutants (Wynn et al. 2011). This finding is in agreement with the seed harvest index and total seed number which was drastically lower under elevated CO_2 in our samples (Franzaring et al. 2011). Moreover, the number of failed pods (carrying no seed) was significantly higher in elevated CO_2 atmosphere (Franzaring et al. 2011), suggesting that the reduced *SEU* expression may, at least partially, be responsible for lower ovule formation.

4.7 N-limitation (leaf #4) and elevated CO_2 induces expression of protein degradation related genes

To be able to perform essential cellular functions and survive under N deficiency condition, plants degrade proteins in old and mature leaves and transport the resulting nitrogen to actively developing organs such as young leaves, flowers and seeds (Peng et al. 2007). Two main protein degradation pathways in plants are autophagy and ubiquitin-proteasome pathways (Smalle and Vierstra 2004). In ubiquitin-proteasome pathway, targeted proteins proceed with consecutive ubiquitin conjugation via ubiquitin activation enzymes, conjugases and ligases. Finally, the ubiquinated proteins end up in proteasomes for degradation (Peng et al. 2007). It was of notice that samples with lower chlorophyll content, leaf #4 under low N, showed extensive gene up-regultion related to protein degradation indicating higher nutrient remobilization in those samples. Most of protein degradation gene expression changes were observed in ubiquitin related pathway (Figure 16).

In the present study it was shown that elevated CO_2 also resulted in up-regulation of the genes involved in protein degradation especially via ubiquitin-proteasome pathway. Most of up-regulated genes belonged to zinc finger (C3H4-type RING finger) family and to a lesser extent to F-box proteins. Moreover, up-regulation of autophagy related genes, especially under low N and elevated CO_2 was observed, however it was not as remarkable as ubiquitin-proteasome pathway due to the lower number of regulated genes. Of notice was up-regulation of different proteasome subunits related genes which was not the case under ambient CO_2 level. It has been

reported that soybean plants (*Glycine max*) grown under elevated CO_2 showed repression of protein synthesis transcripts while transcripts levels for protein degradation (such as ubiquitin-proteasome pathway) was enhanced indicating a link between growth and protein turnover under this condition (Ainsworth et al. 2006). In the afore -mentioned study it was also observed that cell wall biosynthesis, lignin and fatty acid production was also enhanced indicating that elevated CO_2 promote a shift from N-rich proteins to biosynthetic products with higher ratios of C to N.

4.8 N-limitation results in up-regulation of flavonoid biosynthesis genes while elevated CO_2 enhances regulation of glucosinolates and phenylpropanoids genes

Flavonoid related genes showed more up-regulation in leaf #4 under N limitation condition. Flavonoids are a sub-group of phenylpropanoids and have been shown to accumulate in response to nutrient depletion. Though, the physiological function of this accumulation is unclear (Lillo et al. 2008), it has been shown that N limitation in Arabidopsis affects regulation of phenylpropanoid and flavonoid related genes. For instance, re-addition of nitrate to nitrate starved Arabidopsis seedlings results in repression of large number of genes and induction of fewer genes involved in phenylpropanoid and flavonoid metabolism within 30 min to 3 hours of nitrate treatment (Scheible et al. 2004). Moreover, in another study the secondary metabolite response to N starvation was different between roots and shoots so that flavonoid synthesis genes showed differential gene expression changes in shoot while phenylpropanoids were more differentially expressed in the roots. Authors concluded that this is in agreement with the main accumulation of anthocyanin in the shoots after long term N starvation (Krapp et al. 2011). In the current study, in case of leaf #4, more flavonoid related genes showed up-regulation under low N condition. This is correlated with the low level of chlorophyll content in these leaves which can probably lead to production of higher ROS species in the cells. It is possible that up-regulation of flavonoid synthesis genes can results in production of flavonoids scavenging the free radicals and protects the cells from oxidative damage. Interestingly, *PAL2* gene showed up-regulation in leaf #4 under N limitation. *PAL2* encodes phenylalanine ammonia-lyase 2 which catalyzes the first step in the phenylpropanoid pathway and is considered an important regulation point between primary and secondary metabolism. The PAL enzyme catalyses the conversion of phenylalanine to trans-cinnamate which becomes the main precursor for the biosynthesis of various phenylpropanoids required for plant growth, development and adaptation (Wong et al. 2012). It has been shown that PAL2 and PAL1 are involved in lignin

synthesis, and moreover, they play role in abiotic environmental-triggered flavonoid synthesis in *Arabidospsis* (Olsen et al. 2008). Besides the effects of different N treatments on secondary metabolites, elevated CO_2 also caused gene expression changes in several groups of secondary metabolites. For instance, elevated CO_2 resulted in up-regulation of sulfur-containing metabolites (glucosinolates) under both N conditions and in both leaves. However this up-regulation was more pronounced under normal N condition. Concomitantly, it has been shown that treatment of *Brassica oleracea* plants with elevated CO_2 significantly increased the leaf glucosinolate content (Klaiber et al. 2013).

Stronger up-regulation of phenylpropanoids (lignin in cell wall) in leaf #8 was observed under both N conditions in this study which is in accordance with the up-regulation of cell wall related genes in these leaves. Moreover, transcriptome response study of soybeans (*Glycine max*) plants grown under elevated CO_2 revealed an increase in cell wall and lignin biosynthesis in the leaves (Ainsworth et al. 2006). Carbon rich secondary metabolites such as terpens showed more up-regulation at elevated CO_2 compared to ambient CO_2. This is in agreement with soybeans study which is attributed to the higher C: N ratio under this condition which results in a shift from N containing secondary metabolites towards those which contains higher C in their structure.

4.9 Opposite transcriptome response of old leaf (#4) and young leaf (#8) to different N treatments

Leaf SPAD value measurements of oilseed rape plants used in the present study revealed that in general plants grown under normal N exhibited higher SPAD values compared to low N (Figure 9). However, interestingly, at the latest harvest, 106 DAS (H6), which leaf #4 has already been fallen down, leaf #8 under normal N showed lower values compared to low N condition (Figure 21) (Franzaring et al. 2011). This is consistent with gene expression changes observed in this leaf. More senescence associated gene expression changes in leaf #8 under normal N especially belonging to pathways such as protein modification and degradation, secondary metabolites and stronger down-regulation of photosynthesis genes. It has been shown that in young leaves of *B. rapa*, one of *B. napus* ancestors, N starvation is accompanied with delayed senescence and induction of a dual function protein, BnD22, a water-soluble chlorophyll-binding protein that acts against oxidative alterations of chlorophylls and exhibits a protease inhibitor activity. Through its dual function, BnD22 may be involved in sustaining sink growth of stressed plants and contribute to a better utilization of N recycled from senescent leaves, a physiological trait that could improve NUE (Avice and Etienne 2014). In the present study, *BnD22* gene was

significantly up-regulated in leaf #8 compared to leaf #4 under both N conditions and in accordance to the previous report in *B. rapa*, this up-regulation was stronger under low N condition in *B. napus* (Figure S4).

It can be concluded that younger leaves of the plants with N deficiency tend to keep their resources for a longer time compared to well fertilized plants which is correlated with a slower senescence progression in this leaves (Franzaring et al. 2011). Concomitantly, it has been reported that when plants grow under constant low N supply they will produce more compact leaves and strong structure tissues while leaves which are treated with a sudden N limitation after treating with adequate N supply show a rapid degradation of chlorophyll and resource remobilization in order to support other parts of the plant (Desclos et al. 2009, Franzaring et al. 2011). N labeling studies of several Arabidopsis accessions grown under low N exhibited enhanced nitrogen use efficiency (NUE) as well as nitrogen remobilization efficiency (NRE) and ^{15}N partitioning in seeds (^{15}NHI). Under N limitation condition, $^{14}NO_3$ which was absorbed after labeling was mainly allocated to the seeds but to the dry remains at high N. As a consequence, N concentration (N%) in the dry remaining was 4-fold higher under high N compared to N limitation while N% in seeds was poorly affected and showed less differences between low and high N. This new insights are helpful for further understanding of NUE concept in Arabidopsis as well as in crops especially oilseed rape (Masclaux-Daubresse and Chardon 2011). This is consistent with oilseed rape which showed low differences in the amount of nutrients in the seeds between low and normal N conditions despite the difference in dry mass which was significantly higher under normal N (Franzaring et al. 2011).

4.10 Similarities and differences between developmental and N-deficiency induced leaf senescence at the transcriptome level

Leaf Senescence can be controlled by both developmental and various environmental conditions. N deficiency is one of the major causes of premature senescence in different plant species including crops with negative effects on biomass and seed yield (Balazadeh et al. 2014). The overlaps and discrepancies between developmental and N deficiency induced leaf senescence in oilseed rape, as a well-known crop for low NUE, has not yet been addressed. Such knowledge can give insights in to the different machinery involved in regulation of senescence associated processes affecting nutrient remobilization and hence improve our understanding towards managing NUE. Therefore, to have an overview about the senescence up-regulated genes (SAGs) and senescence down-regulated genes (SDGs) under normal (NN) and low (LN) N supply, all the regulated genes in response to different N treatments, regardless

of time of the regulation, were collected and divided in to four different categories based on their regulation direction and N treatment including NN_SAGs, NN_SDGs, LN_SAGs and LN_SDGs.

It is noteworthy to mention that here SAGs and SDGs are used as general terms describing respectively up- and down-regulated genes during senescence in this study and not necessarily the known senescence related genes. From the already known SAGs, *SAG14,* which is involved in aluminium induced oxidative stress (Richards et al. 1998) was up-regulated under NN while this was the case for *SAG12* and *SAG2* under N limitation. *SAG29/SWEET15* was down-regulated under low N and *SAG101* showed repression under both N conditions. *SAG29/SWEET15* has been shown to be one of the direct targets of *ORE1* in *invivo* condition, which the latter is a positive key player of the senescence regulation (Matallana-Ramirez et al. 2013). Moreover, *SAG29/SWEET15* mainly expressed in senescing plant tissues and is involved in an ABA dependent high salinity stress response in the roots of Arabidopsis which propose its role as a molecular linker between environmental stresses with senescence process (Seo et al. 2011).

The number and function of unique and shared genes between these different classes are shown in Figure S5. The Biological pathways related to the unique and common SAGs and SDGs between NN and LN were further identified using SuperViewer tool. In the next step, the unique and common pathways between NN and LN were identified. Of notice were pathways related to energy production including TCA cycle and mitochondrial electron transport which showed up-regulation only under LN. This can be related to the higher metabolic activities for nutrient remobilization under low N condition. Moreover, secondary metabolism and stress pathways also showed unique up-regulation under LN. An important gene involved in secondary metabolism pathway, was *PHENYLALANINE AMMONIA-LYASE 2 (PAL2).* Phenylalanine ammonia-lyase (PAL) catalyzes the first step of the phenylpropanoid pathway, which produces precursors to a variety of important secondary metabolites such as anthocyanin (Huang et al. 2010). It has been shown that nitrogen depletion in Arabidopsis results in up-regulation of *PAL1* and *PAL2* (Olsen et al. 2008). Several pathways were represented as both SAGs and SDGs related under NN or LN. For instance photosynthesis and transport were in the list of both up- and down-regulated pathways under NN while it was the case for pathways such as mitochondrial electron transport/ATP synthesis and stress under LN. This can imply that distinct set of the genes belonging to the same biological functions are playing role during senescence under both N conditions but some are up-regulated while others are repressed.

10 genes were common between NN_SAGs and NN_SDGs and 28 genes were shared between LN_SAGs and LN_SDGs. This is due to their reverse expression regulation at different time points indicating their control by both N and time. One gene might be up-regulated at one time point while shows repression at the other time point. The genes which showed both up- and down-regulation under NN were significantly ($P<0.05$) related to TCA/organic acid transformation while up- and down-regulated genes under low N were significantly ($P<0.05$) belonging to hormone metabolism (ethylene and ABA) (Figure S5).

It was of interest to unravel those genes and their biological functions which show an opposite regulation direction in response to NN and LN. 14 genes related to TCA/org transformation and major CHO metabolism showed up-regulation under NN (NN_SAGs) and down-regulation under LN (LN_SDGs). Both of these pathways are correlated to carbon (C) metabolism indicating the importance of C/N balance in the regulation of senescence in oilseed rape. It has been shown that there is a tight link between N and C metabolism and the C/N ratio is a key signal involved in plant growth and development (McAllister et al. 2012) genes were shown to be down-regulated under NN (NN_SDGs) and up-regulated under LN (LN_SAGs) in the current study. These genes were involved in polyamine metabolism, amino acid metabolism, signaling, RNA and some were not assigned. These observations implies that 1) there is higher number of genes up-regulated under LN while repressed under NN and 2) these genes have N associated functions or are involved in signaling and transcription regulation. The second group is particularly interesting because they provide information about N-deficiency specific signaling and transcription regulation in oilseed rape. The list of signaling related genes contained one leucine rich repeat VIII receptor kinase (At1g29720), four genes involved in calcium signaling (At3g59440, At1g53210, At2g26180, At3g13460) and two map kinases including MAPK17 (At2g01450) and MAPK6 (At2g43790). The genes associated with RNA were mainly transcription factors belonging to different families such as bZIP (At2g21230 and At3g14880), C2H2 zinc finger (At5g22480 and At1g72050) and two unclassified transcription factors (At5g19350 and At5g10770).

4.11 Comparison of oilseed rape transcriptome response to N limitation with other plant species

Several studies of the Arabidopsis transcriptome response to nitrate application or starvation has been reported (Balazadeh et al. 2014, Scheible et al. 2004, Wang M. et al. 2002, Wang R. et al. 2003, Wang Y. H. et al. 2001). From the aforementioned studies, the report from Peng et al.,

2007 was more comparable with the current study with respect to growth condition and N deficiency treatment which focuses on the long term N starvation for plants grown in the soil while in most of the other researches plants were grown hydroponically under low N condition and transcriptome profiling is performed after nitrate re-addition. This is rather immediate plant response to nutrient shock and may not completely reflect the plant adaptive responses to constant low N condition. In addition, immediate gene expression responses are more temporal and they might disappear after nitrate resupply. Several genes including nitrate reductase (NR), nitrite reductase (NiR) and two nitrate transporters which were markedly up-regulated in nitrate starved tomato plants were all disappeared after 48 h of nitrate treatment (Peng et al. 2007, Wang Y. H. et al. 2001). These results propose that adaptive response to low N condition differ from short time N starvation and instant gene responses to nitrate re-addition. In a study conducted on the maize source leaf it was revealed that N deficiency not only affects nitrogen and carbon metabolism but also phosphate homeostasis. Maize N starvation resulted in the accumulation of phosphate and concomitant down-regulation of phosphate starvation response genes, underlining that phosphate homeostasis control is important under stress conditions (Schluter et al. 2012). However, oilseed rape data comparison (this study) with Maize data from aforementioned study revealed that this is not the case in oilseed rape plants in the current study. To have an overall view about the overlaps and discrepancies between Arabidopsis and oilseed rape, the transcriptome data from the study conducted by Peng et al., 2007 was compared with the data from this study. For a more compatible comparison with the other two studies, the data from direct comparison between normal N (NN) and low N (LN) at different time points were combined. Comparison of the genes revealed that there are no overlaps between genes identified in these two studies. Analysis of biological pathways (SuperViewer software) revealed a remarkable similarity between Arabidopsis and oilseed rape. For example pathways such as secondary metabolism, amino acid metabolism and transport were up-regulated while tetrapyrrole synthesis, cell wall and co-factor and vitamin metabolism were down-regulated in both plant species. Fewer pathways showed unique up- or down-regulation in oilseed rape or Arabidopsis. For instance, fermentation and N-metabolism were up-regulated only in Arabidopsis while polyamine metabolism was up-regulated only in oilseed rape. N-metabolism, TCA/org transformation, nucleotide metabolism and glycolysis showed down-regulation only in Arabidopsis while gluconeogenesis/glyoxylate cycle was uniquely down-regulated in oilseed rape. Taken together, in case of biological pathways responsive to N deficiency, this study is in general agreement with Arabidopsis response to N deficiency (Peng et al. 2007). The same biological pathways were responsive to N deficiency in both plant species with minor differences mainly related to N and C associated pathways. This is likely due to the differences

in the seed oil content. In oilseed rape the 42% of the seed content is oil (Franzaring et al. 2011) which is a C rich compound while this is not the case in Arabidopsis with ca. 36% of seed oil content (Li Y. et al. 2006b). It is noteworthy to mention that different statistical approaches were applied in these two experiments which may have impact on some of the identified genes. Peng et al. (2007) performed one-way ANOVA (Welch t-test) while in the current study LIMMA method (moderated t-test) was performed for statistical analysis.

4.12 Comparison of oilseed rape transcriptome response to elevated CO_2 with *Arabidopsis thaliana*

It was of interest to identify similarities and discrepancies in the response to elevated CO_2 between oilseed rape and other species such as Arabidopsis. Such comparison will increase our knowledge about genes and pathways responsive to elevated level of CO_2 and on the other hand will unravel the different molecular mechanisms under this condition in crop plants which can be considered for breeders in order to introduce lines with better adaptability to climate changes correlated with higher level of CO_2. For this purpose, the data from a study conducted on three different *A. thaliana* ecotypes including Col-0, Cvi-0, and WS (Li P. et al. 2006a) was used. It must be noticed that in the aforementioned study there was a great difference between cultivars in response to elevated CO_2, indicating a cultivar specific response. All the genes which were significantly up- or down-regulated at least in one of the cultivars were compared with regulated genes in oilseed rape under elevated CO_2 in this study. Since *A. thaliana* plants were grown under normal N, those oilseed rape genes which were responsive to elevated CO_2 under normal N condition were also considered. Moreover, rosette leaves of Arabidopsis plants around bolting time were analyzed which were more comparable with leaf #4 in this study and therefore it was chosen for this comparison. Data analysis revealed that 49 genes showed up-regulation in both plant species which were related to N and C associated pathways such as polyamine metabolism, N-metabolism, OPP, cell wall, transport and DNA. This is in agreement with the fact that N and C metabolism have a very close connection and changes in one of these nutrients can result in metabolism changes of the other one. 18 genes showed down-regulation in both plant species which pathway analysis revealed tricarboxylic acid (TCA) cycle as being overrepresented among them. Some of the genes showed opposite regulation changes between oilseed rape and *A. thaliana* in response to elevated CO_2. 42 genes showed up-regulation in oilseed rape and down-regulation in *A. thaliana* which were related to photosynthesis, amino acid metabolism, cell wall and signaling. On the other hand, 52 genes belonging to fermentation

and secondary metabolism showed down-regulation in oilseed rape and up-regulation in *A. thaliana* under elevated CO_2. A more detailed comparison revealed that among different *A. thaliana* cultivars, WS showed a rather distinct response compared to the other cultivars which interestingly its response was more similar to oilseed rape in this study. For instance similar to oilseed rape, it showed an overall higher up-regulation in genes belonging to photosynthesis, TCA cycle and mitochondrial electron transport, cell wall, lipid metabolism and secondary metabolism while these pathways showed an overall down-regulation in the other cultivars.

Beside shared genes, high number of uniquely regulated genes in each species was observed which can be attributed to the different adaptability response of species and also different growth conditions. Arabidopsis plants were grown in a system of open-air rings within which CO_2 concentration was elevated to approximately 550 ppm, while our samples were grown in growth chamber, though with the same CO_2 concentration. It is probable that Arabidopsis plants which were grown in the field were affected by other environmental factors while oilseed rape plants were in a more controlled condition which restricted the effect of other factors which may affect the expression of the genes in combination with elevated CO_2. Figure 47 represents a schematic model of molecular players responsive to elevated CO_2 in oilseed rape in the current study and their possible correlation (shown with the same colored circles) with the phenotypic observation reported by Franzaring et al. 2011. The genes which are shown in this model were responsive to elevated CO_2 in the current study in all samples, regardless of N treatment and leaf position. Therefore they might play important roles under this condition.

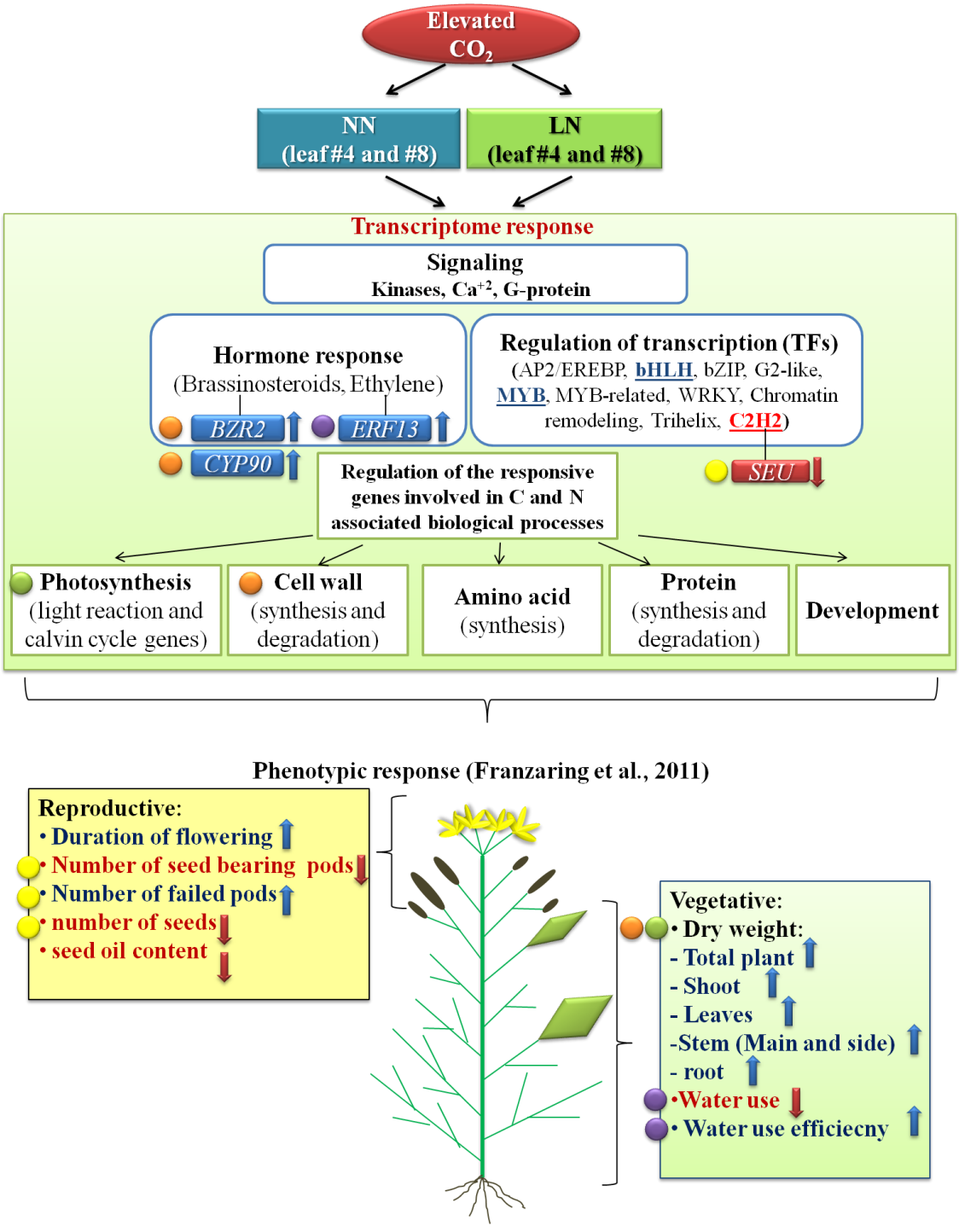


Figure 47. A schematic model of the molecular players responsive to elevated CO_2 in oilseed rape in the current study and their possible correlation (shown with the same colored circles) with the phenotypic observation reported by Franzaring et al. 2011. The details of the represented genes are provided in the text.

4.13 Oilseed rape N-status marker genes show distinct expression levels in response to different N treatments

Oilseed rape is an important crop plant with edible and non-edible uses (Avice and Etienne 2014). Considering its low NUE which results in high demand of N fertilizer application in order to increase seed yield, identification of marker genes sensitive to plant N status and independent of genotype ad growth condition, can improve fertilizer regimes and decrease the economic and environmental consequences of high N fertilizer application. Leaf chlorophyll concentration (SPAD) and photosynthesis rate are suitable markers of leaf senescence but only at rather advanced stages. Moreover, these markers are not N starvation-specific and failed unraveling cultivar differences in stay-green characteristics at early senescence stages. *SAG12-1* is well known molecular marker of leaf senescence progression. However, Koeslin-findeklee et al. 2015 (in press), showed that *SAG12-1* expression is not N starvation-specific. This might be due to the fact that *SAG12-1* encodes a cysteine protease which is involved in degradation of the photosynthetic apparatus which, however, takes place only at advanced stages of the senescence process. In the above-mentioned study, it was observed that four days of senescence inducing treatments (leaf shading, leaf detachment and N-starvation) led to the onset of leaf senescence (indicated by a decrease in SPAD values, photosynthesis rate and an increase in *SAG12-1* expression) in shaded and detached but not in N-starved leaves. Therefore, it is necessary to identify N starvation-specific marker genes which are responsive at earlier stages of N-deficiency, before visible symptoms of leaf senescence (such as leaf yellowing) can be observed.

Using parallel microarray studies on three oilseed rape cultivars including spring (Mozart, in growth chamber) and winter (Apex and Capitol, in hydroponic) grown under sufficient and limited N supply, a set of 46 potential markers with distinct response to different N concentrations was selected. 35 markers were identified in Mozart based on their high expression fold change between different N conditions and 12 markers were previously identified in Apex and Capitol cultivars as being N sensitive and cultivar specific (Koeslin-Findeklee et al. 2015) (in press). One gene (*AMT1.4*) was shared between spring and winter studies. 20 out of 45 marker genes showed distinct expression changes (qPCR assay) dependent on N treatment and not cultivar. Combination of different cultivars provides the opportunity to examine the effect of cultivar on marker gene expression in response to different N status. Therefore, the reliability and robustness of these 20 potential markers were further tested in five different winter cultivars grown in greenhouse including NPZ2, NPZ5, NPZ1005 and MSL101B and a field grown cultivar, Major. Eventually, 13 markers were identified as being

sensitive to plant N status, independent of genotype and growth condition (unconditional markers). 8 out of 13 these markers were able not only to distinguish normal and low N concentration but also showed distinct expression changes between mild and severe N deficiency which indicates their sensitivity to different N levels. Out of 20 marker genes which were identified under controlled condition (growth chamber and greenhouse), five genes showed an opposite regulation in the field grown plants. This is perhaps due to the other environmental factors (such as drought, humidity, temperature, soil pH and etc.) which plants encounter when growing in the field condition. Moreover, in the current study the differences between normal and low N in the field condition was much higher (120 vs. 20 kg N/ ha) compared to the growth chamber (150 vs. 75 kg N/ ha) and greenhouse (1000 vs. 500 or 250 mg N) grown plants. Therefore, lower number of marker genes in the field grown plants can, at least in part, be related to the differences in the N supply. Future studies with more field grown cultivars can provide more information regarding the robustness of identified marker genes in the current study.

3 out of 13 final markers (23%) were belonging to different transcription factor families including NAC (NAC29), ERF/AP2 (ERF72), MYB-like (MYBL2). All of them demonstrated up-regulation under N deficiency except than MYBL2 which was repressed under low N (Figure 27). Different NAC family members are involved in development, auxin signaling, defense, abiotic stress response and leaf senescence (Hickman et al. 2013, Uauy et al. 2006). It has been shown that the expression of NAC029 is highly associated with senescence progression in *Arabidopsis thaliana* rosette leaves (Buchanan-Wollaston V. et al. 2005, Guo et al. 2004). Ethylene-responsive factors (ERFs) exist in the plant kingdom and belong to the AP2/EREBP-type transcription factors with their function as *trans*-acting factors at the last step of transduction (Liu et al. 2011, Ohmetakagi and Shinshi 1995). However, the connection between *ERF72* which is up-regulated under N deficiency condition in this study and senescence or N recycling still needs further studies. MYBL2 has been shown to be involved in regulation of gene expression in anthocyanin biosynthesis pathway (Dubos et al. 2008, Matsui et al. 2008, Ye et al. 2012). Moreover, MYBL2 is a substrate of GSK3-like kinase BIN2 and represses the BR-repressed genes via an interaction with BES1 (Ye et al. 2012).

Out of 34 potential markers which were originally identified via microarray in Mozart plants, 7 (>20%) were responsive in all tested cultivars and conditions while this was the case for 6 (50%) out of 12 N-responsive and cultivar specific potential markers identified in Apex and Capitol. Lower number of validated Mozart markers is perhaps due to the fact that all of tested cultivars were winter cultivars while Mozart is a spring cultivar.

Beside 13 validate markers mentioned before, five markers showed a distinct response to different N conditions under both greenhouse and field study but with an opposite regulation direction indicating that environment can change their regulation direction and therefore were omitted from the list of final markers and categorized as conditional markers. It was of notice that 3 out of 5 conditional markers were TFs including NAC102, TOC1 and MYB77.

The role of NAC102 during senescence has not yet been shown. However, Köslin-Findeklee1 et al., (2014) showed up-regulation of this gene in two early senescing oilseed rape cultivars under N deficiency condition indicating it may has a role during N-deficiency induced leaf senescence. TOC1 is an Arabidopsis circadian clock protein belonging to pseudo ARRB family. It has dual function in controlling circadian and photomorphogenic responses (Mas et al. 2003) and is negatively regulated by two MYB transcription factors, CCA1 and LHY (Gendron et al. 2012) which act as an essential feedback mechanism for clock function (Alabadi et al. 2001). This protein is a linker between circadian clock and plant drought stress response via ABA modulation in collaboration with ABA-related gene, ABAR (Legnaioli et al. 2009). More studies are required to determine if TOC1 play a role not only in drought stress but also in nutrient stress condition such as N limitation.

MYB77 (*MYBR2*) has been shown to be involved in auxin signal transduction (Shin et al. 2007). Moreover, it has recently been reported to have a role in ABA responses and stress signaling in cooperation with *MYBR1* (Jaradat et al. 2013). The direct response of MYB77 to N deficiency is left to be elucidated.

Taken together, this study introduces the first example of oilseed rape N status marker genes which has been validated under controlled and uncontrolled conditions and different cultivars. Regarding the large variation existed in agricultural growth conditions and cultivars; this set of marker genes can be further used by relevant community in order to examine their validity across a broader range of conditions and draw a more global conclusion of their potential values in the agriculture and minimizing the cost and the fertilization input which can, in turn, lead to a more nature friendly oilseed rape production..

4.14 Comparison of N-sensitive expression biomarkers in oilseed rape and Maize

Recently, Yang et al., 2011, performed a study in maize via multiple whole-genome microarray analysis which identified gene expression biomarkers with potential to correctly assess plant

response to different N situations including normal and N deficient growth conditions. It was of interest to check whether there is any overlap between N-responsive expression biomarkers in Maize and oilseed rape. There was no overlap between marker genes identified in oilseed rape and those from maize. This discrepancy can be attributed to the different approaches which were followed in these studies. Considering high number of analyzed arrays, Yang et al., 2011, applied composite gene expression scoring system to identify N responsive markers in maize. However, in the current study alternative approaches were followed based on high expression fold changes between two N treatment in Mozart cultivar and N-responsive and cultivar specific markers in Apex and Capitol. The differences between applied strategies for marker identification can partially explain the lack of overlaps between final set of markers between two species. Moreover, species specific differences must be taken into account. Maize is a monocot with C4 photosynthesis whose response to N deficiency may differ to some extent from oilseed rape as a C3 dicot. Furthermore, to unravel the similarities and discrepancies of the maize and oilseed rape N- responsive markers in the biological context, the biological functions of the identified markers from these two species were compared. Maize marker genes belonged to functional categories including cell wall metabolism, lipid metabolism, electron transport, stress responses, transcription regulation and genes with unknown functions. Oilseed rape markers were correlated with pathways such as tetrapyrrole synthesis, co-factor and vitamin metabolism, secondary metabolism, nucleotide metabolism, transport, stress and signaling. Comparison of biological pathways between maize and oilseed rape showed that cell wall, stress and transcription regulation were shared between both species, indicating that the genes belonging to the certain pathways have a crucial role in response to limited N condition.

A future challenge will be studies with focus on identification of universal N-responsive marker genes which are not only independent of genotypes but also independent of species. This is crucial for both monocot and dicot crop species which are grown worldwide, especially concerning population expanding which leads to the more intense agriculture and fertilizer input. Using similar criterion and statistical approaches for expression marker selection between different species may increase the possibility of finding shared marker genes. However, the effect of the environment in the field and also the species specific differences in response to low N conditions seems to be the main challenges for such studies.

4.15 From the lab to the field: towards a more economic and nature friendly oilseed rape agriculture

Oilseed rape has become the oil crop with the worldwide fastest growing production rate. From 2003 to 2013 the production increased by 97%, with a 12% increase from 2012 to 2013. With a harvest of 72.5 million tons, equivalent to 7.3% of the world oil crop production, it is now the fourth biggest oil crop behind soybean, palm fruit oil and seed cotton (source: http://faostat.fao.org/). Besides its traditional use as source for edible oil (since the breeding of canola varieties in the 1970s) and rapeseed meal as animal feed in recent years it has become the major biofuel crop. In Europe about 80% of the rapeseed oil is refined to biodiesel. Population growth will need that crop yields increase in parallel which in turn leads to higher nutrient input and environmental consequences. This is more critical in oilseed rape which has a low NUE and therefore higher loss of nutrient to the soil which can be transported to the ground water. N fertilizers have been reported to result in overstimulation of algal growth and producing "algal bloom" which harms aquatic ecosystems and it's organisms by preventing oxygen and light penetration through the water. Another disadvantage of excessive fertilizer usage is the cost and economical aspect which can influence producers and consumers. Therefore, a more balanced fertilizer application in the right time and in the right amount can improve both environmental and economical consequences of fertilizer application.
The aim is to export and apply our knowledge from the lab to the real world in the field. One approach will be application of identified N sensitive marker genes in this study which has the potential to distinct between different N levels and therefore can be used as a tool for a more intelligent fertilizer input strategies. This finding supports that using the internal quantification system in plant "plant IQ" for environmental changes which is reflected in gene expression alterations, we will be able to better follow the need of the plant, in this specific case for N, in the more accurate time and amount. Transferring the knowledge from the lab to the filed can be challenging regarding the techniques which need to be applied in the field. In the current study to be able to quantify the gene expression in response to N supply, one needs to perform PCR amplification. This requires a thermocycler machine which is rather expensive and may not be appropriate for field application.

However, there are alternative approaches to overcome this issue. Non-PCR based methods such as isothermal amplification which has been developed in last two decades provide an alternative method for field research. Using isothermal technology for RNA amplification has the advantage of RNA amplification without thermocycler machine. Several isothermal based

techniques for RNA amplification such as Transcription Mediated Amplification (TMA) /Nucleic Acid Sequence-Based Amplification (NASBA), Signal Mediated Amplification of RNA Technology (SMART), Reverse Transcriptase-Strand Displacement Amplification (RT-SDA), Single Primer Isothermal Amplification (Ribo-SPIA) has been reviewed (Gill and Ghaemi 2008). An updated review on isothermal amplification with the latest devices has been addressed recently (Craw and Balachandran 2012). Even though most of these devices are designed for clinical purposes it does not mean they cannot be used in the field. Developing new technologies for molecular applications in the field will help for a better cooperation between lab, where the knowledge is produced, and the field, where it is going to be applied.

Future atmospheric CO_2 enrichment is inevitable and the current increase in CO_2 emission is already above the worst scenarios established by the IPCC (Peters et al. 2012). Atmospheric CO_2 enrichment may affect both developmentally regulated and N deficiency-induced senescence and interact with N supply. Unraveling biological pathways with response to different N treatment during senescence and/or elevated CO_2 and their relevant genes can shed more light on the molecular mechanisms involved in oilseed rape response to environmental stresses. More studies including knock out and overexpressing lines are needed to clarify the function and impact of the newly identified genes in this study in response to N deficiency or elevated CO_2. Oilseed rape transformation is still technically more difficult than Arabidopsis transformation which may hinder the progression of transformation studies directly in oilseed rape. However, the winter oilseed rape (*Brassica napus* var Cabriolet) has been mutagenised using EMS (ethylmethanesulfonate) to generate the population JBnCAB_E (The JIC Consortium *Brassica napus* CABRIOLET EMS) (https://www.jic.ac.uk/staff/ian-bancroft/population.html). This provides an opportunity to study the function of certain genes in oilseed rape which is more reliable, informative and straightforward than transferring the knowledge from model plants such as Arabidopsis to oilseed rape. Oilseed rape is a polyploidy plant with multiple copies of the genes it must be noted that traditional forward genetic screens has limitations for homologous genes with overlapping functions (Hauser et al. 2013). Small RNAs can reduce gene function post-transcriptionally. In this context, one approach is using artificial microRNAs (amiRNAs) for simultaneous targeting of several sequence-related genes (for example, homologue genes in oilseed rape) (Ossowski et al. 2008). Moreover, recent progression in genome editing techniques such as clustered regularly interspersed short palindromic repeats (CRISPR)/Cas system provides new possibilities for understanding gene function and for developing valuable new traits in plants (Shan et al. 2014). This system has been successfully used for mutagenesis and gene targeting in crop plants such as rice and wheat (Shan et al. 2014). CRISPR/Cas system enable precise genome engineering by introducing

DNA double-strand breaks that subsequently trigger DNA repair pathways by either non-homologous end joining or homologous recombination which can generate gene knockouts, replacements, insertions and chromosome rearrangements (Chen K. L. and Gao 2014).

Furthermore, it is important to study the proteome and metabolome changes beside the transcriptome response and estimate their correlations. Combination of different "omics" studies will provide a more realistic image of the plant response to environmental changes.

Breeding strategies are required to consider different aspects of climate changes in to account, including elevated CO_2 and its potential negative impact on the quantity and quality of oilseed rape grain yield (Franzaring et al. 2011). Moreover, a high nitrogen index (HI) can be achieved by better nutrient remobilization from source to the sink and eventually to the seeds which requires understanding the molecular network underlying this process. Combination of knowledge about plant response to both N deficiency and its impact on precocious senescence in parallel with understanding the molecular players in response to elevated CO_2 will provide a broader spectrum for breeders to produce cultivars which have higher NUE and therefore higher HI and at the same time, it can minimize the possible negative effects of future level of CO_2 on the yield. Figure 48 summarizes the possible contribution of the results from the current study in improving oilseed rape agriculture via three main strategy: 1) identification of the genes involved in the initiation of N-deficiency induced leaf senescence and therefore N remobilization from old source leaves to sink organs including young leaves and seeds. 2) Improving fertilizer management by identification of N-statues marker genes for early diagnose of plant N deficiency before visible symptoms of induced senescence (such as leaf yellowing) can be observed. 3) Unraveling the molecular players involved in the response of oilseed rape plants to the higher level of CO_2 in the future. Different functional categories including three main master regulatory networks (signaling components, hormone metabolism and transcription factors) and the down-stream pathways involved in initiation of N-deficiency induced leaf senescence or response to elevated CO_2 are shown. Examples of the related genes with highest regulation changes (up-regulation) or important known functions (*EFB1* involved in protein degradation and *SEUESS* involved in ovule initiation which can be a candidate gene to be studied in reproductive organs) are shown in red. Considering lower yield in oilseed rape under elevated CO_2 (Franzaring et al. 2011). One important aspect which is still remaining to be elucidated is the transcriptome response of reproductive organs (flowers and pods) of oilseed rape to elevated CO_2. This will broaden our knowledge of the molecular mechanisms in both vegetative (leaves in this study) and reproductive parts of this important crop for adaptation to the future climate change.

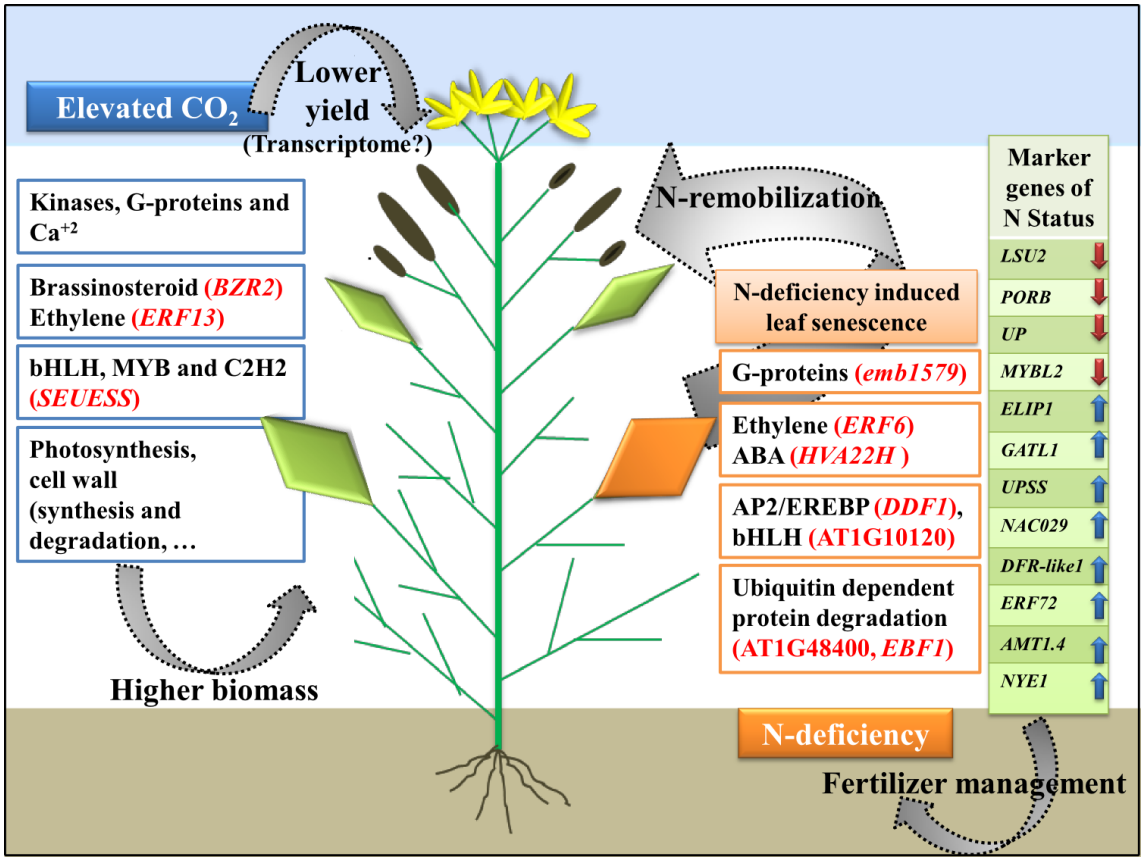


Figure 48. A schematic model summarizing the main possible contributions of the current study in improving oilseed rape agriculture. Examples of the related genes with highest regulation changes (up-regulation) or important known functions (e.g. EFB1: protein degradation and SEUESS: ovule initiation, a candidate gene to be studied in reproductive organs) are shown in red. The dotted arrows indicate the potential influence of each part on oilseed rape agriculture. Genes associated with N-deficiency induced leaf senescence (up-regulated between 85 and 92 DAS, orange boxes) can be considered as potentially important molecular players underlying N-remobilization and consequently NUE in oilseed rape. N-statues marker genes (shown in green boxes) can improve fertilizer managements and therefore minimize the negative environmental and financial consequences of excess fertilizer application. Understanding the molecular players in response to elevated CO_2 will be beneficial for considerations of the effects of climate change on the oilseed rape agriculture in the future (higher biomass and lower yield). The order of orange and blue boxes from top to bottom are signaling genes, hormone metabolism, transcription factor encoding genes and the genes involved in different down-stream physiological pathways.

5 Summary

Oilseed rape (*Brassica napus* L.) is an important crop plant primarily grown for oil production. Despite its high nitrogen (N) uptake, the overall nitrogen use efficiency (NUE) of oilseed rape is rather low. To achieve optimal oilseed yields, high amounts of N fertilizer must be applied to the field which is economically adverse and has negative environmental effects. The major fraction of N that the plant requires for pod development derives from remobilization of N which has accumulated during the vegetative phase in the stem and in leaves. One critical factor determining the NUE is the timing of leaf senescence initiation and the efficiency of N remobilization to the sink organs. At the time of abscission, the lower oilseed rape leaves still have a high N content, resulting in a return to the soil during winter of up to 100 kg N ha^{-1} of the approximately 200 kg fertilizer N ha^{-1} required for optimal seed yield. Nitrogen remobilization is significantly more efficient in younger leaves in the upper canopy that senesce during pod development. At the time of abscission their nitrogen content is only 1% of dry weight. The aims of this work were (1) to advance the understanding of N-remobilization and -allocation under normal and limiting N supply, (2) to identify sensitive early expression markers for monitoring the N status of oilseed rape plants, and (3) to investigate the consequences of future atmospheric conditions with elevated CO_2 concentrations on oilseed rape gene expression at varying N supply.

1) Leaf transcriptome analyses under adequate and limited N supply during development. Old source leaves (leaf #4) and young sink leaves (leaf #8) of oilseed rape spring variety 'Mozart' plants grown under normal (150 kg N ha^{-1}) and limiting N conditions (75 kg N ha^{-1}) were harvested at 4 different developmental stages (78, 85, 92 and 99 days after sowing, DAS). Labeled cRNA of the samples was hybridized to a newly developed custom *B. napus* microarray carrying 59,577 oligonucleotide probes from *B. napus* EST contigs. To annotate them, a BLAST search against the *Arabidopsis thaliana* genome sequence (TAIR version 10) was performed and the (putative) gene function of the most closely related Arabidopsis ortholog was assigned to the *B. napus* EST contigs. Microarray data revealed that under low N condition most of gene expression changes occur at earlier time points (between 85 and 92 DAS) when compared with normal N supply. Most of the regulated genes were senescence-associated transcription factors or related to senescence-associated activities, such as up-regulation of protein degradation. This indicates that low N promotes earlier senescence initiation in old oilseed rape leaves. Furthermore, the transcriptome response of young leaves to different N

conditions was opposite to that of old leaves. In young leaves (#8) under normal N more senescence associated transcriptome alterations were observed, indicating a delayed senescence in sink leaves under N limitation which can be correlated with a more economic use of available resources under N limitation.

2) Identification of expression markers for the plant N status.

Using two independent microarray studies on oilseed rape spring variety Mozart (growth chamber condition) and winter varieties Apex and Capitol (hydroponic condition) grown under sufficient and limited N supply, 46 potential markers were selected. 35 of these potential markers were identified in Mozart based on their high expression changes (>10 fold) between different N conditions. In addition 12 potential markers which were identified by our collaborators (Prof. Horst, Leibnitz-University of Hannover) as N-sensitive and cultivar-specific (Apex and Capitol cultivars) were included. Between these two set of genes, one potential marker was shared. The expression of all 46 potential marker genes was further analyzed by qPCR in the five winter cultivars NPZ2, NPZ5, NPZ1005 and MSL101B, grown under greenhouse condition and Major, grown under field condition to identify the most reliable markers. Eventually, 13 markers showing distinct expression levels dependent on N supply but independent of cultivar and growth condition were identified.

3) Leaf transcriptome response of oilseed rape plants to elevated CO_2 levels under different N conditions. Oilseed rape plants (cv. Mozart) were grown in growth chamber under ambient (380 ppm) or elevated (550 ppm) atmospheric CO_2 levels in combination with normal or low N conditions. By using the same *B. napus* microarray as in 1), transcriptome responses of both old leaf (#4) and young leaf (#8) were analyzed at 92 DAS under normal and low N conditions. The most striking observation under elevated CO2 (in young and old leaves under both N conditions) was up-regulation of the genes belonging to C-associated pathways such as photosynthesis, cell wall (synthesis and degradation) lipid metabolism and biosynthesis of C-containing secondary metabolites. The coordinated regulation of these pathways correlates with and might explain the phenotypic alterations of oilseed rape plants grown at high CO_2 levels, namely higher vegetative growth and biomass production, as was reported by our collaborator (Franzaring et al. 2011).

6 Zusammenfassung

Raps (*Brassica napus* L.) stellt eine wichtige Nutzpflanze, die hauptsächlich zur Produktion von Öl verwendet wird, dar. Trotz der effizienten Stickstoffaufnahme ist die Effizienz der Stickstoffnutzung (*Nitrogen Use Efficiency*, NUE) von Raps eher gering. Um dennoch eine optimale Samenausbeute für die Gewinnung von Öl zu erhalten, müssen große Mengen an Stickstoffdünger auf den Feldern für den Rapsanbau ausgebracht werden, was zu wirtschaftlichen Nachteilen und negativen Effekten für die Umwelt führt. Der Großteil des für die Entwicklung der Schoten benötigten Stickstoffs (N), wird durch die Remobilisierung von, sich während der vegetativen Phase, im Stamm und in den Blättern akkumulierten Stickstoff gewonnen. Ein kritischer Faktor, um die NUE zu bestimmen, ist der Zeitpunkt der Initiierung der Blattseneszenz und die Effektivität der Remobilisierung von Stickstoff zu den verbrauchenden Organen (Stickstoffsenken) in der Pflanze. Zum Zeitpunkt der Abszission haben die niedriger gelegenen Blätter weiterhin einen hohen Stickstoffgehalt. Dies führt während des Winters zu einer *Rückzufuhr* in den Boden von bis zu 100 kg Stickstoff ha^{-1} der schätzungsweise 200 kg an benötigtem Stickstoffdünger, die für eine optimale Samenausbeute benötigt werden. Die Remobilisierung von Stickstoff ist in den jüngeren Blättern des oberen Teils der Pflanze, die während der Schotenentwicklung seneszieren, wesentlich effizienter. Zum Zeitpunkt der Abszission macht ihr Stickstoffgehalt lediglich 1 % der Trockenmasse aus. Die Ziele dieser Arbeit waren (1) das Verständnis zur Remobilisierung und Verteilung von Stickstoff in Raps unter normaler und limitierter Stickstoffversorgung zu verbessern, (2) die Identifizierung von Markergenen, die während der Frühphase der Seneszenz exprimiert werden, um den Stickstoffstatus von Rapspflanzen zu überwachen, und (3) die Untersuchung der Auswirkungen von zukünftigen atmosphärischen Bedingungen mit erhöhten CO_2-Konzentrationen auf die Genexpression von Rapspflanzen unter variierendem Stickstoffangebot.

1) Transkriptomanalysen in Blättern unter günstigem und limitierendem Stickstoffangebot während der Entwicklung der Pflanze.

Alte *Source*-Blätter (Blatt #4) und junge *sink*-Blätter (Blatt #8) von Rapspflanzen der Sommervarietät „Mozart“, die unter normalem (150 kg N ha^{-1}) und limitierendem Stickstoffangebot (75 kg N ha^{-1}) wuchsen, wurden zu 4 unterschiedlichen Entwicklungszeitpunkten (78, 85, 92 und 99 Tage nach der Aussaat, *Days After Sowing*, *DAS*) geerntet. Markierte cRNA der Proben wurde gegen 59.577 Oligonukleotid-Sonden eines neu

erstellten Microarrays hybridisiert. Die Oligonukleotid-Sonden wurden aus *B. napus* EST-Contigs erstellt. Um sie zu annotieren, wurde ein BLAST-Suchlauf gegen die Arabidopsis *thaliana* Genomsequenz (TAIR Version 10) durchgeführt und die (putative) Genfunktion des nächst-verwandten Arabidopsis-Orthologs den *B. napus* EST-Contigs zugeordnet.

Der Vergleich der Microarray-Daten zwischen dem normalen und niedrigen Stickstoffangebot zeigte, dass die meisten Veränderungen der Genexpression bei einem niedrigen Stickstoffangebot zu frühen Zeitpunkten (zwischen 85 und 92 DAS) auftraten. Die meisten der regulierten Gene waren Seneszenz-assoziierte Transkriptionsfaktoren oder standen in Verbindung zu Seneszenz-assoziierten Prozessen, wie z.B. der Hochregulierung der Proteindegradierung. Dies weist darauf hin, dass ein niedriges Stickstoffangebot zu einer früheren Initiierung der Seneszenz in Blättern von Rapspflanzen führt. Weiterhin waren Veränderungen im Transkriptom als Antwort auf verschiedene Stickstoffbedingungen in jungen Blättern gegensätzlich zu denen in älteren Blättern. In jungen Blättern (#8) wurden unter normalen Stickstoffbedingungen mehr Seneszenz-assoziierte Veränderungen des Transkriptoms beobachtet, was auf eine verzögerte Seneszenz in *Sink*-Blättern unter Stickstofflimitierung hinweist. Dies kann möglicherweise auf einen ökonomischeren Verbrauch von verfügbaren Ressourcen unter Stickstoffmangel zurückgeführt werden.

2) Identifizierung von Expressionsmarkern für den Stickstoffstatus in Rapspflanzen.

Basierend auf zwei unabhängigen Microarray-Studien zu der Sommerraps-Varietät Mozart (Anzucht in der Klimakammer) und den Winterraps-Varietäten Apex und Capitol (Anzucht in hydroponischen Systemen), die unter ausreichender und limitierender Stickstoffversorgung wuchsen, wurden 46 potenzielle Marker ausgewählt. 35 dieser potenziellen Marker wurden in Mozart, basierend auf hohen Expressionsänderungen (>10-fach) bei verschiedenen Stickstoffangeboten, identifiziert. Weitere 12 potenzielle Marker, die zuvor durch unseren Kooperationspartner (Prof. Horst, Leibnitz-Universität Hannover) in Apex und Capitol identifiziert wurden, vervollständigten die Auswahl. Für alle potenziellen Marker wurde gezeigt, dass sie sensibel gegenüber dem Stickstoffangebot und Varietätsspezifisch sind. Einer der potenziellen Marker kam in beiden Auswahlen von Genen vor. Um die zuverlässigsten und robustesten Marker zu identifizieren, wurde die Expression aller 46 potenziellen Markergene in 5 verschiedenen Wintervarietäten (NPZ2, NPZ5, NPZ1005 und MSL101B, angezogen unter Gewächshaus- und Major, angezogen unter Feldbedingungen) per qPCR analysiert. Letztendlich wurden 13 Marker identifiziert, die deutliche Expressionsänderungen abhängig vom Stickstoffangebot aber unabhängig von Rapsvarietät und Wachstumsbedingungen zeigen.

3) Veränderungen des Blatttranskriptoms von Rapspflanzen bei erhöhtem CO_2-Gehalt und unterschiedlichen Stickstoffkonzentrationen.

Rapspflanzen der Varietät „Mozart“ wurden in Klimakammern unter atmosphärischem (380 ppm) oder erhöhtem (550 ppm) CO_2-Gehalt in Kombination mit normalen oder niedrigen Stickstoffkonzentrationen angezogen. Unter Verwendung des gleichen *B. napus* Microarray-Setups wie in 1), wurden Expressionsänderungen in Blatt #4 und Blatt #8 (altes bzw. junges Blatt) nach 92 DAS untersucht. Das auffälligste Ergebnis bei erhöhtem CO_2 Gehalt (in jungen und alten Blättern) war die Hochregulierung der Expression von Genen, die an C-assoziierten Prozessen wie Photosynthese, Zellwandsynthese und -abbau, Lipidmetabolismus und Biosynthese von C-enthaltenden sekundären Metaboliten beteiligt sind. Die koordinierte Regulierung dieser Prozesse korreliert mit phänotypischen Änderungen, wie verstärktes vegetatives Wachstum und erhöhte Biomasse der Rapspflanzen (Franzaring et al. 2011), die unter einem erhöhten CO_2-Gehalt wuchsen und kann diese möglicherweise erklären.

7 References

Acharya BR, Assmann SM. 2009. Hormone interactions in stomatal function. Plant Mol Biol 69: 451-462.

Adam Z, Clarke AK. 2002. Cutting edge of chloroplast proteolysis. Trends in Plant Science 7: 451-456.

Adam Z, Rudella A, van Wijk KJ. 2006. Recent advances in the study of Clp, FtsH and other proteases located in chloroplasts. Curr Opin Plant Biol 9: 234-240.

Aguera E, Cabello P, de la Haba P. 2010. Induction of leaf senescence by low nitrogen nutrition in sunflower (Helianthus annuus) plants. Physiol Plant 138: 256-267.

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy. New Phytologist 165: 351-371.

Ainsworth EA, Rogers A. 2007. The response of photosynthesis and stomatal conductance to rising [CO2]: mechanisms and environmental interactions. Plant Cell and Environment 30: 258-270.

Ainsworth EA, Rogers A, Vodkin LO, Walter A, Schurr U. 2006. The effects of elevated CO2 concentration on soybean gene expression. An analysis of growing and mature leaves. Plant Physiol 142: 135-147.

Ainsworth EA, Davey PA, Hymus GJ, Osborne CP, Rogers A, Blum H, Nosberger J, Long SP. 2003. Is stimulation of leaf photosynthesis by elevated carbon dioxide concentration maintained in the long term? A test with Lolium perenne grown for 10 years at two nitrogen fertilization levels under Free Air CO(2) Enrichment (FACE). Plant Cell and Environment 26: 705-714.

Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, Kay SA. 2001. Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science 293: 880-883.

Arvidsson S, Kwasniewski M, Riano-Pachon DM, Mueller-Roeber B. 2008. QuantPrime - a flexible tool for reliable high-throughput primer design for quantitative PCR. BMC Bioinformatics 9.

Aukerman MJ, Sakai H. 2003. Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell 15: 2730-2741.

Avice JC, Etienne P. 2014. Leaf senescence and nitrogen remobilization efficiency in oilseed rape (Brassica napus L.). J Exp Bot.

Bai MY, Shang JX, Oh E, Fan M, Bai Y, Zentella R, Sun TP, Wang ZY. 2012. Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in Arabidopsis. Nat Cell Biol 14: 810-817.

Balazadeh S, Riano-Pachon DM, Mueller-Roeber B. 2008. Transcription factors regulating leaf senescence in Arabidopsis thaliana. Plant Biol (Stuttg) 10 Suppl 1: 63-75.

Balazadeh S, Schildhauer J, Araujo WL, Munne-Bosch S, Fernie AR, Proost S, Humbeck K, Mueller-Roeber B. 2014. Reversal of senescence by N resupply to N-starved Arabidopsis thaliana: transcriptomic and metabolomic consequences. J Exp Bot 65: 3975-3992.

Ballicora MA, Iglesias AA, Preiss J. 2004. ADP-glucose pyrophosphorylase: a regulatory enzyme for plant starch synthesis. Photosynthesis Research 79: 1-24.

Bancroft I, et al. 2011. Dissecting the genome of the polyploid crop oilseed rape by transcriptome sequencing. Nat Biotechnol 29: 762-766.

Beguerisse-Diaz M, Hernandez-Gomez MC, Lizzul AM, Barahona M, Desikan R. 2012. Compound stress response in stomatal closure: a mathematical model of ABA and ethylene interaction in guard cells. BMC Syst Biol 6: 146.

Besseau S, Li J, Palva ET. 2012. WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence in Arabidopsis thaliana. Journal of Experimental Botany 63: 2667-2679.

Bishop KA, Betzelberger AM, Long SP, Ainsworth EA. 2014. Is there potential to adapt soybean (Glycine max Merr.) to future [CO]? An analysis of the yield response of 18 genotypes in free-air CO enrichment. Plant Cell Environ.

Brearley J, Venis MA, Blatt MR. 1997. The effect of elevated CO2 concentrations on K+ and anion channels of Vicia faba L. guard cells. Planta 203: 145-154.

Breeze E, et al. 2011. High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation. Plant Cell 23: 873-894.

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D. 2003. The molecular analysis of leaf senescence – a genomics approach. Plant Biotechnology Journal 1: 3-22.

Buchanan-Wollaston V, et al. 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant Journal 42: 567-585.

Cao Q, Miao YX, Wang HY, Huang SY, Cheng SS, Khosla R, Jiang RF. 2013. Non-destructive estimation of rice plant nitrogen status with Crop Circle multispectral active canopy sensor. Field Crops Research 154: 133-144.

Chalhoub B, et al. 2014. Plant genetics. Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. Science 345: 950-953.

Chen KL, Gao CX. 2014. Targeted genome modification technologies and their applications in crop improvements. Plant Cell Reports 33: 575-583.

Chen YF, Li LQ, Xu Q, Kong YH, Wang H, Wu WH. 2009. The WRKY6 Transcription Factor Modulates PHOSPHATE1 Expression in Response to Low Pi Stress in Arabidopsis. Plant Cell 21: 3554-3566.

Cheung F, et al. 2009. Comparative analysis between homoeologous genome segments of Brassica napus and its progenitor species reveals extensive sequence-level divergence. Plant Cell 21: 1912-1928.

Cosgrove DJ. 2000. Loosening of plant cell walls by expansins. Nature 407: 321-326.

Craw P, Balachandran W. 2012. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. Lab Chip 12: 2469-2486.

Daughtry CST, Walthall CL, Kim MS, de Colstoun EB, McMurtrey JE. 2000. Estimating corn leaf chlorophyll concentration from leaf and canopy reflectance. Remote Sensing of Environment 74: 229-239.

Davies PJ, Gan S. 2012. Towards an integrated view of monocarpic plant senescence. Russian Journal of Plant Physiology 59: 467-478.

De la Mata L, De la Haba P, Alamillo JM, Pineda M, Aguera E. 2013. Elevated CO2 concentrations alter nitrogen metabolism and accelerate senescence in sunflower (Helianthus annuus L.) plants. Plant Soil and Environment 59: 303-308.

De Souza AP, Gaspar M, Da Silva EA, Ulian EC, Waclawovsky AJ, Nishiyama MY, Jr., Dos Santos RV, Teixeira MM, Souza GM, Buckeridge MS. 2008. Elevated CO2 increases photosynthesis, biomass and productivity, and modifies gene expression in sugarcane. Plant Cell Environ 31: 1116-1127.

Dejoux JF, Recous S, Meynard JM, Trinsoutrot I, Leterme P. 2000. The fate of nitrogen from winter-frozen rapeseed leaves: mineralization, fluxes to the environment and uptake by rapeseed crop in spring. Plant and Soil 218: 257-272.

Desclos M, Etienne P, Coquet L, Jouenne T, Bonnefoy J, Segura R, Reze S, Ourry A, Avice JC. 2009. A combined 15N tracing/proteomics study in Brassica napus reveals the chronology of proteomics events associated with N remobilization during leaf senescence induced by nitrate limitation or starvation. Proteomics 9: 3580-3608.

Desimone M, Henke A, Wagner E. 1996. Oxidative Stress Induces Partial Degradation of the Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in Isolated Chloroplasts of Barley. Plant Physiol 111: 789-796.

di Toppi LS, Marabottini R, Badiani M, Raschi A. 2002. Antioxidant status in herbaceous plants growing under elevated CO2 in mini-FACE rings. Journal of Plant Physiology 159: 1005-1013.

Diaz C, Purdy S, Christ A, Morot-Gaudry JF, Wingler A, Masclaux-Daubresse C. 2005. Characterization of markers to determine the extent and variability of leaf senescence in Arabidopsis. A metabolic profiling approach. Plant Physiol 138: 898-908.

Diaz C, Lemaitre T, Christ A, Azzopardi M, Kato Y, Sato F, Morot-Gaudry JF, Le Dily F, Masclaux-Daubresse C. 2008. Nitrogen Recycling and Remobilization Are Differentially Controlled by Leaf Senescence and Development Stage in Arabidopsis under Low Nitrogen Nutrition. Plant Physiology 147: 1437-1449.

Dong Q, Schlueter SD, Brendel V. 2004. PlantGDB, plant genome database and analysis tools. Nucleic Acids Research 32: D354-359.

Druart N, Rodríguez-Buey M, Barron-Gafford G, Sjödin A, Bhalerao R, Hurry V. 2006. Molecular targets of elevated [CO2] in leaves and stems of Populus deltoides: implications for future tree growth and carbon sequestration. Functional Plant Biology 33: 121.

Dubos C, Le Gourrierec J, Baudry A, Huep G, Lanet E, Debeaujon I, Routaboul JM, Alboresi A, Weisshaar B, Lepiniec L. 2008. MYBL2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana. Plant Journal 55: 940-953.

Egle K, Beschow H, Merbach W. 2008. Assessing post-anthesis nitrogen uptake, distribution and utilisation in grain protein synthesis in barley (Hordeum vulgare L.) using N-15 fertiliser and N-15 proteinogenic and non-proteinogenic amino acids. Annals of Applied Biology 152: 209-221.

Etalo DW, Stulemeijer IJ, van Esse HP, de Vos RC, Bouwmeester HJ, Joosten MH. 2013. System-wide hypersensitive response-associated transcriptome and metabolome reprogramming in tomato. Plant Physiol 162: 1599-1617.

Fan SC, Lin CS, Hsu PK, Lin SH, Tsay YF. 2009. The Arabidopsis nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. Plant Cell 21: 2750-2761.

Fangmeier A, De Temmerman L, Mortensen L, Kemp K, Burke J, Mitchell R, van Oijen M, Weigel HJ. 1999. Effects on nutrients and on grain quality in spring wheat crops grown under elevated CO2 concentrations and stress conditions in the European, multiple-site experiment 'ESPACE-wheat'. European Journal of Agronomy 10: 215-229.

Fariduddin Q, Yusuf M, Ahmad I, Ahmad A. 2014. Brassinosteroids and their role in response of plants to abiotic stresses. Biologia Plantarum 58: 9-17.

Feller A, Machemer K, Braun EL, Grotewold E. 2011. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. Plant J 66: 94-116.

Fischer JJ, Beatty PH, Good AG, Muench DG. 2013. Manipulation of microRNA expression to improve nitrogen use efficiency. Plant Science 210: 70-81.

Franzaring J, Weller S, Schmid I, Fangmeier A. 2011. Growth, senescence and water use efficiency of spring oilseed rape (Brassica napus L. cv. Mozart) grown in a factorial combination of nitrogen supply and elevated CO2. Environmental and Experimental Botany 72: 284-296.

Frink CR, Waggoner PE, Ausubel JH. 1999. Nitrogen fertilizer: retrospect and prospect. Proc Natl Acad Sci U S A 96: 1175-1180.

Fukayama H, Fukuda T, Masumoto C, Taniguchi Y, Sakai H, Cheng WG, Hasegawa T, Miyao M. 2009. Rice plant response to long term CO2 enrichment: Gene expression profiling. Plant Science 177: 203-210.

Fukayama H, et al. 2011. Gene expression profiling of rice grown in free air CO2 enrichment (FACE) and elevated soil temperature. Field Crops Research 121: 195-199.

Galmes J, Aranjuelo I, Medrano H, Flexas J. 2013. Variation in Rubisco content and activity under variable climatic factors. Photosynth Res.

Gendron JM, Pruneda-Paz JL, Doherty CJ, Gross AM, Kang SE, Kay SA. 2012. Arabidopsis circadian clock protein, TOC1, is a DNA-binding transcription factor. Proceedings of the National Academy of Sciences of the United States of America 109: 3167-3172.

Gibeaut DM, Cramer GR, Seemann JR. 2001. Growth, cell walls, and UDP-Glc dehydrogenase activity of Arabidopsis thaliana grown in elevated carbon dioxide. Journal of Plant Physiology 158: 569-576.

Gill P, Ghaemi A. 2008. Nucleic acid isothermal amplification technologies: a review. Nucleosides Nucleotides Nucleic Acids 27: 224-243.

Goh HH, Sloan J, Dorca-Fornell C, Fleming A. 2012. Inducible Repression of Multiple Expansin Genes Leads to Growth Suppression during Leaf Development. Plant Physiology 159: 1759-1770.

Gombert J, Etienne P, Ourry A, Le Dily F. 2006. The expression patterns of SAG12/Cab genes reveal the spatial and temporal progression of leaf senescence in Brassica napus L. with sensitivity to the environment. Journal of Experimental Botany 57: 1949-1956.

Gray JE, Holroyd GH, van der Lee FM, Bahrami AR, Sijmons PC, Woodward FI, Schuch W, Hetherington AM. 2000. The HIC signalling pathway links CO2 perception to stomatal development. Nature 408: 713-716.

Gregersen PL, Culetic A, Boschian L, Krupinska K. 2013. Plant senescence and crop productivity. Plant Mol Biol 82: 603-622.

Griffin KL, Anderson OR, Gastrich MD, Lewis JD, Lin G, Schuster W, Seemann JR, Tissue DT, Turnbull MH, Whitehead D. 2001. Plant growth in elevated CO2 alters mitochondrial number and chloroplast fine structure. Proc Natl Acad Sci U S A 98: 2473-2478.

Guo Y, Gan S. 2005. Leaf senescence: signals, execution, and regulation. Curr Top Dev Biol 71: 83-112.

Guo Y, Cai Z, Gan S. 2004. Transcriptome of Arabidopsis leaf senescence. Plant Cell and Environment 27: 521-549.

Gupta N, Gupta AK, Gaur VS, Kumar A. 2012. Relationship of Nitrogen Use Efficiency with the Activities of Enzymes Involved in Nitrogen Uptake and Assimilation of Finger Millet Genotypes Grown under Different Nitrogen Inputs. Scientific World Journal.

Gupta P, Duplessis S, White H, Karnosky DF, Martin F, Podila GK. 2005. Gene expression patterns of trembling aspen trees following long-term exposure to interacting elevated CO2 and tropospheric O3. New Phytol 167: 129-141.

Halford NG, Hey S, Jhurreea D, Laurie S, McKibbin RS, Paul M, Zhang Y. 2003. Metabolic signalling and carbon partitioning: role of Snf1-related (SnRK1) protein kinase. J Exp Bot 54: 467-475.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windowns 95/98/NT. Nucleic Acids Symposium Series 42: 95-98.

Hanstein SM, Felle HH. 2002. CO2-triggered chloride release from guard cells in intact fava bean leaves. Kinetics of the onset of stomatal closure. Plant Physiology 130: 940-950.

Harada A, Sakai T, Okada K. 2003. Phot1 and phot2 mediate blue light-induced transient increases in cytosolic Ca2+ differently in Arabidopsis leaves. Proc Natl Acad Sci U S A 100: 8583-8588.

Hauser F, Chen WX, Deinlein U, Chang K, Ossowski S, Fitz J, Hannon GJ, Schroeder JI. 2013. A Genomic-Scale Artificial MicroRNA Library as a Tool to Investigate the Functionally Redundant Gene Space in Arabidopsis. Plant Cell 25: 2848-2863.

He Y, Fukushige H, Hildebrand DF, Gan S. 2002. Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol 128: 876-884.

Hendrey GR, Lewin KF, Nagy J. 1993. Free Air Carbon-Dioxide Enrichment - Development, Progress, Results. Vegetatio 104: 17-31.

Hickman R, et al. 2013. A local regulatory network around three NAC transcription factors in stress responses and senescence in Arabidopsis leaves. Plant J 75: 26-39.

Hirayama T, Shinozaki K. 2007. Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. Trends Plant Sci 12: 343-351.

Hogy P, Fangmeier A. 2008. Effects of elevated atmospheric CO(2) on grain quality of wheat. Journal of Cereal Science 48: 580-591.

Hörtensteiner S. 2006. Chlorophyll degradation during senescence. Annu Rev Plant Biol 57: 55-77.

Hu H, Boisson-Dernier A, Israelsson-Nordstrom M, Bohmer M, Xue S, Ries A, Godoski J, Kuhn JM, Schroeder JI. 2010. Carbonic anhydrases are upstream regulators of CO2-controlled stomatal movements in guard cells. Nat Cell Biol 12: 87-93; sup pp 81-18.

Hu J, Zhong C, Ding C, Chi Q, Walz A, Mombaerts P, Matsunami H, Luo M. 2007. Detection of near-atmospheric concentrations of CO2 by an olfactory subsystem in the mouse. Science 317: 953-957.

Huang J, Gu M, Lai Z, Fan B, Shi K, Zhou YH, Yu JQ, Chen Z. 2010. Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. Plant Physiol 153: 1526-1538.

Humbeck K. 2013. Epigenetic and small RNA regulation of senescence. Plant Mol Biol 82: 529-537.

Hymus GJ, Baker NR, Long SP. 2001. Growth in elevated CO2 can both increase and decrease photochemistry and photoinhibition of photosynthesis in a predictable manner. Dactylis glomerata grown in two levels of nitrogen nutrition. Plant Physiology 127: 1204-1211.

Ishida H, Shimizu S, Makino A, Mae T. 1998. Light-dependent fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in chloroplasts isolated from wheat leaves. Planta 204: 305-309.

Isopp H, Frehner M, Long SP, Nosberger J. 2000. Sucrose-phosphate synthase responds differently to source-sink relations and to photosynthetic rates: Lolium perenne L. growing at elevated p(CO2) in the field. Plant Cell and Environment 23: 597-607.

Jackson ST, Overpeck JT. 2000. Responses of plant populations and communities to environmental changes of the late Quaternary. Paleobiology 26: 194-220.

Jaradat MR, Feurtado JA, Huang D, Lu Y, Cutler AJ. 2013. Multiple roles of the transcription factor AtMYBR1/AtMYB44 in ABA signaling, stress responses, and leaf senescence. BMC Plant Biol 13: 192.

Jing HC, Schippers JHM, Hille J, Dijkwei PP. 2005. Ethylene-induced leaf senescence depends on age-related changes and OLD genes in Arabidopsis. Journal of Experimental Botany 56: 2915-2923.

Kant S, Seneweera S, Rodin J, Materne M, Burch D, Rothstein SJ, Spangenberg G. 2012. Improving yield potential in crops under elevated CO(2): Integrating the photosynthetic and nitrogen utilization efficiencies. Front Plant Sci 3: 162.

Kasajima I, Ide Y, Hirai MY, Fujiwara T. 2010. WRKY6 is involved in the response to boron deficiency in Arabidopsis thaliana. Physiologia Plantarum 139: 80-92.

Kato M, Takashima K, Kakutani T. 2004. Epigenetic control of CACTA transposon mobility in Arabidopsis thaliana. Genetics 168: 961-969.

Kiba T, Kudo T, Kojima M, Sakakibara H. 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. J Exp Bot 62: 1399-1409.

Kiba T, et al. 2012. The Arabidopsis Nitrate Transporter NRT2.4 Plays a Double Role in Roots and Shoots of Nitrogen-Straved Plants. Plant Cell 24: 245-258.

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG. 2009. Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. Science 323: 1053-1057.

Kim JI, Murphy AS, Baek D, Lee SW, Yun DJ, Bressan RA, Narasimhan ML. 2011. YUCCA6 over-expression demonstrates auxin function in delaying leaf senescence in Arabidopsis thaliana. Journal of Experimental Botany 62: 3981-3992.

Kim TH, Bohmer M, Hu H, Nishimura N, Schroeder JI. 2010. Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. Annu Rev Plant Biol 61: 561-591.

Kim YS, Sakuraba Y, Han SH, Yoo SC, Paek NC. 2013. Mutation of the Arabidopsis NAC016 transcription factor delays leaf senescence. Plant and Cell Physiology 54: 1660-1672.

Klaiber J, Dorn S, Najar-Rodriguez AJ. 2013. Acclimation to Elevated CO2 Increases Constitutive Glucosinolate Levels of Brassica Plants and Affects the Performance of Specialized Herbivores from Contrasting Feeding Guilds. Journal of Chemical Ecology 39: 653-665.

Koeslin-Findeklee F, Safavi Rizi V, Becker MA, Parra-Londono S, Arif M, Balazadeh S, B. M-R, R. K, J. HW. 2015. Transcriptomic analysis of nitrogen starvation- and cultivar-specific leaf senescence in winter oilseed rape (Brassica napus L.). Plant Science http://dx.doi.org/10.1016/j.plantsci.2014.11.018.

Kontunen-Soppela S, et al. 2010. Gene expression responses of paper birch (Betula papyrifera) to elevated CO2 and O-3 during leaf maturation and senescence. Environmental Pollution 158: 959-968.

Krapp A, Berthome R, Orsel M, Mercey-Boutet S, Yu A, Castaings L, Elftieh S, Major H, Renou JP, Daniel-Vedele F. 2011. Arabidopsis roots and shoots show distinct temporal adaptation patterns toward nitrogen starvation. Plant Physiol 157: 1255-1282.

Kurai T, Wakayama M, Abiko T, Yanagisawa S, Aoki N, Ohsugi R. 2011. Introduction of the ZmDof1 gene into rice enhances carbon and nitrogen assimilation under low-nitrogen conditions. Plant Biotechnology Journal 9: 826-837.

Lake JA, Woodward FI, Quick WP. 2002. Long-distance CO2 signalling in plants. Journal of Experimental Botany 53: 183-193.

Lamesch P, et al. 2012. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic Acids Research 40: D1202-1210.

Larkum AW. 2010. Limitations and prospects of natural photosynthesis for bioenergy production. Curr Opin Biotechnol 21: 271-276.

Leakey AD, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR. 2009. Elevated CO2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. J Exp Bot 60: 2859-2876.

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

Li F, Wu X, Tsang E, Cutler AJ. 2005. Transcriptional profiling of imbibed Brassica napus seed. Genomics 86: 718-730.

Li P, Ainsworth EA, Leakey AD, Ulanov A, Lozovaya V, Ort DR, Bohnert HJ. 2008. Arabidopsis transcript and metabolite profiles: ecotype-specific responses to open-air elevated [CO2]. Plant Cell Environ 31: 1673-1687.

Li P, Sioson A, Mane SP, Ulanov A, Grothaus G, Heath LS, Murali TM, Bohnert HJ, Grene R. 2006a. Response diversity of Arabidopsis thaliana ecotypes in elevated [CO2] in the field. Plant Mol Biol 62: 593-609.

Li Y, Beisson F, Pollard M, Ohlrogge J. 2006b. Oil content of Arabidopsis seeds: the influence of seed anatomy, light and plant-to-plant variation. Phytochemistry 67: 904-915.

Li Z, Peng J, Wen X, Guo H. 2012. Gene network analysis and functional studies of senescence-associated genes reveal novel regulators of Arabidopsis leaf senescence. J Integr Plant Biol 54: 526-539.

Liang YK, Dubos C, Dodd IC, Holroyd GH, Hetherington AM, Campbell MM. 2005. AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in Arabidopsis thaliana. Current Biology 15: 1201-1206.

Lillo C, Lea US, Ruoff P. 2008. Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. Plant Cell and Environment 31: 587-601.

Lim PO, Woo HR, Nam HG. 2003. Molecular genetics of leaf senescence in Arabidopsis. Trends Plant Sci 8: 272-278.

Lim PO, Kim HJ, Nam HG. 2007. Leaf senescence. Annu Rev Plant Biol 58: 115-136.

Liu J, Wu YH, Yan JJ, Liu YD, Shen FF. 2008. Protein degradation and nitrogen remobilization during leaf senescence. Journal of Plant Biology 51: 11-19.

Liu J, Li J, Wang H, Fu Z, Yu Y. 2011. Identification and expression analysis of ERF transcription factor genes in petunia during flower senescence and in response to hormone treatments. J Exp Bot 62: 825-840.

Long SP, Ainsworth EA, Rogers A, Ort DR. 2004. Rising atmospheric carbon dioxide: plants FACE the future. Annu Rev Plant Biol 55: 591-628.

Long SP, Zhu XG, Naidu SL, Ort DR. 2006. Can improvement in photosynthesis increase crop yields? Plant Cell and Environment 29: 315-330.

Ludewig F, Sonnewald U. 2000. High CO2-mediated down-regulation of photosynthetic gene transcripts is caused by accelerated leaf senescence rather than sugar accumulation. FEBS Lett 479: 19-24.

Makino A, Mae T. 1999. Photosynthesis and plant growth at elevated levels of CO2. Plant and Cell Physiology 40: 999-1006.

Malagoli P, Laine P, Rossato L, Ourry A. 2005. Dynamics of nitrogen uptake and mobilization in field-grown winter oilseed rape (Brassica napus) from stem extension to harvest. II. An 15N-labelling-based simulation model of N partitioning between vegetative and reproductive tissues. Ann Bot (Lond) 95: 1187-1198.

Marga F, Grandbois M, Cosgrove DJ, Baskin TI. 2005. Cell wall extension results in the coordinate separation of parallel microfibrils: evidence from scanning electron microscopy and atomic force microscopy. Plant J 43: 181-190.

Martinez DE, Costa ML, Gomez FM, Otegui MS, Guiamet JJ. 2008. 'Senescence-associated vacuoles' are involved in the degradation of chloroplast proteins in tobacco leaves. Plant J 56: 196-206.

Mas P, Alabadi D, Yanovsky MJ, Oyama T, Kay SA. 2003. Dual role of TOC1 in the control of circadian and photomorphogenic responses in Arabidopsis. Plant Cell 15: 223-236.

Masclaux-Daubresse C, Chardon F. 2011. Exploring nitrogen remobilization for seed filling using natural variation in Arabidopsis thaliana. Journal of Experimental Botany 62: 2131-2142.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of Botany 105: 1141-1157.

Matallana-Ramirez LP, Rauf M, Farage-Barhom S, Dortay H, Xue GP, Droge-Laser W, Lers A, Balazadeh S, Mueller-Roeber B. 2013. NAC transcription factor ORE1 and senescence-induced BIFUNCTIONAL NUCLEASE1 (BFN1) constitute a regulatory cascade in Arabidopsis. Mol Plant 6: 1432-1452.

Matsui K, Umemura Y, Ohme-Takagi M. 2008. AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant Journal 55: 954-967.

May P, Liao W, Wu Y, Shuai B, Richard McCombie W, Zhang MQ, Liu QA. 2013. The effects of carbon dioxide and temperature on microRNA expression in Arabidopsis development. Nat Commun 4: 2145.

McAllister CH, Beatty PH, Good AG. 2012. Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J 10: 1011-1025.

Melotto M, Underwood W, He SY. 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. Annu Rev Phytopathol 46: 101-122.

Menke FL, Kang HG, Chen Z, Park JM, Kumar D, Klessig DF. 2005. Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. Mol Plant Microbe Interact 18: 1027-1034.

Miao Y, Laun T, Zimmermann P, Zentgraf U. 2004. Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. Plant Mol Biol 55: 853-867.

Miao YX, Mulla D, Randall G, Vetsch J, Vintila R. 2009. Combining chlorophyll meter readings and high spatial resolution remote sensing images for in-season site-specific nitrogen management of corn. Precision Agriculture 10: 45-62.

Mitsuda N, Ohme-Takagi M. 2009. Functional analysis of transcription factors in Arabidopsis. Plant and Cell Physiology 50: 1232-1248.

Miyake C, Shinzaki Y, Miyata M, Tomizawa K. 2004. Enhancement of cyclic electron flow around PSI at high light and its contribution to the induction of non-photochemical quenching of chl fluorescence in intact leaves of tobacco plants. Plant and Cell Physiology 45: 1426-1433.

Mondini L, Nachit M, Porceddu E, Pagnotta MA. 2012. Identification of SNP mutations in DREB1, HKT1, and WRKY1 genes involved in drought and salt stress tolerance in durum wheat (Triticum turgidum L. var durum). OMICS 16: 178-187.

Munekaga Y, Hashimoto M, Miyaka C, Tomizawa KI, Endo T, Tasaka M, Shikanai T. 2004. Cyclic electron flow around photosystem I is essential for photosynthesis. Nature 429: 579-582.

Musse M, De Franceschi L, Cambert M, Sorin C, Le Caherec F, Burel A, Bouchereau A, Mariette F, Leport L. 2013. Structural Changes in Senescing Oilseed Rape Leaves at Tissue and Subcellular Levels Monitored by Nuclear Magnetic Resonance Relaxometry through Water Status. Plant Physiology 163: 392-406.

Nakashima K, Shinwari ZK, Sakuma Y, Seki M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K. 2000. Organization and expression of two Arabidopsis DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. Plant Molecular Biology 42: 657-665.

Nemhauser JL, Mockler TC, Chory J. 2004. Interdependency of brassinosteroid and auxin signaling in Arabidopsis. Plos Biology 2: E258.

New DC, Wong JT. 1998. The evidence for G-protein-coupled receptors and heterotrimeric G proteins in protozoa and ancestral metazoa. Biol Signals Recept 7: 98-108.

Ohmetakagi M, Shinshi H. 1995. Ethylene-Inducible DNA-Binding Proteins That Interact with an Ethylene-Responsive Element. Plant Cell 7: 173-182.

Olsen KM, Lea US, Slimestad R, Verheul M, Lillo C. 2008. Differential expression of four Arabidopsis PAL genes; PAL1 and PAL2 have functional specialization in abiotic environmental-triggered flavonoid synthesis. Journal of Plant Physiology 165: 1491-1499.

Onoda Y, Hikosaka K, Hirose T. 2004. Allocation of nitrogen to cell walls decreases photosynthetic nitrogen-use efficiency. Functional Ecology 18: 419-425.

Ossowski S, Schwab R, Weigel D. 2008. Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J 53: 674-690.

Parlitz S, Kunze R, Müller-Röber B, Balazadeh S. 2011. Regulation of photosynthesis and transcription factor expression by leaf shading and re-illumination in *Arabidopsis thaliana* leaves. Journal of Plant Physiology 168: 1311-1319.

Parry MAJ, Andralojc PJ, Scales JC, Salvucci ME, Carmo-Silva AE, Alonso H, Whitney SM. 2013. Rubisco activity and regulation as targets for crop improvement. Journal of Experimental Botany 64: 717-730.

Peng M, Bi YM, Zhu T, Rothstein SJ. 2007. Genome-wide analysis of Arabidopsis responsive transcriptome to nitrogen limitation and its regulation by the ubiquitin ligase gene NLA. Plant Mol Biol 65: 775-797.

Peters GP, Marland G, Le Quere C, Boden T, Canadell JG, Raupach MR. 2012. CORRESPONDENCE: Rapid growth in CO2 emissions after the 2008-2009 global financial crisis. Nature Climate Change 2: 2-4.

Portis AR, Jr., Parry MA. 2007. Discoveries in Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase): a historical perspective. Photosynth Res 94: 121-143.

Prost L, Jeuffroy MH. 2007. Replacing the nitrogen nutrition index by the chlorophyll meter to assess wheat N status. Agronomy for Sustainable Development 27: 321-330.

Provart NJ, Zhu TA. 2003. A browser-based functional classification SuperViewer forArabidopsis genomics. Curr. Computational Mol. Biol: 271-272.

Rana D, van den Boogaart T, O'Neill CM, Hynes L, Bent E, Macpherson L, Park JY, Lim YP, Bancroft I. 2004. Conservation of the microstructure of genome segments in Brassica napus and its diploid relatives. Plant Journal 40: 725-733.

Reddy AR, Rasineni GK, Raghavendra AS. 2010. The impact of global elevated CO2 concentration on photosynthesis and plant productivity. Current Science 99: 46-57.

Reich PB, Hobbie SE, Lee T, Ellsworth DS, West JB, Tilman D, Knops JM, Naeem S, Trost J. 2006. Nitrogen limitation constrains sustainability of ecosystem response to CO2. Nature 440: 922-925.

Ribeiro DM, Mueller-Roeber B, Schippers JH. 2013. Promotion of growth by elevated carbon dioxide is coordinated through a flexible transcriptional network in Arabidopsis. Plant Signal Behav 8.

Ribeiro DM, Araujo WL, Fernie AR, Schippers JH, Mueller-Roeber B. 2012. Action of gibberellins on growth and metabolism of Arabidopsis plants associated with high concentration of carbon dioxide. Plant Physiol 160: 1781-1794.

Richards KD, Schott EJ, Sharma YK, Davis KR, Gardner RC. 1998. Aluminum induces oxidative stress genes in Arabidopsis thaliana. Plant Physiology 116: 409-418.

Richet N, et al. 2012. Elevated CO2 and/or ozone modify lignification in the wood of poplars (Populus tremula x alba). J Exp Bot 63: 4291-4301.

Riechmann JL, Meyerowitz EM. 1998. The AP2/EREBP family of plant transcription factors. Biological Chemistry 379: 633-646.

Robatzek S, Somssich IE. 2002. Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev 16: 1139-1149.

Rojdestvenski I, Ivanov AG, Cottam MG, Borodich A, Huner NPA, Oquist G. 2002. Segregation of photosystems in thylakoid membranes as a critical phenomenon. Biophysical Journal 82: 1719-1730.

Rossato L, MacDuff JH, Laine P, Le Deunff E, Ourry A. 2002. Nitrogen storage and remobilization in Brassica napus L. during the growth cycle: effects of methyl jasmonate on nitrate uptake, senescence, growth, and VSP accumulation. J Exp Bot 53: 1131-1141.

Salon C, Munier-Jolain NG, Duc G, Voisin AS, Grandgirard D, Larmure A, Emery RJN, Ney B. 2001. Grain legume seed filling in relation to nitrogen acquisition: A review and prospects with particular reference to pea. Agronomie 21: 539-552.

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M. 2004. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiology 136: 2483-2499.

Schildhauer J, Wiedemuth K, Humbeck K. 2008. Supply of nitrogen can reverse senescence processes and affect expression of genes coding for plastidic glutamine synthetase and lysine-ketoglutarate reductase/saccharopine dehydrogenase. Plant Biology 10: 76-84.

Schjoerring JK, Bock JGH, Gammelvind L, Jensen CR, Mogensen VO. 1995. Nitrogen incorporation and remobilization in different shoot components of field-grown winter oilseed rape (Brassica napus L) as affected by rate of nitrogen application and irrigation. Plant and Soil 177: 255-264.

Schluter U, Mascher M, Colmsee C, Scholz U, Brautigam A, Fahnenstich H, Sonnewald U. 2012. Maize source leaf adaptation to nitrogen deficiency affects not only nitrogen and carbon metabolism but also control of phosphate homeostasis. Plant Physiol 160: 1384-1406.

Schwartz A, Ilan N, Grantz DA. 1988. Calcium Effects on Stomatal Movement in Commelina communis L. : Use of EGTA to Modulate Stomatal Response to Light, KCl and CO(2). Plant Physiol 87: 583-587.

Schwartz C, Amasino R. 2013. Nitrogen recycling and flowering time in perennial bioenergy crops. Front Plant Sci 4: 76.

Seltmann MA, Hussels W, Berger S. 2010. Jasmonates during senescence: signals or products of metabolism? Plant Signal Behav 5: 1493-1496.

Seo PJ, Park JM, Kang SK, Kim SG, Park CM. 2011. An Arabidopsis senescence-associated protein SAG29 regulates cell viability under high salinity. Planta 233: 189-200.

Sewelam N, Kazan K, Thomas-Hall SR, Kidd BN, Manners JM, Schenk PM. 2013. Ethylene Response Factor 6 Is a Regulator of Reactive Oxygen Species Signaling in Arabidopsis. PLoS ONE 8.

Shan QW, Wang YP, Li J, Gao CX. 2014. Genome editing in rice and wheat using the CRISPR/Cas system. Nature Protocols 9: 2395-2410.

Sharkey TD, Laporte M, Lu Y, Weise S, Weber APM. 2004. Engineering plants for elevated CO2: A relationship between starch degradation and sugar sensing. Plant Biology 6: 280-288.

Shen J-r, Wu J-y, Zhang J, Liu P-w, Yang G-s. 2006. Analysis of Differential Gene Expression Pattern in Brassica napus Hybrid Huayouza 6 and Its Parents Using Arabidopsis cDNA Microarray. Agricultural Sciences in China 5: 33-38.

Shimazaki K, Doi M, Assmann SM, Kinoshita T. 2007. Light regulation of stomatal movement. Annu Rev Plant Biol 58: 219-247.

Shimono H, Suzuki K, Aoki K, Hasegawa T, Okada M. 2010. Effect of panicle removal on photosynthetic acclimation under elevated CO2 in rice. Photosynthetica 48: 530-536.

Shin R, Burch AY, Huppert KA, Tiwari SB, Murphy AS, Guilfoyle TJ, Schachtman DP. 2007. The Arabidopsis transcription factor MYB77 modulates auxin signal transduction. Plant Cell 19: 2440-2453.

Smalle J, Vierstra RD. 2004. The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol 55: 555-590.

Smyth GK. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3.

Spreitzer RJ, Salvucci ME. 2002. Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. Annu Rev Plant Biol 53: 449-475.

Stitt M, Krapp A. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell and Environment 22: 583-621.

Stone SL. 2014. The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling. Front Plant Sci 5: 135.

Sun H, et al. 2014. Heterotrimeric G proteins regulate nitrogen-use efficiency in rice. Nat Genet 46: 652-656.

Sun J, Gibson KM, Kiirats O, Okita TW, Edwards GE. 2002. Interactions of nitrate and CO2 enrichment on growth, carbohydrates, and rubisco in Arabidopsis starch mutants. Significance of starch and hexose. Plant Physiol 130: 1573-1583.

Szekeres M, Nemeth K, KonczKalman Z, Mathur J, Kauschmann A, Altmann T, Redei GP, Nagy F, Schell J, Koncz C. 1996. Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in arabidopsis. Cell 85: 171-182.

Tatusova TA, Karsch-Mizrachi I, Ostell JA. 1999. Complete genomes in WWW Entrez: data representation and analysis. Bioinformatics 15: 536-543.

Taub DR, Miller B, Allen H. 2008. Effects of elevated CO2 on the protein concentration of food crops: a meta-analysis. Global Change Biology 14: 565-575.

Taylor G, Ranasinghe S, Bosac C, Gardner SDL, Ferris R. 1994. Elevated Co2 and Plant-Growth - Cellular Mechanisms and Responses of Whole Plants. Journal of Experimental Botany 45: 1761-1774.

Taylor G, Street NR, Tricker PJ, Sjodin A, Graham L, Skogstrom O, Calfapietra C, Scarascia-Mugnozza G, Jansson S. 2005. The transcriptome of Populus in elevated CO2. New Phytol 167: 143-154.

Teng N, Wang J, Chen T, Wu X, Wang Y, Lin J. 2006. Elevated CO2 induces physiological, biochemical and structural changes in leaves of Arabidopsis thaliana. New Phytol 172: 92-103.

Trick M, Cheung F, Drou N, Fraser F, Lobenhofer EK, Hurban P, Magusin A, Town CD, Bancroft I. 2009. A newly-developed community microarray resource for transcriptome profiling in Brassica species enables the confirmation of Brassica-specific expressed sequences. BMC Plant Biol 9: 50.

Turck F, Zhou A, Somssich IE. 2004. Stimulus-dependent, promoter-specific binding of transcription factor WRKY1 to its native promoter and the defense-related gene PcPR1-1 in parsley. Plant Cell 16: 2573-2585.

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J. 2006. A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314: 1298-1301.

Usadel B, et al. 2005. Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of corresponding genes, and comparison with known responses. Plant Physiol 138: 1195-1204.

Usuda H. 2006. Effects of elevated CO2 on the capacity for photosynthesis of a single leaf and a whole plant, and on growth in a radish. Plant and Cell Physiology 47: 262-269.

Usuda H, Shimogawara K. 1998. The effects of increased atmospheric carbon dioxide on growth, carbohydrates, and photosynthesis in radish, Raphanus sativus. Plant and Cell Physiology 39: 1-7.

Valdes AE, Overnas E, Johansson H, Rada-Iglesias A, Engstrom P. 2012. The homeodomain-leucine zipper (HD-Zip) class I transcription factors ATHB7 and ATHB12 modulate abscisic acid signalling by regulating protein phosphatase 2C and abscisic acid receptor gene activities. Plant Mol Biol 80: 405-418.

van der Graaff E, Schwacke R, Schneider A, Desimone M, Flügge UI, Kunze R. 2006. Transcription analysis of Arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol 141: 776-792.

Van Sandt VS, Guisez Y, Verbelen JP, Vissenberg K. 2007. Xyloglucan endotransglycosylase/hydrolase (XTH) is encoded by a multi-gene family in the primitive vascular land plant Selaginella kraussiana. Plant Biol (Stuttg) 9: 142-146.

Vidal EA, Gutierrez RA. 2008. A systems view of nitrogen nutrient and metabolite responses in Arabidopsis. Current Opinion in Plant Biology 11: 521-529.

Wang F, Cui X, Sun Y, Dong CH. 2013. Ethylene signaling and regulation in plant growth and stress responses. Plant Cell Rep 32: 1099-1109.

Wang H, Xu Q, Kong YH, Chen Y, Duan JY, Wu WH, Chen YF. 2014. Arabidopsis WRKY45 Transcription Factor Activates PHT1;1 Expression in Response to Phosphate Starvation. Plant Physiol.

Wang M, Hao Z, Ji L, Zhou G. 2002. [Effect of elevated atmospheric CO2 concentrations on photosynthesis light response characteristics of three coniferous tree species seedlings]. Ying Yong Sheng Tai Xue Bao 13: 646-650.

Wang R, Okamoto M, Xing X, Crawford NM. 2003. Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol 132: 556-567.

Wang XL, Peng FT, Li MJ, Yang L, Li GJ. 2012. Expression of a heterologous SnRK1 in tomato increases carbon assimilation, nitrogen uptake and modifies fruit development. Journal of Plant Physiology 169: 1173-1182.

Wang YH, Garvin DF, Kochian LV. 2001. Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. Plant Physiology 127: 345-359.

Weaver LM, Amasino RM. 2001. Senescence is induced in individually darkened Arabidopsis leaves, but inhibited in whole darkened plants. Plant Physiol 127: 876-886.

Webb AAR, McAinsh MR, Mansfield TA, Hetherington AM. 1996. Carbon dioxide induces increases in guard cell cytosolic free calcium. Plant Journal 9: 297-304.

Wei H, Gou J, Yordanov Y, Zhang H, Thakur R, Jones W, Burton A. 2013. Global transcriptomic profiling of aspen trees under elevated [CO2] to identify potential molecular mechanisms responsible for enhanced radial growth. J Plant Res 126: 305-320.

Wicker T, et al. 2007. A unified classification system for eukaryotic transposable elements. Nat Rev Genet 8: 973-982.

Wingler A, Purdy S, MacLean JA, Pourtau N. 2006. The role of sugars in integrating environmental signals during the regulation of leaf senescence. J Exp Bot 57: 391-399.

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ. 2007. An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One 2: e718.

Wong JH, Namasivayam P, Abdullah MP. 2012. The PAL2 promoter activities in relation to structural development and adaptation in Arabidopsis thaliana. Planta 235: 267-277.

Woo HR, Kim HJ, Nam HG, Lim PO. 2013. Plant leaf senescence and death - regulation by multiple layers of control and implications for aging in general. J Cell Sci 126: 4823-4833.

Wynn AN, Rueschhoff EE, Franks RG. 2011. Transcriptomic characterization of a synergistic genetic interaction during carpel margin meristem development in Arabidopsis thaliana. PLoS ONE 6: e26231.

Xu H, Wang X, Chen J. 2010. Overexpression of the Rap2.4f transcriptional factor in Arabidopsis promotes leaf senescence. Sci China Life Sci 53: 1221-1226.

Xu YH, Liu R, Yan L, Liu ZQ, Jiang SC, Shen YY, Wang XF, Zhang DP. 2012. Light-harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in Arabidopsis. J Exp Bot 63: 1095-1106.

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T. 2004. Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. Proceedings of the National Academy of Sciences of the United States of America 101: 7833-7838.

Yang B, Srivastava S, Deyholos MK, Kav NNV. 2007. Transcriptional profiling of canola (Brassica napus L.) responses to the fungal pathogen Sclerotinia sclerotiorum. Plant Science 173: 156-171.

Yang TJ, et al. 2006. Sequence-level analysis of the diploidization process in the triplicated FLOWERING LOCUS C region of Brassica rapa. The Plant Cell 18: 1339-1347.

Yang XS, et al. 2011. Gene expression biomarkers provide sensitive indicators of in planta nitrogen status in maize. Plant Physiology 157: 1841-1852.

Yanhui C, et al. 2006. The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol 60: 107-124.

Yao YK, et al. 2012. Active canopy sensor-based precision N management strategy for rice. Agronomy for Sustainable Development 32: 925-933.

Ye HX, Li L, Guo HQ, Yin YH. 2012. MYBL2 is a substrate of GSK3-like kinase BIN2 and acts as a corepressor of BES1 in brassinosteroid signaling pathway in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 109: 20142-20147.

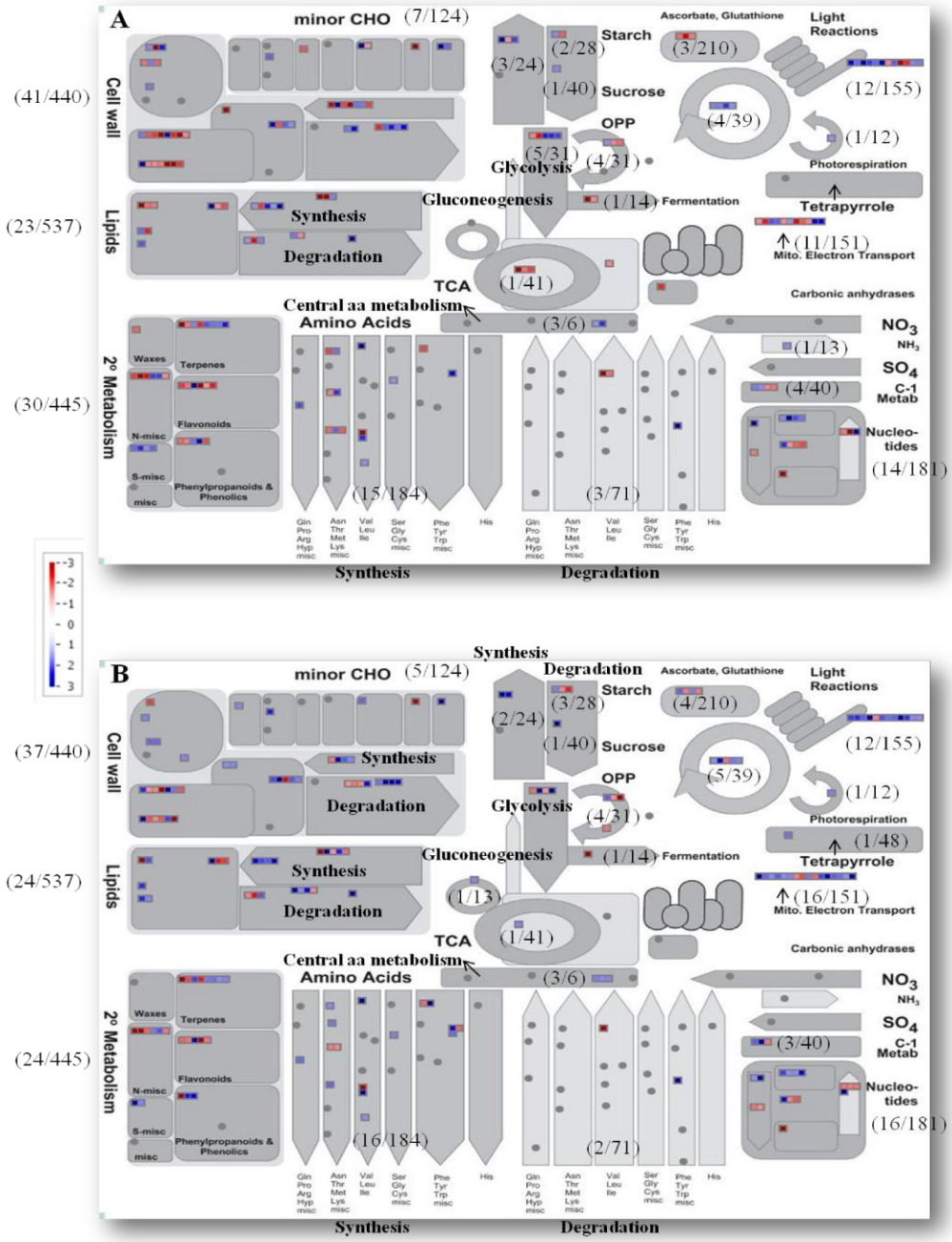
Yoo CY, Pence HE, Jin JB, Miura K, Gosney MJ, Hasegawa PM, Mickelbart MV. 2010. The Arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. Plant Cell 22: 4128-4141.

Young JJ, Mehta S, Israelsson M, Godoski J, Grill E, Schroeder JI. 2006. CO2 signaling in guard cells: Calcium sensitivity response modulation, a Ca2+-independent phase, and CO2 insensitivity of the gca2 mutant. Proceedings of the National Academy of Sciences of the United States of America 103: 7506-7511.

Zentgraf U, Jobst J, Kolb D, Rentsch D. 2004. Senescence-related gene expression profiles of rosette leaves of Arabidopsis thaliana: leaf age versus plant age. Plant Biology 6: 178-183.

Zheng ZL. 2009. Carbon and nitrogen nutrient balance signaling in plants. Plant Signal Behav 4 584-591.

8 Appendices


A
minor CHO (7/124)
Starch
(2/28)
(3/24)
(1/40) Sucrose
OPP
(5/31)
(4/31)
Glycolysis
Gluconeogenesis
(1/14) Fermentation
TCA
(1/41)
Ascorbate, Glutathione
(3/210)
Light Reactions
(12/155)
(4/39)
(1/12)
Photorespiration
Tetrapyrrole
(11/151)
Mito. Electron Transport
Carbonic anhydrases
(41/440)
Cell wall
(23/537)
Lipids
Synthesis
Degradation
Central aa metabolism
Amino Acids
(3/6)
(30/445)
2° Metabolism
Waxes
Terpenes
Flavonoids
N-misc
S-misc
misc
Phenylpropanoids & Phenolics
(15/184)
(3/71)
NO3
NH3
(1/13)
SO4
(4/40)
C-1 Metab
Nucleo-tides
(14/181)
Gln Pro Arg Hyp misc
Asn Thr Met Lys misc
Val Leu Ile
Ser Gly Cys misc
Phe Tyr Trp misc
His
Synthesis
Degradation
-3
-2
-1
0
1
2
3
B
minor CHO (5/124)
Starch
(3/28)
(2/24)
(1/40) Sucrose
OPP
Glycolysis
(4/31)
Gluconeogenesis
(1/14) Fermentation
(1/13)
TCA
(1/41)
Ascorbate, Glutathione
(4/210)
Light Reactions
(12/155)
(5/39)
(1/12)
Photorespiration
(1/48)
Tetrapyrrole
(16/151)
Mito. Electron Transport
Carbonic anhydrases
(37/440)
Cell wall
(24/537)
Lipids
Synthesis
Degradation
Central aa metabolism
Amino Acids
(3/6)
(24/445)
2° Metabolism
Waxes
Terpenes
Flavonoids
N-misc
S-misc
misc
Phenylpropanoids & Phenolics
(16/184)
(2/71)
NO3
NH3
SO4
(3/40)
C-1 Metab
Nucleo-tides
(16/181)
Gln Pro Arg Hyp misc
Asn Thr Met Lys misc
Val Leu Ile
Ser Gly Cys misc
Phe Tyr Trp misc
His
Synthesis
Degradation

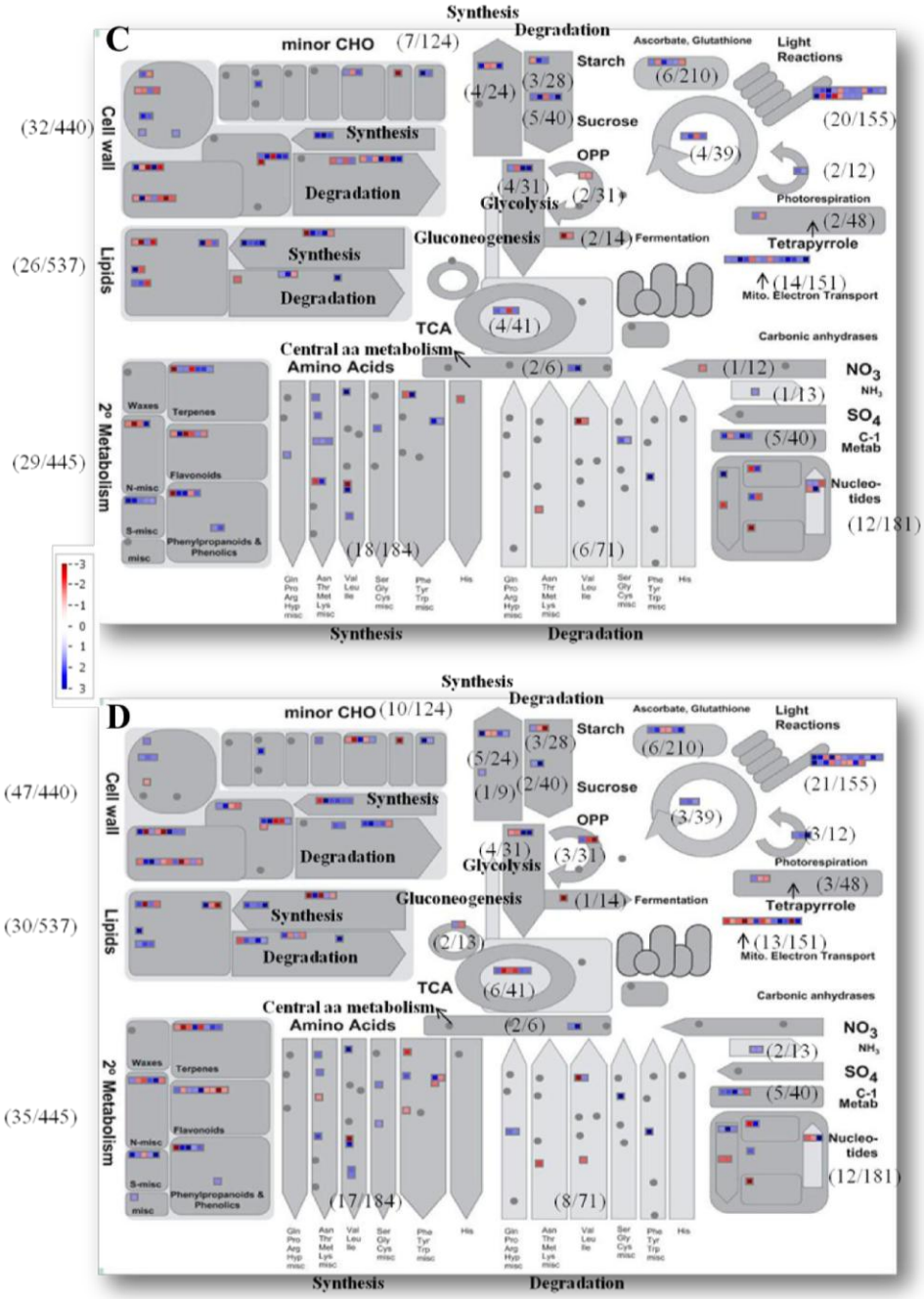


Figure S2. Overview of biological pathways and related regulated genes with differential expression changes in response to elevated CO_2 in leaf #4 under normal N (A), leaf #4 under low N (B), leaf #8 under normal N (C) and leaf #8 under low N (D). The first and the second numbers indicate the number of regulated genes and the total number of genes belonging to that category available in MapMan (TAIR10), respectively. Image is retrieved from MapMan software. Blue and red blocks denote up- and down-regulated genes, respectively

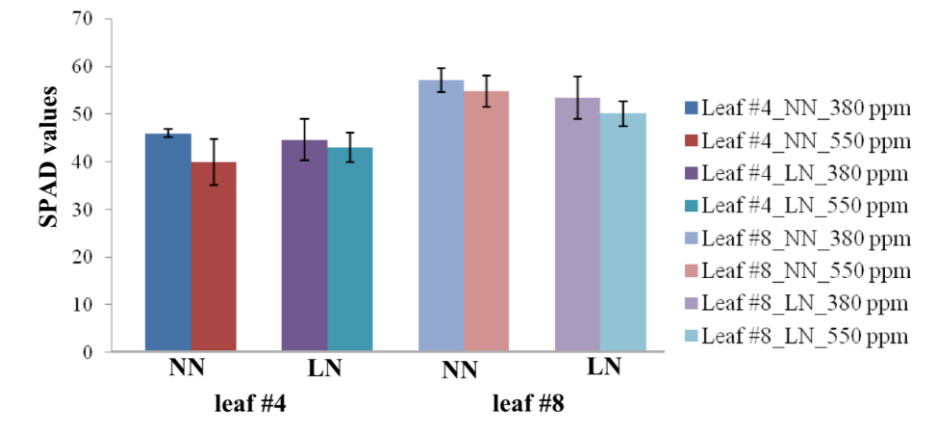


Figure S3. SPAD values of leaf #4 and leaf #8 at 92 DAS in response to ambient (380ppm) and elevated (550ppm) under normal (NN) and low (LN) N conditions. Error bars represent the standard deviation of the means (n=3).

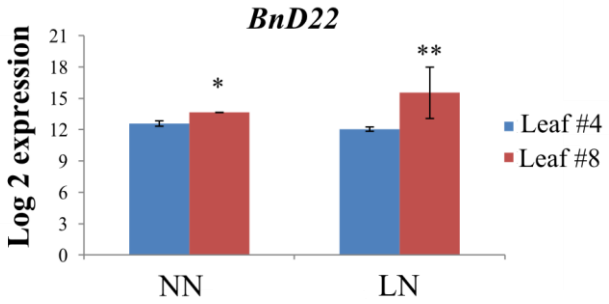
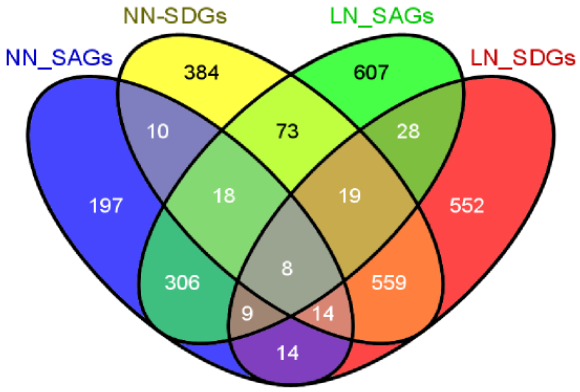


Figure S4. *BnD22* expression in leaf #4 and leaf #8 under normal (NN) and low (LN) N conditions. The data are log2 expression values from microarray. Asterisks denote statistically significant differences between leaf #4 and leaf #8 by Student's *t*-test (** $P < 0.001$, * $P < 0.01$). Error bars represent the standard deviation of the means (n=3).



NN_SAGs (197)

PS (4)
Transport (11)
Signaling (12)
RNA (25)
Protein: synthesis, degradation, post translational modification, targeting (35)
Not assigned (43)

LN_SAGs (607)

TCA/org transformation (4)
Mitochondrial electron transport/ATP synthesis (7)
PS (7)
Cell (14)
Secondary metabolism (12)
Development (20)
Transport (25)
RNA (73)
Stress (28)
Signaling (30)
Protein: synthesis, degradation, post translational modification, targeting (101)
Not assigned (168)
DNA: synthesis and repair (16)
Micro RNA, natural antisense etc. (2)

NN_SDGs (384)

Glycolysis (4)
Co-factor and vitamin metabolism (4)
PS (9)
Mitochondrial electron transport/ATP synthesis (6)
Hormone metabolism (20)
Nucleotide metabolism (5)
Amino acid metabolism (7)
Secondary metabolism (11)
Cell wall (11)
Cell (16)
Development (15)
Transport (18)
RNA (45)
Not assigned (91)
DNA (10)

LN_SDGs (552)

Mitochondrial electron transport/ATP synthesis (11)
TCA/org transformation (5)
Nucleotide metabolism (9)
Cell wall (21)
Redox (8)
Transport (33)
Lipid metabolism (13)
Cell (24)
Development (22)
RNA (68)
Signaling (27)
Protein: synthesis, degradation, post translational modification, targeting (91)
Not assigned (130)
Stress (12)
DNA (11)
Micro RNA, natural antisense etc. (1)

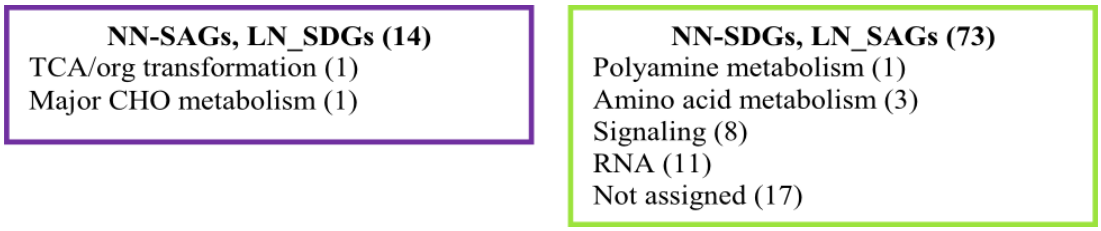


Figure S5. Comparison of transcriptome response during developmental and N-deficiency induced leaf senescence in oilseed rape. Significantly represented biological pathways ($p<0.05$, hypergeometric distributions) associated with SAGs and SDGs under normal (NN) and low (LN) N conditions are retrieved from SuperViewer software with MapMan as classification source. P-value of the hypergeometric distribution is calculated as: $p = BC(M,x) * BC(N-M, n-x) / BC(N,n)$ with BC as the binomial coefficient calculated as follows: $BC(n,k) = n! / (k! * (n-k)!)$, x as the number of input genes with the selected classification, n as the total number of input genes, M as the number of genes with the selected classification in the database (MapMan, TAIR10) and N as the total number of genes in the database (TAIR10). (Source: http://bar.utoronto.ca). Unique pathways in response to LN or NN are underlined. Number of the regulated genes belonging to each biological category is provided in parenthesis.

TableS4. Number of regulated genes belonging to significantly represented (p<0.05, based on hypergeometric distribution in SuperViewer tool) biological categories only under normal N (NN) or low N (LN) during development (in leaf #4, see figure 13, page 40). P-value of the hypergeometric distribution is calculated as: p = BC(M,x) * BC(N-M, n-x) / BC(N,n) with BC as the binomial coefficient calculated as follows: BC(n,k) = n! / (k! * (n-k)!), x as the number of input genes with the selected classification, n as the total number of input genes, M as the number of genes with the selected classification in the database (MapMan, TAIR10) and N as the total number of genes in the database (TAIR10). (Source: http://bar.utoronto.ca). Each time point was compared with the previous time point under normal and low N, separately. H: harvest. DAS: Days after sowing.

Normal N (NN)	
Up-regulated under normal N (NN)	
Between 78 and 85 DAS (H2 and H3)	
Biological category	**Nr. of regulated genes**
Amino acid metabolism	**4**
synthesis	4
Stress	**8**
Biotic stress	3
Abiotic stress	5
protein	**24**
synthesis	8
degradation	8
posttranslational modification	7
targeting	1
Between 85 and 92 DAS (H3 and H4)	
Polyamine metabolism	**1**
Minor carbohydrate metabolism	**2**
Between 92 and 99 DAS (H4 and H5)	
Photosynthesis	**7**
Amino acid metabolism	**8**
synthesis	5
degradation	3
Stress	21
Biotic stress	6
Abiotic stress	15
Down-regulated under normal N (NN)	
Between 78 and 85 DAS (H2 and H3)	
Glycolysis	**4**
tetrapyrrole synthesis	**2**
Hormone metabolism	**6**
Stress	**7**
Biotic stress	2
Abiotic stress	5

DNA	**3**
Synthesis	2
unspecified	1
Between 85 and 92 DAS (H3 and H4)	
Protein	43
synthesis	8
Degradation	16
targeting	5
posttranslational modification	14
Between 92 and 99 DAS (H4 and H5)	
Photosynthesis	**14**

Low N (LN)

Up-regulated under low N (LN)

Between 85 and 92 DAS (H3 and H4)

Biological category	**Nr. of regulated genes**
Mitochondrial electron transport	**4**
Amino acid metabolism	**9**
Synthesis	7
Degradation	2
Photosynthesis	**6**
RNA	**71**
Processing	9
Transcription	2
Regulation of transcription	55
RNA binding	5
Development	**16**
Stress	**22**
Biotic stress	6
Abiotic stress	16
Signaling	**25**
DNA	**11**
Synthesis	9
Repair	1
Unspecified	1
Protein	**76**
Folding	1
Amino acid activation	3
Synthesis	19
Targeting	3
Posttranslational modification	13
Degradation	37

Between 92 and 99 DAS (H4 and H5)	
Mitochondrial electron transport	**6**
TCA/organic acid transformation	**5**
Secondary metabolism	**14**
Cell	**25**
Organisation	13
Cell division	5
Cell cycle	1
Vesicule transport	6
Down-regulated under low N (LN)	
Between 78 and 85 DAS (H2 and H3)	
Major carbohydrate metabolism	**2**
Cell wall	**5**
Synthesis	2
Degradation	2
Modification	1
RNA	**11**
Processing	4
Rrgulation of transcription	6
RNA binding	1
Between 85 and 92 DAS (H3 and H4)	
Nucleotide metabolism	**5**
Cell	**16**
Cell organisation	6
Cell division	1
Cell cycle	1
Vesicule transport	7
Cell death	1
Development	**14**
Transport	**19**
Between 92 and 99 DAS (H4 and H5)	
N-metabolism	**3**
Stress	**29**
Abiotic stress	20
Biotic stress	9
Secondary metabolism	8
Cell wall	**21**
Synthesis	8
Protein	3
Modification	4
Pectin esterase	6

Table S5. Number of regulated genes belonging to significantly represented (p<0.05, based on hypergeometric distribution in SuperViewer tool) biological categories under both N conditions (leaf #4) during development (in leaf #4, see figure 14, page 42). P-value of the hypergeometric distribution is calculated as: p = BC(M,x) * BC(N-M, n-x) / BC(N,n) with BC as the binomial coefficient calculated as follows: BC(n,k) = n! / (k! * (n-k)!), x as the number of input genes with the selected classification, n as the total number of input genes, M as the number of genes with the selected classification in the database (MapMan, TAIR10) and N as the total number of genes in the database (TAIR10). (Source: http://bar.utoronto.ca). Each time point was compared with the previous time point under normal and low N, separately. H: harvest. DAS: Days after sowing.

Normal N (NN) and low N (LN)		
Between 92 and 99 DAS (H4 and H5)		
Up-regulated		
	NN	**LN**
Glycolysis	6	**6**
Minor carbohydrate metabolism	5	**7**
Development	15	**24**
Major carbohydrate metabolism	6	**6**
RNA	**56**	**67**
RNA processing	7	7
transcription	1	4
regulation of transcription	44	51
RNA binding	4	4
DNA		**18**
synthesis	7	13
repair	3	2
unspecified	2	3
Protein	**8**	**113**
amino acid activation	0	1
amino acid synthesis	5	28
targeting	0	10
posttranslational modification	0	28
amino acid degradation	3	43
folding	0	3
Down-regulated		
Between 78 and 85 DAS (H2 and H3)		
DNA	**3**	**2**
synthesis	2	1
unspecified	1	1
Between 85 and 92 DAS (H3 and H4)		
Cofactor and vitamine metabolism	**3**	**3**
Photosynthesis	**4**	**5**
Cell wall	**12**	**14**
cell wall synthesis	3	2
cell wall proteins	2	5

cell wall degradation	2	1
cell wall modification	2	3
pectin esterase	3	3
RNA	**27**	**43**
RNA processing	4	5
transcription	2	1
regulation of transcription	20	32
RNA binding	1	5
DNA	**10**	**8**
synthesis	5	5
repair	1	1
unspecified	4	2
Between 92 and 99 DAS (H4 and H5)		
TCA/organic acid trasformation	**8**	**8**
Mitochondrial electron transport	**8**	**13**
Nucleotide metabolism	**14**	**15**
Redox	**10**	**14**
Hormone metabolism	**25**	**25**
DNA	**25**	**26**
synthesis	18	18
repair	3	4
unspecified	4	4
Transport	**37**	**52**
Amino acid metabolism	**16**	**17**
synthesis	13	13
degradation	3	4
Lipid metabolism	**19**	**26**
cell	**33**	**39**
organization	14	17
division	4	6
cell cycle	6	8
vesicle transport	7	7
cell death	2	1
RNA	**107**	**121**
RNA processing	10	11
transcription	5	4
regulation of transcription	83	98
RNA binding	9	8
Development	**27**	**29**
MicroRNA/natural antisense	**2**	**2**