A wider perspective on the barley leaf senescence connecting whole plant development and nitrogen availability

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium
(Dr.rer.nat.)

vorlegt der
Naturwissenschaftlichen Fakultät I
Biowissenschaften, Institut für Biologie
der Martin-Luther-Universität Halle- Wittenberg

von

Herrn Nazeer Fataftah
geboren am 26.11.1985 in Palästina, Tarqumia

Gutachter 1: Prof. Dr. Klaus Humbeck

Gutachter 2: Prof. Dr. Nicolaus von Wirén

Gutachter 3: Prof. Dr. Per L. Gregersen

Halle (Saale), February 2016

Tag der öffentlichen Promotionsverteidigung: 06.07.2016

ACKNOWLEDGEMENTS

Towards the accomplishment of this thesis I have been accompanied and supported by many people. Now, I have the opportunity to express my gratitude for all of them.

Heartily thanks to my lively supervisor Prof. Dr. Klaus Humbeck for providing me the opportunity to carry out my PhD at Martin-Luther University Halle-Wittenberg. I would also like to thank him for his trust, support, and helpful comments.

Thanks to Prof. Dr. Nicolaus von Wirén, Dr. Mohammad Hajirezaei, Dr. Winfriede Weschke, and Dr. Stefan Kohl at IPK for providing facilities and helpful comments to accomplish this work.

I would also like to express my appreciation to my colleagues, Dr. Olaf Barth, Dr. Wiebke Zschiesche, Dr. Nicole Ay, Stefan Ehnert, Christina Mohr, Paula Paramon, Ulrike Raum, and Stefanie Stöckhardt, for supporting me in my scientific and personal life.

For my friends and colleagues, many thanks for making my stay at Germany enjoyable.

Contents

List of Figures

List of tables

1. Summary

Leaf senescence, the last step of leaf development, is a highly regulated process, modulated by a number of internal and external factors. During the senescence process resources like nitrogen (N) are remobilized from senescent tissues to sink tissues. This intrinsically depends on the accurate dispersion of resources according to sink strength of various organs competing with each other. Consequently, N deficiency accelerates barley leaf senescence and its resupply can delay the senescence progression. In order to identify genetic and metabolic factors that regulate leaf senescence in response to whole plant development and N supply, chlorophyll content, root/shoot ratio, hormonal changes, anion levels, C/N ratio, transcriptomic and global metabolic rearrangements were analyzed in barley primary and 4th leaves at different nitrogen regimes. Moreover, phloem exudates of primary leaf were collected in EDTA solution and analyzed for sugar and amino acid levels to monitor export activity. The results showed that leaves undergoing senescence have a capacity to sense the availability of nitrogen and accordingly to modulate senescence progression and sustain barley plant growth. This is reflected by global hormonal (tZ and ABA), transcriptomic, and metabolic rearrangement in response to leaf development and N status. Especially, TCA cycle-components were affected under N deficiency condition. This metabolic rearrangement was also reflected by transcriptomic change. Such as, genes coding pyruvate kinase, pyruvate dehydrogenase, and pyruvate dikinase were regulated by senescence and N status.

Keywords: Barley, leaf senescence, nitrogen, transcriptome, metabolites, phytohormones, phloem exodate

2. Zusammenfassung

Das Altern von Blättern, auch Blattseneszenz genannt, ist auf molekularer Ebene ein geordneter, streng regulierter Prozess. Dabei nehmen sowohl interne als auch externe Faktoren Einfluss auf den zeitlichen Ablauf der Blattalterung. Während der Blattseneszenz werden wichtige Ressourcen, wie organische Stickstoffverbindungen, remobilisiert und aus den seneszenten Geweben in junge, sich entwickelnde Bereiche (Sink) der Pflanze transportiert. Eine exakte Verteilung der entsprechend verfügbaren Ressourcen erfolgt anhand des jeweiligen Bedarfs (Sink-Stärke) der verschiedenen, dabei konkurrierenden Organe. Folgerichtig beschleunigt Stickstoffmangel die Blattseneszenz. Interessanterweise kann eine Wiederversorgung mit Stickstoff nach einer solchen Mangelperiode die bereits in Gang gesetzten Seneszenzprozesse stark verzögern. Um genetische und metabolische Faktoren zu identifizieren, welche die Blattseneszenz während der Entwicklung einer Pflanze in Abhängigkeit von der Stickstoffverfügbarkeit regulieren, wurden die Primärblätter und die 4. Blätter von Gersten-pflanzen mit unterschiedlicher Stickstoffversorgung vergleichend untersucht. Neben den Chlorophyll-gehalten, dem Wurzel/Spross-Verhältnis und Phytohormonveränderungen sind auch die Mengen verschiedener Anionen, das C/N-Verhältnis, globale Transkriptmuster und die globale metabolische Reorganisation analysiert worden. Des Weiteren wurden Phloemsäfte von Primärblättern hinsichtlich der entsprechenden Kohlenhydrat- und Aminosäuregehalte analysiert, um die jeweilige Exportaktivität zu untersuchen. Die im Rahmen dieser Arbeit erhobenen Daten zeigen, dass Gerstenblätter im Zuge des Seneszenzprozesses Veränderungen der Stickstoffverfügbarkeit wahrnehmen, den Ablauf der Blattseneszenz entsprechend anpassen und auf diese Weise das Wachstum der gesamten Gersten-pflanze ermöglichen. Dies wird vor allem durch die Veränderungen der Phytohormonspiegel (trans-Zeatin und ABA), des Transkriptoms und des Metaboloms in Abhängigkeit vom jeweiligen Ent-wicklungszeitpunkt und Stickstoffstatus deutlich sichtbar. So werden unter Stickstoffmangel die Komponenten des Citratzyklus besonders

stark beeinflusst. Mit dieser metabolischen Reorganisation gehen entsprechende Veränderungen auf Transkriptebene einher. So wird die Expression der Gene, welche für die Pyruvatkinase, die Pyruvatdehydrogenase und die Pyruvat-Phosphat-Dikinase codieren, in Abhängigkeit von der Seneszenzphase und dem jeweiligen Stickstoffstatus reguliert.

Schlagwort: Gerste, Blattseneszenz, Stickstoff, Transkriptom, Metaboliten, Phytohormone, Phloemsaft

3. Introduction

3.1. Leaf Senescence

3.1.1. Developmental senescence and senescence concepts

Leaf senescence is one of the fundamental biological questions and was a puzzle for centuries. Leaf senescence can be defined as the last developmental stage of the leaf life ended by leaf death. It is characterized by loss of chlorophyll (yellowing) and photosynthetic capacity. Plant species or even cultivars differ greatly in their life span. Thus, leaf senescence is a genetically controlled trait. Nevertheless, leaf senescence is a highly regulated process, which includes the visible color changes, dismantling of chloroplasts, degradation of proteins, RNA, and DNA and translocation of macro/micromolecules to other plant parts, mainly sink tissues, (Bleeker and Patterson, 1997).

Leaf senescence, even under ideal growth conditions, can be regulated by internal factors (e.g. epigenetic controller and hormones) dependent on developmental age. This regulation includes global changes in gene transcription. Genes which are up regulated during the senescence process, are termed senescence associated genes (SAGs). In contrast, genes repressed with leaf senescence are senescence downregulated genes (SDGs). In transcriptome analysis, it was shown that more than 800 SAGs were upregulated during developmental senescence (Buchanan-Wollaston et al., 2005). The plethora of differentially regulated genes makes the senescence puzzle solving highly complicated.

Chloroplast contains the most reduced nitrogen available for remobilization. About one-third of total reduced nitrogen is present in Rubisco, which is often referred to as the most abundant protein on earth (Feller et al., 2008). Once leaf senescence has been initiated, it leads to a massive degradation of macromolecules by catalytic enzymes, and remobilization of phloem-mobile nutrients from the senescing leaf to developing sinks, such as grains and roots. In this context, nitrogen is an important nutrient that is remobilized during

senescence. Nitrogen-containing macromolecules are degraded and converted to amino acids, mainly glutamate and glutamine, prior to be loaded to phloem and transported to sink tissues. Senescence timing and remobilization efficiency influences agronomic traits including nutrient use efficiency and yield (quantity or quality). While late senescence varieties often, but not always exhibit higher yields (Gregersen et al., 2013), early induced senescence is associated with higher grain proteins and minerals content (Uauy et al., 2006; Waters et al., 2009). Due to the economic importance of crop plants, more researches are needed to understand the physiological and molecular aspects of senescence and nutrients remobilization in aim to increase crops yield quantity and/or quality. In fact, barley has smaller (~5.1 Gb) and simpler genome (2n= 14) than wheat (Distelfeld et al., 2014). So, it is a useful model to study crop physiology, and it has extensively been used for studying nitrogen metabolism and senescence (Kohl et al., 2012, Krupinska et al., 2012).

It is important to distinguish between organ (e.g. leaf) senescence syndrome and terminal monocarpic senescence. Organ senescence is mostly associated with leaf age during vegetative growth, and involves remobilization of the nutrients to younger plant parts or roots. On the other hand, the whole plant senescence is initiated during the reproductive stage of monocarpic plants and the nutrients are remobilized from senescing plant parts, mainly flag leaf, to the developing seeds (Davis and Gan, 2012). There exists a negative correlation between yield and nutrient values. Late senescence may lead to lower proteins and nutrients content in grains, that can be explained by proteins and micronutrients are diluted by prolonged carbohydrate accumulation (Gregersen, 2011), or the synthesis of storage protein in grains consume more carbohydrates (Munier-Jolain and Salon, 2005).

Although, there are numerous investigations which led to a better understanding of the senescence program in plants and animals, the central questions regarding how leaf senescence is initiated, what is the nature of developmental factors that trigger leaf senescence, and how the developmental age is determined to initiate the senescence remain unanswered.

3.1.2. Abiotic stresses induce leaf senescence

While leaf senescence is initiated by developmental age, many unsuitable environmental factors, such as drought, insufficient light, and nutrients deficiency can also prematurely trigger the senescence program, which leads a decrease in crops yield. Comparison of transcriptome data reveals a crosstalk between developmental and stress induced leaf senescence at molecular level. For example, among the 43 transcription factors that are induced during developmental senescence, 28 genes are also induced by various stresses (Chen et al., 2002b). Guo and Gan (2012) have shown by analysing microarray expression data from developmental and 27 different treatments inducing senescence process that at early induction of senescence showed limited common regulation in the induction of gene expression to that of developmental leaf senescence. Once the senescence is started, the different senescence inducers share a huge proportion of SAGs expression. The induction of senescence by theses environmental stresses contributes to nutrient remobilization. Thus, allowing the rest of the plant (e.g. young leaves, roots, and seeds) to benefit from this remobilization. Nevertheless, to introduce delaying senescence cultivars may lead to induce stresses tolerant plants (Rivero et al., 2007).

3.2. Molecular and genetic regulation of leaf senescence

The internal factors triggering leaf senescence include developmental age, epigenetic control levels, phytohormones and sugars level, as well as reproductive timing (Fig.3.1). This indicates multiple pathways responding to various internal and external factors are interconnected to form regulatory pathways for senescence (He et al., 2001). Leaf growth and maturation could intimately regulate leaf senescence. Thus, it is possible that genes controlling these processes, including meristematic activity, could influence age-dependent senescence (Lim et al., 2007). It was shown that the blade on petiole 1-1 (bop1-1) mutant that showed enhanced meristematic activity exhibited a prolonged leaf life span (Ha et al., 2003). It is now clear that the senescence program involves highly complicated molecular regulation pathways that are

controlled at multiple layers of regulation, including chromatin structuring (epigenetic), and transcription, as well as by post-transcriptional, translational and post-translational regulation (reviewed by Woo et al., 2013).

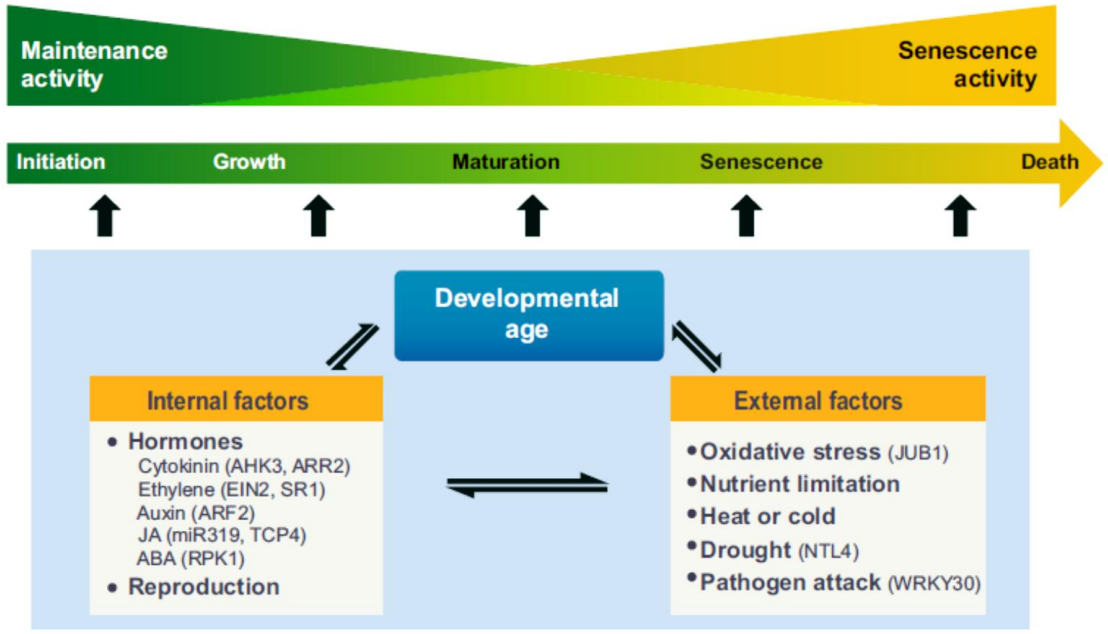

Figure 3. 1. Overview of internal and external regulators of leaf growth and senescence. Internal factors include various phytohormones and reproduction. External factors that modulate leaf senescence include oxidative stress, high or low temperature, drought, nutrient deficiency and pathogen infection. In brackets: Protein or factors related to the different regulators sensing or response (Woo et al., 2013).

Beside the regulatory genes of phytohormones and sugars metabolism that will be discussed in the next sections, several senescence regulatory genes were identified by genetic and molecular studies, as well as 'omics' analyses. 96 transcription factor genes were found to be upregulated in Arabidopsis senescing leaves. These belong to transcription factor families including WRKY, NAC, MYB, C2H2-type zinc finger, and AP2/EREBP (Lim et al., 2007). For example, an At-WRKY53 knockout mutant showed delayed leaf senescence, whereas over expression line induced the senescence process. It has been reported that WRKY53 targets W-box sequences in promotor of genes including SAGs, RR genes, stress-related genes, and other transcription factors as also other WRKY genes (Miao et al., 2004). Moreover, WRKY53 can be induced by H_2O_2 and can regulate its own expression in a negative feedback

loop (Miao et al., 2004). Another example is WRKY6 which is upregulated during leaf senescence as well as during pathogen infection (Robatzek and Somssich, 2002). Target genes of WRKY6 are many genes associated with pathogen and senescence response, including the senescence –induced receptor like kinase gene (SIRK).

A total of 20 genes from 100 NAC members were found to be upregulated during natural and dark-induced leaf senescence (Guo and Gan, 2006). A list of 48 barley NAC genes (*HvNACs*) has been obtained through searches in publicly available barley sequence databases. Phylogenetic comparisons to Brachypodium, rice, and Arabidopsis NAC proteins indicate that the barley NAC family includes members from all of the eight NAC subfamilies, although by comparison to these species presumably a number of *HvNACs* still remains to be identified. Comparison of expression profiles of selected barley NAC genes with closely related NAC genes from other plant species, including both monocots and dicots, suggests conserved functions in the areas of secondary cell wall biosynthesis, leaf senescence, root development, seed development, and hormone regulated stress responses (Christiansen et al., 2011). In addition, grain protein content (GPC) locus on barley chromosome 6 strongly influences the timing of post-anthesis flag leaf senescence (Lacerenza et al., 2010). It has been demonstrated by map-based cloning that the wheat high-GPC/fast-senescence phenotype is due to the presence of a functional NAC transcription factor, while the gene is deleted or truncated in low-GPC tetraploid or hexaploid germplasm (Uauy et al., 2006). Distelfeld et al. (2008) have demonstrated the presence of a homologous gene (HvNAM-1) at this locus in barley plant. The subsequent identification of the upstream regulatory factors and downstream targets has uncovered the gene regulatory networks that control leaf senescence (Guo and Gan, 2006; Zhang and Gan, 2012; Hickman et al., 2013). Expression of ORE1 (ANAC092) is induced during Arabidopsis leaf aging by ETHYLENE INSENSITIVE 2 (EIN2), and is negatively regulated by miR164. It has been shown that ORE1 controls the expression of 170 genes, including 78 SAGs (Balazadeh et al., 2010).

At chromatin regulating level, Brusslan et al., (2012) revealed by using chromatin immunoprecepitation sequencing (Chip-seq) and gene expression analyses that there is a genome-wide change in histone methylation which is associated with Arabidopsis leaf senescence. They found that genes with increased level of histone H3 trimethyl lysine 4 (H3K4me3), a mark of actively transcribed chromatin, are induced with leaf senescence. Whereas histone H3 trimethyl lysine 27 (H3K27me3), an inactive histone mark, showed contrary behavior.

Recently, small –interfering RNAs (siRNAs) and micro RNAs (miRNAs) have been reported to control mRNA stability. miR164 transcription gradually decreased with leaf aging, through the activation of EIN2, which results in upregulating of its target, ORE1 (Kim et al., 2009). In addition overexpressing miR319, targets TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP), delayed leaf senescence, partially by modulating JA biosynthesis (Schommer et al., 2008). Nevertheless, we currently have only an incomplete picture of molecular processes underlying leaf senescence, and not much is known about the regulation of leaf senescence at translational level. To figure this regulation will be an important step to understand the leaf senescence process. Therefore, to understand the senescence process might require a paradigm in analysing the process. Thus, it is crucial to use multiple ‘omics’ approaches, such as transcriptome, proteome, global metabolic rearrangement and phenome, coupled with computational modeling (Woo et al., 2013).

3.3. N assimilation and the plants response to N supply conditions

N is the most important macronutrient for plants growth and development. It is required for the synthesis of amino acids and nucleotides, as well as for the synthesis of phospholipids and many secondary metabolites. Plants obtain N as nitrate and ammonium, with organic amino acids also making a contribution (Miller et al., 2007). Annually, 85–90 million metric tons of nitrogenous fertilizers are added to the soil worldwide (Good et al., 2004). N fertilizers represent the major expenses in plant production. Furthermore, incomplete capture and poor conversion of nitrogen fertilizer also causes soil and water pollution, as well as

global warming through emissions of nitrous oxide. Lowering fertilizer input and breeding plants with better nitrogen use efficiency (NUE) is one of the main goals of research on plant nutrition (Hirel et al., 2007).

3.3.1. N transporters, metabolism and signaling

Plants uptake N form compounds through special transporters in root. Though, the preferred form of N is taken up depends on plant adaptation to soil condition, such as soil pH. Plants prefer nitrate when are grown in high pH condition (Maathuis, 2009). Four transport systems have been found for nitrate uptake in plants. NRT1 genes family is low-affinity transport system (LATS). 53 genes belonging to this family were found in Arabidopsis, and show expressed pattern in different plant tissues (Tsay et al., 2007). While, high-affinity transport system (HATS; NRT2, seven members) genes family is acting when the nitrate concentration in soil is low (William and Miller, 2001). In addition, chloride channels (CLC, seven members) and slow anion channel- associated 1 homologues (SLAC1/SLAH, five members) also can transport nitrate (reviewed by Krapp et al., 2014). On other hand, 6 genes for ammonium transporters (AMTs) were found in Arabidopsis (Gazzarini et al., 1999), and 10 in rice (Sonoda et al., 2003).

After the N taken up by plant through roots, the N is assimilated in roots or transferred to shoot as inorganic (nitrate and ammonium) compounds, where it is assimilated to the organic compounds. The first step of N assimilation is to convert nitrate to nitrite, which is catalyzed in the cytosol by nitrate reductase (NR) enzyme (Meyer and Stitt, 2001). Then after, nitrite is translocated to the chloroplast where it is reduced to ammonium by nitrite reductase (NiR), followed by assimilation of ammonium into glutamate and glutamine in the chloroplast by GS/GOGAT cycle (Fig.3.2) (Lea and Forde, 1994). 2-oxoglutarate react with ammonium to synthesize glutamate, which is catalyzed by glutamate synthase (GOGAT). Two classes of nuclear genes code for glutamine synthetase (GS), known as GS1 and GS2. The chloroplastic GS2 is involved in the primary N assimilation, and in the re-assimilation of ammonium produced from photorespiration. Conversely, the cytosolic GS (GS1) is involved

in ammonium recycling during leaf senescence (Fig.3.2) (Bernard and Habash, 2009). Furthermore, glutamate can be the N donor to synthesize the other amino acids and other N containing compounds such as chlorophyll.

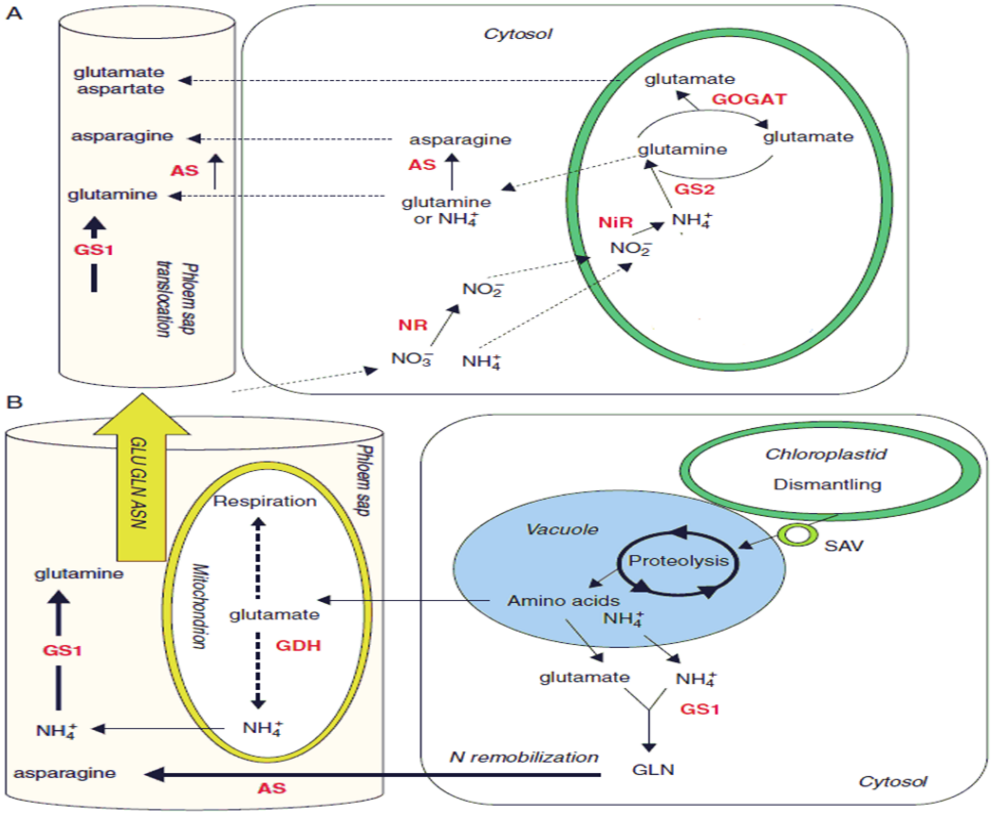

Figure 3. 2. Schematic presentation of key enzymes involved in nitrogen management in (A) young and (B) senescing leaves. (A) Nitrate reductase (NR) and asparagine synthetase (AS) are localized in the cytosol, and nitrite reductase (NiR), glutamine synthetase 2 isoenzyme (GS2), glutamate synthase (GOGAT) 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 senescence-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. (Masclaux-Daubresse et al., 2010)

N uptake and assimilation is highly regulated to match the demand of whole plant. Nutrient status is sensed by special receptors, and the activity of transporters and metabolic enzymes is modulated. N status can be detected directly by nitrate or endogenous N assimilates such as amino acids. It was found that under N deficiency condition many of ammonium and high-affinity

nitrate transporters is transcriptionally induced, while several NRT2 and AMT1 transporters were transcriptionally repressed by N metabolites such as amino acids (reviewed by Tsay et al., 2007; Meyer and Sitt, 2001). Using global transcription analyses after nitrate feeding (Scheible et al., 2004) confirmed that N uptake and assimilation is directly regulated by nitrate. And by using nitrate reductase null mutants, it was able to confirm that much of this regulation is regulated by nitrate itself (Wang et al., 2004). Nitrate as a signal should be identified by special sensor. The nitrate transporter (NRT1.1/CHL1) was shown to play, in addition to its transporter function, also role as such a nitrate sensor (Wang et al., 2009). Plant can response to availability of nitrate in minutes. This early responses of roots (3-9 min) involved genes are required to set up the condition for using or reducing nitrate, such as ribosomes and the oxidative pentose phosphate (OPP) pathway, and after an early nitrate-specific response (up to 9 min), interactions with other signals such as hormones occur (Krouk et al., 2010b). However, none of global transcriptome analyses performed has identified nitrate-responsive promoter elements (Krapp et al., 2014). It has been characterized recently that NIN-like protein (NLPs) transcription factor is a master regulator of nitrate signal (Marchive et al., 2013). Other transcription factors involved in nitrate signaling such as the squamosa promoter-binding-like protein 9 (SPL9) (Krouk et al., 2010b), MADS box transcription factor ANR1 (Zhang and Forde, 1998) or the LOB domain-binding proteins LDB37/38/39 (Rubin et al., 2009) are involved in the regulation of nitrate-related response. Nevertheless, the putative ionotropic glutamate receptor, 1.1 (AtGLR1.1), and isopentenyl transferase 3 (AtIPT3) of Arabidopsis thaliana, which is a key enzyme of cytokinin biosynthesis, are two factors that are thought to link nitrogen nutrition to hormonal signaling (Weaver et al. 1998; Kang & Turano 2003; Takei et al. 2004). But the exact mechanisms of this link are still unknown.

3.3.2. Effect of N deficiency on plant development and leaf senescence

N is a macronutrient in plants and a limiting factor for their growth and development. When N supply is limited, the plant “tries” to be more efficient in N uptake and assimilation. In addition, plants have a strategy to induce under

these conditions senescence in older leaves to recycle N and translocate it to sink tissues. This allows the plants to be more efficient and fit under unsuitable environmental conditions. Plants respond to nitrogen availability by changing their root/ shoot ratio, and the resources favored to be remobilized from shoot to roots. This allows the plants to cover more soil area by their roots and search for more N resources (Shangguan et al., 2004; Agren and Franklin 2003). Moreover, this phenotype could be controlled by sugars, auxin, and CK (Forde 2002).

It is well reported that N deficiency induces leaf senescence, though leaf senescence is delayed when N is supplied (Egli et al., 1976; Schildhauer et al., 2008; Gregersen et al., 2013; Balazadeh et al., 2014). But not much is known about the metabolic and complex regulatory network of N induced leaf senescence in the model plant *Arabidopsis thaliana*, and in economically important plants like crop plants. It is not clear if the senescence signal is mainly established in old leaves or is a systematic signal form starved sink tissues.

N and carbon (C) metabolism are closely connected. For example, carbon acts as skeleton for amino acids and other N containing compounds. N deficiency causes high sugars accumulation, and many studies showed that sugars modulate N uptake and metabolic enzymes, but it seems to be independent of the known sugar signaling pathways, such as hexokinase (Lillo, 2008). In this condition the C/N ratio appears to be a major signal affecting plant development and leaf senescence (McAllister et al., 2012; O'Hara et al., 2013). Schildhauer et al., (2008) showed that the time of nitrogen supply regulates the course of leaf senescence in flag leaves of *Hordeum vulgare*. Although both, addition of nitrate or ammonium, effectively reversed nitrogen depletion-induced primary leaf senescence, addition of urea did not. But to identify key players of the N-dependent regulation of leaf senescence and for making a comparative study with Arabidopsis regulation (Balazadeh et al., 2014), we study the wider transcriptomic, hormonal, and metabolic rearrangement during reversal of barley leaf senescence by N resupplied.

3.4. Sugars signaling in integrating environmental signal

The sugars levels in leaves are affected by many environmental factors including CO_2 concentration, light, and biotic stress, as well as abiotic stress, such as insufficient nutrient supply and drought. Plants have mechanism to regulate the photosynthetic rate in response to environmental factors and sink demand. It is known that photosynthesis genes are sugar-repressible (Jang and sheen, 1994). High CO_2 concentration suppressed photosynthesis mostly when combined with low N condition. Paul and Driscoll (1997) suggested that sugar repressed photosynthesis may depend more crucially on C/N ratio of leaves, rather than sugar status alone. It was found that glucose is a more potent signal in regulating transcription of genes related with N metabolism than N itself (Price et al., 2004). In addition, sugars induced GS1 gene, which is involved in N remobilization during senescence (Wingler et al., 2006).

Leaf senescence can be triggered by two conditions related to sugar levels, higher carbohydrate accumulation or sugars starvation by dark. And to figure if developmental senescence is related to sugar accumulation or starvation, a comparison of the global transcriptomic data of Arabidopsis plant found difference between developmental and sugar starvation-induced senescence (Buchanan Wollaston et al., 2005). For example, the pathway of nitrogen remobilization during senescence process differed in dark-induced and developmental senescence. While glutamate decarboxylase and GS1 genes were induced in developmental senescence, asparagine synthetase and glutamate dehydrogenase genes were induced in dark-induced senescence. However, there are differences in this regulation between plant species or even between cultivars. For example, sugar levels are described to fall during late senescence as shown in tobacco (Masclaux et al., 2000), while hexoses accumulate until late senescence stages in Arabidopsis leaves (Stessman et al., 2002). Moreover, it has been argued that early SAGs are sugar-inducible, whereas late SAGs are sugar-repressible (Paul and Pellny, 2003). It has been shown that phloem blockage by callose deposition could lead to an age-dependent sugar accumulation (Jongebloed et al., 2004). However, it is not

clear how the export of amino acids is achieved during leaf senescence. In addition, steam girdling of barley leaves led to carbohydrates accumulation and induced leaf senescence (Parrott et al., 2007).

It was shown that developmental and external supplied glucose induced- leaf senescence of Arabidopsis was delayed in hexokinase-1 (hxk1) mutant, indicating that HXK1 is involved in sugar-induced leaf senescence (Moore et al., 2003). However, there is crosstalk between sugar and ABA signaling in controlling many developmental processes. It has been shown that ABA is not required for sugar signaling in the regulation of leaf senescence (Pourtau et al., 2004). Although, transcription factor AB15, involved in ABA signaling, could play a role in interaction of these two signals. The finding that the overexpression of an extracellular invertase gene under control of the senescence-induced SAG12 promoter delayed leaf senescence in transgenic tobacco (Nicotiana tabacum) plants, This coincided with the finding that elevated cytokinin production correlates with an increased extracellular invertase activity. These results demonstrate that extracellular invertase activity is required for the delay of senescence by cytokinins (Balibrea Lara et al., 2004).

Sucrose-nonfermentation1-related protein kinase1 (SnRK1) is an energy sensor protein that modulates developmental processes through regulating gene expression for metabolic rearrangement in response to sugar status in plants. Moreover, SnRK1 modulates stress-inducible gene expression and the induction of stress tolerance in Arabidopsis and rice (Cho et al., 2012). It was shown that phosphate starvation and trehalose-6-phosphate (T6P) application inhibit the activity of AtSnRK1 (Fragoso et al., 2009; Zhang et al., 2009). Cho et al., (2012) showed that the leaves of transgenic Arabidopsis expressing wild-type OsSnRK1 and KIN10 senesced more slowly compared to the wild type plants. While transgenic plants expressing inactive forms of SnRK1s (OsSnRK1_IN and KIN10_IN) displayed accelerated leaf senescence.

Recently, a sugar compound (trehalose-6-p (T6P)) received more attention to have an important role in modulation of leaf senescence, which is considered

to be a signal for carbohydrate availability in plant cells. In T6P phosphatase gene (otsB)-expressing Arabidopsis plants, T6P accumulated less strongly during senescence than in wild-type plants. These plants showed a similar phenotype as described for plants overexpressing the SnRK1 gene, KIN10, including reduced anthocyanin accumulation and delayed senescence (Cho et al., 2012; Wingler et al., 2012), but T6P doesn't inhibit SnRK1 activity to the same extent in mature and senescing leaves as in young leaves (Zhang et al., 2009; Wingler et al., 2012). Nevertheless, the plants with altered T6P content showed more obvious phenotypic differences during the later developmental stages of leaf senescence. It was interesting to observe that the senescence was delayed in otsB –expressing lines independent of the higher glucose, fructose, and sucrose levels compared to wild type plants. Thus, this indicates that T6P is required, and hexoses accumulation alone may not suffice to induce the senescence program (Wingler et al., 2012).

3.5. Sink/source interconnection through systemic signals

Plants optimize their developmental processes in relation to nutrients supply and sink demand to assimilates. The adjustment of plant growth, root/shoot ratio, flowering time, and development under different environmental conditions needs a complicated systemic signaling network and a close coordination between sink and source organs during plant development to ensure the highest adaptation mechanisms. This includes various molecular signals which can be transferred through the phloem and xylem, such as phytohormones, sugars, amino acids, and miRNAs. Novel local and systemic signals that adjust root and shoot development in response to N and P starvation were recently discovered (Puig et al., 2012). Cytokinins (CKs) are mainly synthesized in roots and considered as root-to-shoot signal that can control the shoot development (Domagalska and leyser, 2011). While auxin is produced in shoot and transported to roots where it regulates root development (reviewed by Coudert et al., 2010; Puig et al., 2012). Many of the systemic signals between root and shoot were studied and confirmed by using grafting experiment, such as MORE AXILLARY GEOWTH (MAX) which inhibits axillary development (Ongaro and

Leyser, 2008; Dun et al., 2009) and BYPASS1 that regulates shoot development (Van Norman et al., 2004).

Nitrate status in plant can be regulated by local and/or by systemic signals depending on its supply and the demand from the different plant organs. When the plant was grown in heterogeneous N content or split-root experiments, initiation and development of lateral roots toward the higher nitrate concentration was stimulated (Zhang and Forde, 2000; Wang et al., 2002). In addition, another systemic signal from shoot is needed to consider the high demand for N. Interestingly, it was shown that NRT1.1 is able to transport auxin, in addition to nitrate, which at higher nitrate concentration, nitrate competes with the auxin and prevents basipetal auxin transport out of the lateral roots, which auxin accumulated and stimulates lateral root growth (Krouk et al., 2010a). Moreover, when plant is repleted with high nitrate, it was suggested that high nitrate concentration inhibits through systemic signal pathway the translocation of auxin from shoot to roots, which inhibits the lateral roots formation (Wlach-Liu et al., 2006). N metabolites, such as glutamate have been also proposed to be involved in shoot-to-root signals that regulate root development and nitrate assimilation in roots (reviewed by Miller et al., 2007). While, sugars transportation levels from leaves can be a systemic signal to roots, which induce nitrate and ammonium transporter genes (Lejay et al., 2003).

Sink activity required different types of resources include the photoassimilates, which are supplied by source organs. Under sink limitation in co-ordination with altered whole plant carbon to nitrogen balance, carbohydrates start to accumulate in leaves, which leads to decreased expression of photosynthetic genes and induce leaf senescence. In addition, sugars accumulation also represses the expression of sucrose transporters (Chiou and Bush, 1998). This interrelationship between sinks and sources establishes a balance between photosynthesis and use by growth processes. Plants that can increase sink size such as potato suffer less from the photosynthetic end-product accumulation and from its feedback inhibition (Paul and Foyer, 2001).

Improvement in crop yields during twentieth century have been achieved through altered assimilates partitioning and increased harvest index without increase in overall biomass (Paul and Foyer, 2001). This opens a discussion for the efficient strategies, through increased carbon fixation per unit leaf area or increased sink activity, should be used for further increase in crops yields. Nevertheless, improvement of crop yield depends on our understanding of the nature of the regulation mechanisms for photosynthetic rate and sink/source interconnection coupled with better understanding for environmental and metabolic factors.

3.6. Phytohormones regulate plant development including leaf senescence

Plant adaptation to different environmental stresses is determined by hormonal signaling pathways that allow the co-ordination of shoots and roots growth. The regulation of senescence by cytokinin and ethylene is conserved; however, the action of other hormones varies between plant species (Schippers et al., 2007). Abscisic acid (ABA) and cytokinins (CKs) has been considered as signals to mediate leaf senescence. While ABA was shown to induce senescence in natural and stress- induce leaf senescence (Pourtau et al., 2004; Schippers et al., 2007), CKs could delay leaf senescence (Gan and Amasino, 1995). Four approaches are used to identify the enzymes and regulatory factors related to phytohormones biosynthesis and response including genetic, biochemistry, cell biology, and bioinformatics approaches.

3.6.1. Cytokinins biosynthesis and signaling

Cytokinins (CKs) can be found in plants naturally as isoprenoid and aromatic forms. Isoprenoid CKs are naturally available in plant as N^6-(Δ^2-isopentenyl)-adenine (iP), trans-zeatin (tZ), cis-zeatin (cZ), and dihydrozeatin (DZ). The most abundant among them generally are tZ and iP as well as their sugar conjugates, but there is a lot of variation depending on plant species, tissue, and developmental stage (Sakaibara, 2006). For example, tZ- and iP-type CKs are the most abundant forms in Arabidopsis, whereas substantial amount of cZ-form is found in maize (Veach et al., 2003).

CKs biosynthesis starts by N-prenylation of adenosine 5'-phosphates (AMP, ADP, or ATP) at the N^6-terminus with dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP); that catalyze by adenosine phosphate-isopentenyltransferase (IPT). And riboside 5'-phosphates (iPRMP, iPRDP, or iPRTP) are produced from this reaction. The spatial expression analyses of AtIPTs genes revealed tissue-specific patterns (Miyawaki et al., 2004). tZ can be biosynthesized in plants in two pathways, the iP nucleotide-dependent and the iP nucleotide-independent. In the iP nucleotide- dependent pathway, tZ is synthesized by cytochrome P450 monooxygenase that mainly expressed in roots. Recently, two such enzymes were identified in Arabidopsis, CYP735A1 and CYP735A2 (Takei et al., 2004) (Fig.3.3). In the iP nucleotide-independent pathway, it is assumed that tz nucleotide is produced directly by IPT using an unknown hydroxylated side-chain precursor (Åstot et al., 2000; reviewed by Sakakibara, 2006). Since the CKs play a crucial role in plant signaling and development, the steady-state of active CKs levels is determined by glycosylation and degradation. O-glucosylated CKs is a storage inactive CKs forms in plant tissues, and this glucosylation is reversed by β-glucosidase (Brzobohaty et al., 1993). On other hand, CKs are irreversibly degraded by cleavage the side chain, which is catalyzed by CK oxidase (CKX) (Armstrong, 1994). In barley plant, silencing HvCKX1 gene decreased the CK oxidase/dehydrogenase level and led to a higher plant yield and root weight (Zalewski et al., 2010).

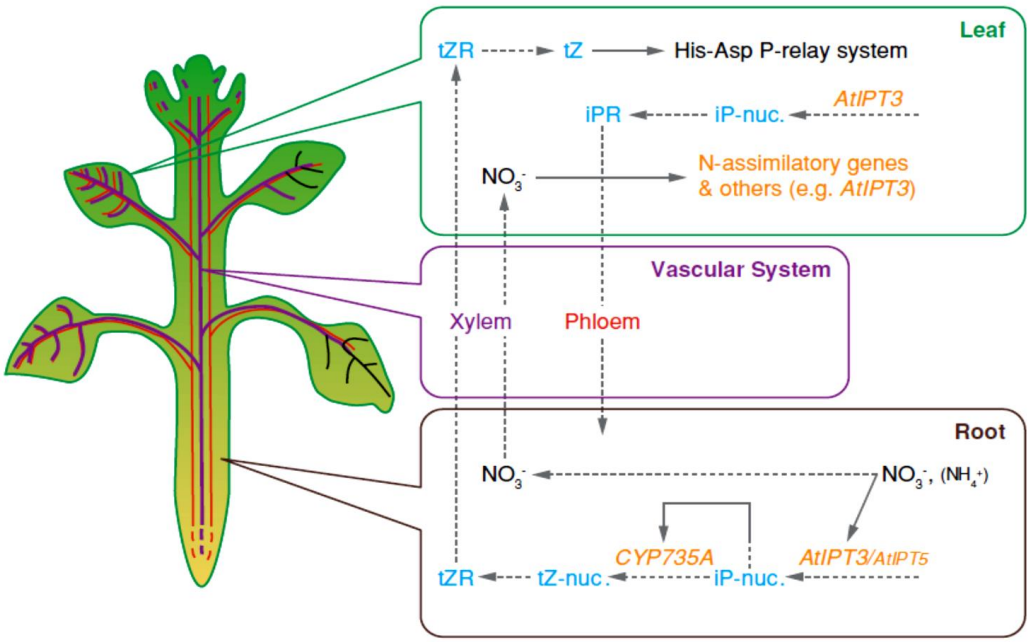

Figure 3. 3. Nitrogen-dependent regulation of cytokinin (CK) biosynthesis and root/shoot communication via xylem and phloem. Solid arrows indicate positive regulation of gene expression. Broken lines with arrowheads show metabolic flow or translocation of CKs. Xylem stream and phloem stream are indicated in violet and red lines, respectively. iP-nuc., iP nucleotides; tZ-nuc., tZ nucleotides. (Sakakibara, 2006).

CKs play as systemic signal, which is transported between different plant organs through xylem and phloem (Fig.3.3), and it was reported that iP- and tZ-type CKs accumulate in phloem and xylem, respectively, which play a role in systemic signals to control roots and shoot growth and development (Corbesier et al., 2003; Lejeune et al., 1994; Takei et al., 2001). Samuelson and Larsson (1993) showed that the root zeatin riboside (ZR) level of N-limited barley is largely non-responsive to nitrate dose in the long term, but positively responded to increased nitrate supply. The authors also showed that pretreatment the barely plant with cycloheximide inhibited nitrate-induced ZR response, but the level of ZR wasn't affected. This means N metabolites are required for this response. In addition, it was considered that the nucleosides (CKs riboside; as tZR and IPR) are the major translocation form of CKs (Hill-Cottingham and Lloyd-Jones, 1968). Foo et al., (2007) showed by using grafting method between ramosus (rms2) mutant and wild type plants that the systemic

feedback from shoot to root, which regulates the xylem sap CKs export, is conserved between pea and Arabidopsis plants. Two groups of plant gene families were shown to act in transport of CKs and their ribosides forms, equilibrative nucleoside transporter (ENT) family in rice (Hirose et al., 2005) and purine permeases (PUPs) in Arabidopsis (Burkle et al., 2003). Nevertheless, more physiological characterization is needed to understand their role in CKs transportation and plant development. It is worth to mention, it was shown that a decrease in zeatin riboside (ZR) content and increase in ABA content were observed earlier in roots than leaves for rice plants during grain filling, which may cause the decline of leaves photosynthetic function (Shu-Qing et al., 2004).

The expression of the different genes related to CK biosynthesis and homeostasis is modulated by phytohormones such as auxin and abscisic acid (ABA), as well as CKs itself. In Arabidopsis roots, auxin induced the transcription of AtIPT5 and AtIPT7, whereas CKs negatively regulated the expression of AtIPT1, AtIPT5, and AtIPT7 (Miyawaki et al., 2004). Auxin and ABA downregulated the expression of the CYP735A1 and CYP735A2 in roots. But they were upregulated by CKs (Takei et al., 2004). Moreover, ABA and CKs upregulated genes for CKX in maize (Brugiere et al., 2003). Up to now, it is known that CK induces autophosphorylation of histidine kinase (AHK) receptors, which is considered as two-component signaling pathway. The receptors show differing sensitivity to different CK types (Romanov et al., 2006). The phosphoryl is then transferred to a histidine phosphotransferase (HP) protein, and then activates a response regulators (ARRs) (reviewed by El-Showk et al., 2013). In Arabidopsis, 23 response regulators (RRs) were found, which divided into three groups, two of them (type A and B) are involved in CK signaling. It was shown that the transcription of type A ARRs are upregulated by CKs (D'Agostino et al., 2000).

It was shown that CKs could delay leaf senescence (Gan and Amasino, 1995). The application of CKs and increasing its production could delay leaf senescence (Richmond and Lang, 1957; Ori el al., 1999), as well as reducing

CKs levels accelerated leaf senescence (Masferrer et al., 2002). In addition, type–A ARRs and CKs biosynthesis genes were transcriptionally downregulated during leaf senescence (Buchanan-Wollaston et al., 2005). While the most evident for the direct role of CKs in delaying leaf senescence than the effect of whole plant development was by inducing IPT expression under SAG12 promotor in transgenic tobacco plant, which the CKs biosynthesis was increased only when SAG12 was induced after initiation senescence syndrome (Ori el al., 1999; Gan and Amasino, 1995). However, CKs no longer can inhibit leaf senescence in SAG12-IPT line, when extracellular invertase activity is inhibited (Balibrea Lara et al., 2004). This means that one way of delaying leaf senescence by CKs could be through regulation of invertase enzymes activity. But the whole picture of CKs signaling and its complex pathways is still unclear and needs more studies to be elucidated. Hoth et al., (2003) showed that 823 up- and 917 downregulated in Arabidopsis seedling stage after 24h of endogenous IPT induction.

3.6.2. ABA biosynthesis and signaling

ABA was identified in early 1960s, which regulates several aspects of plant growth and development, such as embryo maturation, seed dormancy, stomatal closure, floral induction, and external biotic and abiotic stress responses, as well as promoting senescence syndrome. The level of biological active ABA in plant cell is modulated by a balance of biosynthesis and inactivation by reversal conjugation and irreversible degradation, as well as by compartmentation and transport. The biosynthetic pathway for ABA was first identified by using radioactive of density-labeled precursors (reviewed by Zeevaart and Creelman, 1988). While the genetic and molecular tools helped us to identify the enzymes, catalyzing the different steps in ABA biosynthesis. The ABA biosynthetic pathway starts in plastids with MEP pathway, which then after the carotenoids is produced (Fig. 3.4). Only β-carotene is further metabolized to ABA via zeaxanthin, which is catalyzed by β-carotene hydroxylases. Zeaxanthin epoxidase (ZEP), encoded only by ABA1 single locus in Arabidopsis, converts zeaxanthin to violaxanthin via the intermediate compound antheraxanthin (Fig.3.4; reviewed by Finkelstein, 2013). 9-cis-

violaxanthin is converted to xanthoxin in plastids, and this reaction is catalyzed by a rate limiting enzyme in ABA biosynthesis, 9-cis-epoxycarotenoid dioxygenase (NCED).

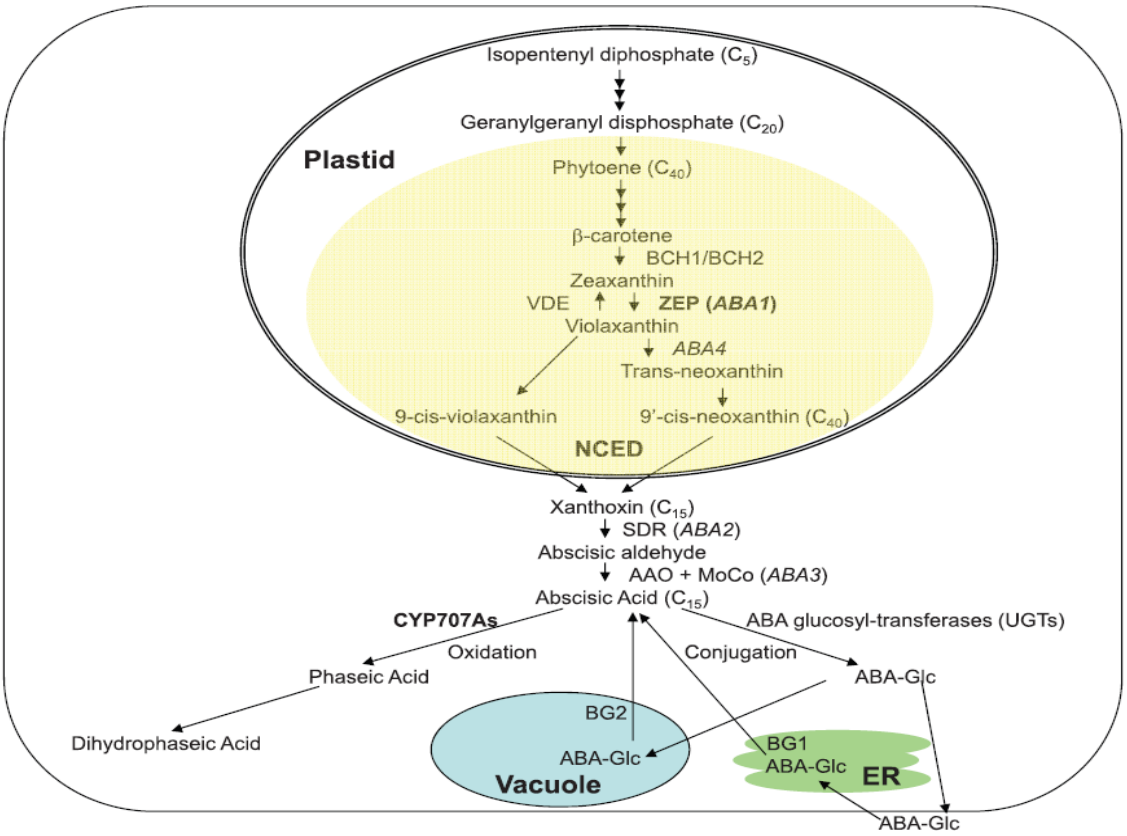

Figure 3. 4. ABA metabolic pathways. ABA biosynthesis, degradation and conjugation pathways are shown in relation to the cellular compartments. Carotenoid intermediates are highlighted in yellow. Enzymes regulating key regulatory steps are shown in bold. Individual loci identified based on ABA deficiency are shown in italics. (Finkelstein, 2013).

Then xanthoxin is transported to the cytosol and converted to ABA via the intermediate abscisic aldehyde. The final step is catalyzed by abscisic aldehyde oxidase (AAO). ABA is synthesized in vascular tissues of roots and shoots, which transported in xylem and phloem. Drought stress results in an increased in ABA transportation from the roots to shoot leading to reduce the transpiration in leaves (reviewed by Davies and Zhang, 1991). Moreover, ABA-conjugate concentrations increased in barley xylem sap under salinity (Dietz et al., 2000). Nevertheless, it was argued that hydraulic signals reflecting changes in water potential due to soil and root drying can induce local biosynthesis of ABA in

leaves before transport from roots is observed (Christmann et al., 2005; Christmann et al., 2007).

The active ABA level is regulated by catabolism, which ABA is hydroxylated at the 8' position by P-450 monoxygenases (CYP707A; ABA-8'-hydroxylases) to produce unstable intermediate compound 8'-OH-ABA that isomerized to phaseic acid (PA), which may then converted to diphaseic acid (DPA) (Fig.3.4). 4 genes of ABA-8'-hydroxylases in Arabidopsis show different spatial, temporal, and stresses patterns of expression. Interestingly, ABA was accumulated higher in ABA-8'-hydroxylases mutant than ABA biosynthesis overexpressing lines. Another mechanism is esterification of ABA to form ABA-glucose ester (ABA-GE) (reviewed by Finkelstein, 2013). While ABA-GE is cleaved by β-glucosidases (BG1: AT1G52400) that are activated by dehydration-induced polymerization (Lee et al., 2006). The activity of β-glucosidase was strongly increased under salinity stress in barley plants (Diez et al., 2000).

Whereas the possibility for existence of extracellular perception came from studies using ABA-protein conjugates, unable to enter the cell, but it is still biological active and modulates ABA-induced genes expression (Jeannette et al., 1999). Many approaches were used to identify the ABA receptors. Up to now, the best identified receptor that might bind to ABA is PYRABACTIN RESISTANT 1 (PYR1) (Park et al., 2009). Thirteen members of this family were found in Arabidopsis (Zhang et al., 2013). ABA binding to receptors causes inhibition of SNF1-related kinases (SnRKs), which induces ABA-response genes such as, ABA insensitive transcription factors, ABI3, ABI4, and ABI5 (reviewed by Finkelstein, 2013). 1-10% of the plant genome is ABA-regulated in any given experiment, nearly 6000 ABA-induced genes (Choudhury and Lahiri, 2010; Wang et al., 2011). In addition, it was shown that ABA also binds to plastid-localized receptor called Mg-chelatase H subunit (CHLH) in Arabidopsis (Shen et al., 2006), but not in barley (Müller and Hansson, 2009).

When plants are faced severe drought stress, high ABA inhibits both roots and shoots growth, but promotes lateral roots formation (Varanian et al., 1994). In contrast, mild drought stress slightly elevated ABA level, which promotes roots

growth and inhibit shoot growth, leading to higher root/shoot ratio (reviewed by Moriwaki et al., 2012). ABA levels and the genes encoding its biosynthetic enzymes, NECD and AAO, increase during developmental- or stress-induced senescence (reviewed by Lim et al., 2007). Recently, it has been argued that a receptor kinase (RPK1) mediates ABA-induced senescence of old leaves (Lee et al., 2011). In addition, abi5 mutant showed delayed leaf senescence under low N and high sugar conditions (Buchanan-Wollaston et al., 2005). Interestingly, it seems that ABA modulates a balance between senescence program and protection activity such as induces expression of antioxidant genes and inhibiting ethylene production under drought stress (reviewed by Lim et al., 2007; Sharp, 2002).

ABA content wasn't changed in root of ammonium, and low N stressed pea plant. However, the ABA level and AAO activity increased in the leaves especially in old ones in the absence of both increased xylem loading and enhanced AAO activity in roots (Zdunek and Lips, 2001). In addition, tZR delivery rate into xylem was decreased under these stresses. The activity of AAO in barley roots was enhanced in plants grown with ammonium comparing to nitrate-grown plants. While ABA concentration increased in leaf of ammonium-grown plants in absence of a significant increase of leaf AAO activity (Omarov et al., 1998). This opened the discussion about if the involvement of ABA in systemic signaling under N stresses is conserved in the different plant species.

3.7. Aim of present work

Leaf senescence is one of the fundamental biological questions and was a puzzle for centuries. Leaf senescence, even under ideal growth conditions, can be regulated by internal factors (e.g. epigenetic controller and hormones) dependent on developmental age. This regulation includes global changes in gene transcription and metabolism to achieve the highest remobilization efficiency (Buchanan-Wollaston et al., 2005). N deficiency can also prematurely trigger the senescence program. However, program of N deficiency- induced senescence can be delayed when N is resupplied to N starved plants (Schildhauer et al., 2008). But not much is known about the metabolic and complex regulatory network of N availability on leaf senescence in the model plant *Arabidopsis thaliana*, and in economically important plants like crop plants. It is not clear if the senescence signal is mainly established in old leaves or is a systematic signal form starved sink tissues. Based on the hypothesis that N status modulates senescence program in old leaves via its impact on global transcriptomic, phytohormonal, and metabolic rearrangement in connection with whole plant development, it was the aim of present thesis:

1) To investigate the role N Status (N deficiency and N resupply) in global transcriptomic, phytohormonal, and metabolic rearrangement.
2) To identify signals involved in delaying of leaf senescence by N resupply.
3) To investigate phloem export activity under natural and N deficiency-induced senescence.
4) To investigate the different effect of N status on old and young leaves.

For this purpose, wild type barley genotype was grown hydroponically, and was treated by three different N regimes. This includes optimum amount (control), low N amount, and optimum amount of N was resupplied to N starved plants. Further the primary and 4^{th} leaves were analyzed to transcriptomic, phytohormonal, and metabolic as shown in scheme (Fig.3.5). In addition, the phloem exudate of primary leaf was collected in EDTA solution and was analyzed to sucrose and amino acid levels.

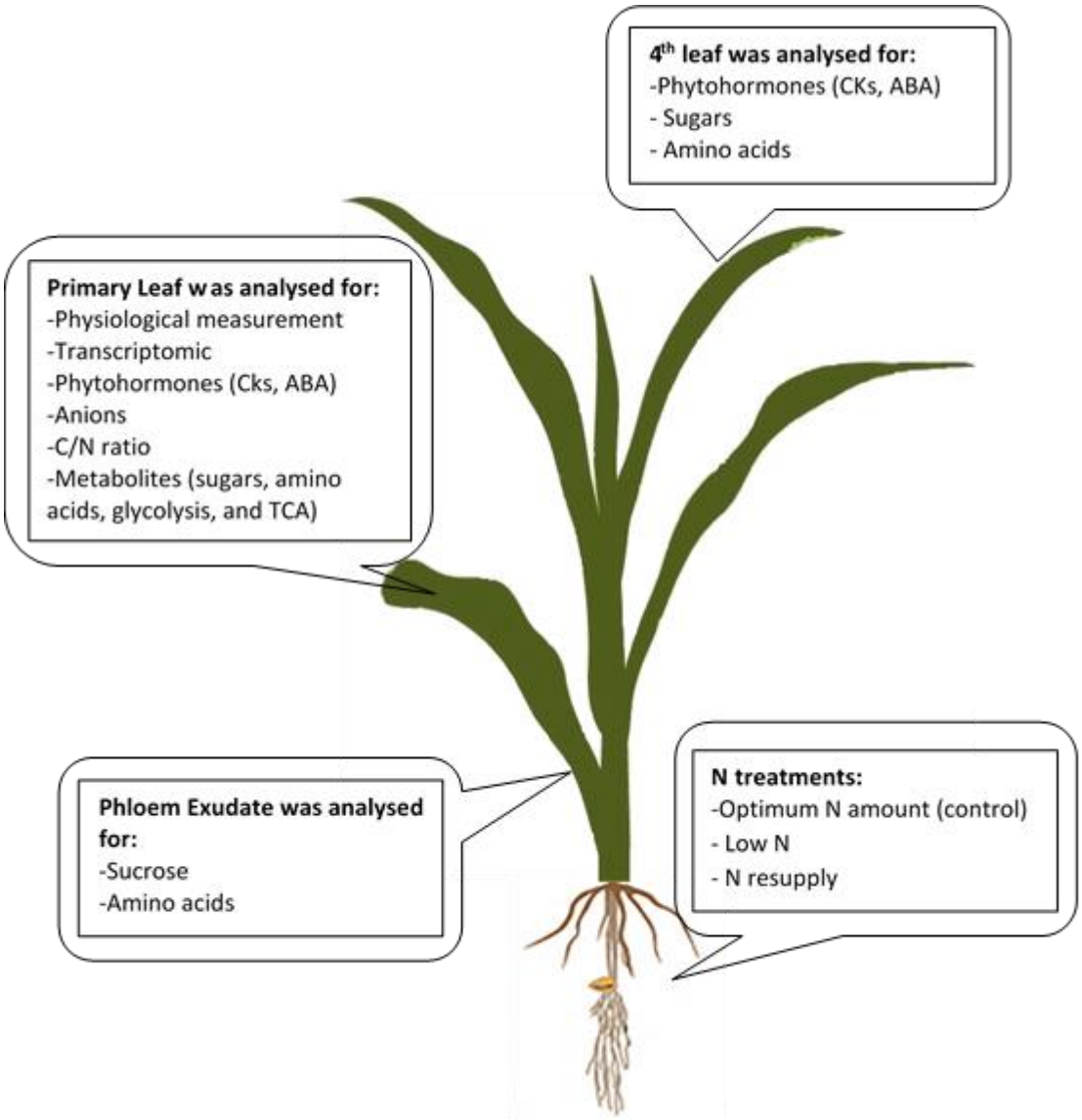

Figure 3. 5. Schematic representation of this thesis work. This includes three N regimes, sampling tissues (primary leaf, 4th leaf, and phloem exudate of primary leaf), and the analyses that have been carried out.

4. Materials and Methods

4.1. Plant culture and sampling

Barley seeds (*Hordeum vulgare* L. cv Golden Promise) were germinated and grown in 0.5X Hogland solution, at 8 day after germination (DAG) the seedlings were transferred to 5 liter pots supplied Hogland nutrients (10 plants each; Fig.4.1). A group of plants were supplied with full Hogland solution including 2 mM $Ca(NO_3)_2$, 0.5 mM K_2SO_4, 0.5 mM MgSO4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 1.0 µM H_3BO_3, 2.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, and 100 µM Fe(III)-EDTA. This group of plants will be mentioned as control. While other group of barley plants were treated with N deficiency condition (0.06 mM $Ca(NO_3)_2$) and $Ca(NO_3)_2$ was replaced by $CaCl_2$. The N deficient plants were kept for 15 d in order to induce leaf senescence syndrome (Fig.5.1). After N deficiency-induced senesces initiated, a group of 10 d N deficient plants were resupplied at 18 DAG by optimum amount of N (2 mM $Ca(NO_3)_2$) (Fig.5.1). The hydroponic system was permanently aerated and maintained in a controlled-environment chamber at 70% humidity, 8 h darkness at 18°C, and 16 h light at 210 µmol photons m^{-2} s^{-1} and 20°C. The Hogland medium was changed every three days (2 d before harvesting). The primary and 4th leaves were harvested (after 8 h of light) in three days interval, and stored at -80°C for further analyses.

Figure 4. 1. Plant culture in hydroponic culture. The seedlings were grown in 5 liter pots supplied Hogland nutrients (10 plants each).

4.2. Chlorophyll content and root: shoot ratio

Relative chlorophyll content per unit at the middle of leaf area was determined using a SPAD (Soil Plant Analysis Development) analyser (Min-olta, by Hydro Agri, Dülmen, Germany) which measures trans-mission of wavelengths lengths (650 and 940 nm, measuring area = 6 mm^2) absorbed by chlorophylls in intact leaves. Each data point represents the mean value of 10 independent measurements. For root: shoot ratio calculation, the plant materials were dried out at 80 ºC for two days and then they were weighted.

4.3. RNA isolation

At each defined time point, 2ml Eppendorf tube was filled up to 0.5 ml by homogenized grinded primary leaves material for RNA preparation. Total RNA was isolated from leaves with a TRIzol-based method according to Chomczynski & Mackey (1995) and quantified NanoDrop-spectrophotometer (NanoDRop Technologies Inc., USA). TRIzol reagent (38% phenol, 0.8 M guanidinium thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, pH 5, 5% glycerol) was used. To verify the quality of RNA, 1 µg of total RNA was fractionated on a 1% (w/v) TAE agarose gel, stained with ethidium bromide and visualized under UV light.

4.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated as described above and treated with RNase-free DNaseI (MBI Fermentas, St Leon-Rot, Germany). One microgram of total RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a volume of 20 µl to generate first-strand cDNA, according to the supplier's instructions. PCR was performed in the iCycler (BioRad, Munich, Germany) in a total volume of 15 µl, including 1X Platinium® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 0.3 µM of each gene-specific primer and 10 µM fluorescein (BioRad) as passive reference dye for well factor calibration. The following PCR program was used: 2 min incubation at 50°C, 1 cycle at 95°C for 2 min to activate the included HotStart-Taq- Polymerase, followed by 40 cycles at 95°C for 15 s, 58°C for 15 s and extension phase at 60°C for 15 s. Subsequent to the normal PCR, determination of a melt curve of the amplified PCR products was carried out. The relative expression rate of

genes of interest in senescing leaves and N supply treatment relative to the mature controls at 11 DAG was calculated as described by Pfaffl (2001). Each data point is based on 3-7 biological replicates. Data were normalized to the reference gene actin expression; therefore, the primers list is mentioned in appendix.

4.5. Microarray analysis

4.5.1. Array design

The array has been designed as was described in Kohl et al., (2015). Transcript data from HarvEST assembly 35 (www.harvest.ucr.edu), two RNAseq experiments, and a full-length cDNA collection were assembled to 46114 unique barley contigs using TGICL pipeline (http://compbio.dfci.harvard.edu/tgi/) as described previously (Kohl et al., 2012).Unambiguous 60 bp oligomer probes were derived using eArray (Agilent Technologies, Santa Clara, USA) and a part of this probe set was replicated.

4.5.2. RNA labelling, and array hybridization

RNA integrity was confirmed using the Bioanalyser system (Agilent Technologies). 100 ng RNA was used for cRNA synthesis and Cy3-labelling with a Low Input Quick Amp Labelling Kit (Agilent Technologies). Labelling efficiency, and amount and quality of cRNA, were assured using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and Bioanalyser system. 600 ng labelled cRNA was used for fragmentation and array loading (Gene Expression Hybridization Kit, Agilent Technologies). Hybridization was done for 17 h at 65°C. After washing (Gene Expression Wash Buffer Kit, Agilent Technologies) and drying, arrays were scanned at 5 µm resolution using an Agilent Technologies Scanner G2505C. Resulting images were evaluated (determination of spot intensities, background correction) with Feature Extraction V11.5 (Agilent Technologies).

4.5.3. Data evaluation

Data evaluation was done with Genespring V12.5 (Agilent Technologies). Values were $\log_2$ transformed and quantile normalized, before relative expression values were calculated by subtracting the median expression of each probe from the other values of this specific probe (baseline

transformation). After removing outliers and transcripts without significant expression at any time point, ANOVA ($P \leq 0.05$, $FC \geq 2$) and FDR correction (Benjamini-Hochberg) was performed. These stringent parameters were chosen in order to identify important transcripts without (unnecessarily) expanding the data set. The results were visualized on MapMan program.

4.6. Phytohormone measurements

4.6.1. Hormone extraction

Hormones were extracted from frozen primary and 4th leaves material in 6 independent biological replications for each measurement. The leaf material was ground in liquid nitrogen and 150 mg of finely powdered fresh material were extracted using 1 ml extraction buffer containing (v/v/v)(15:1:4) ice-cold methanol:formic acid:water. The homogenate was mixed thoroughly and stored at -20°C for 16 h and centrifuged after incubation at 13,000 rpm and 4ºC for 20 min. The pellet was re-extracted using 300 µl of extraction buffer. Subsequently, supernatants were dried at 38ºC for 3h in a speed vacuum concentrator (Christ, Germany). Pellets were re-suspended in 100 µl 80% methanol.

4.6.2. Separation and elution of different hormones using a MCX column

Solid phase MCX columns were equilibrated first with 1 ml acetonitrile followed by 1 ml methanol (MS grade). Columns were further washed by 1 ml of 1 M formic acid and hydrogen chloride (HCl). Re-suspended samples (from section 3.11.1) were mixed with 900 µl of 1 M formic acid and added to the MCX columns. ABA and ABA degradation products were collected with 1 ml pure methanol (MeOH), whereas cytokinins were eluted with 1 ml 0.35 M ammonia (NH_3) dissolved in 60 % MeOH. The dried eluents were re-solved in 50 µl of 25% MeOH and used for LC-MS-MS analysis.

4.6.3. Determination of hormones by UPLC MS-MS

All UPLC-ESI-MS/MS experiments were carried out using an Agilent 1290 infinity system connected to an Agilent triple quadruple mass spectrometer QQQ6490 (Agilent Germany). Separated compounds were ionized at atmospheric pressure via electrospray and directed to the mass spectrometer. The control of the complete system and recording of the spectra were performed with the MassHunter, software B.04.00 (B4038).

To separate the individual cytokinines and ABA, a UPLC system was used including a gradient pump, an autosampler, and a column compartment. Separation was carried out using a high capacity column (Eclipse Plus C18, RRHD 1.8 µm, 2.1x50 mm). A gradient was accomplished with LC-MS grade water (Chem. solute, Geyer, Germany) containing 0.1% formic acid (Fluka, Germany) as buffer A and LC-MS grade methanol (Chem. solute, Geyer, Germany) including 0.1% formic acid as buffer B. The column was equilibrated with a mixture of buffer A (86.5%) and buffer B (13.5%) at a flow rate of 0.4 ml per min and heated to 40°C during the whole measurement. The gradient was produced by changes of the buffer B as follows: 0 to 5 min at 18%, 5 to 6 min at 70 %, 6 to 7 min at 99%, 7 min at 13.5% and kept up to 9 min at 13.5%. The whole duration of the run was 9.0 min.

Phytohormone analysis was performed using a MS/MS triple quadruple mass spectrometer (Agilent 6490, USA). The following parameters were employed: dissolving temperature 350°C, desolation nitrogen gas of 720 l h-1, capillary voltage 2.0 KV, detection in positive ion mode and different dwell times between 40 and 200 seconds. Collision energy (CE) differed among the compounds (Table 10.1). Protonated ions [M-H]+ were monitored with a span of 1 amu. Multiple reactions monitoring (MRM) was performed to identify individual compounds accurately. This allows minimizing parallel monitoring and enhancing the sensitivity.

4.7. Determination of C/N ratio

Relative contents of total carbon, nitrogen and sulfur in dried, powdered samples of leaves were measured using an elemental analyzer (Vario EL; Elementaranalysensysteme, Hanau, Germany) as described in Ahkami et al (2008).

4.8. Measurement of soluble and insoluble carbohydrates

Soluble sugars and starch were determined in primary and 4th leaves according to the method of Chen et al. (2005). 50 mg frozen leaf material was homogenized in liquid nitrogen, dissolved in 0.75 ml of 80% (v/v) ethanol and incubated at 80°C for 60 min. Crude extracts were centrifuged at 14,000 rpm at

4ºC for 5 min and the upper phase was concentrated in a speed vacuum concentrator (Christ, Germany) at 45°C for 180 min, while The remaining insoluble material was kept for starch measurements. The pellet was re-suspended in 0.3 ml HPLC-grade water and shaken for 15 min at 4°C.

A buffer containing 100 mM imidazol-HCl (pH 6.9), 5 mM MgCl2, 2.25 mM NAD, 1 mM ATP (as final concentrations) was used for the measurement of soluble sugars using an EL808 ultramicroplate reader (BioTeK Inc., Germany) at 340 nm. The sequential addition of auxiliary enzymes allowed detecting glucose (Glc), fructose (Fru) and sucrose (Suc). Glucose-6-phosphate dehydrogenase (G6PDH) was first added to remove endogenous hexose-phosphates. Subsequently, hexokinase (HK), phosphoglucoisomerase (PGI) and β-fructosidase were added successively to measure Glc, Fru and Suc as described in Hajirezaei et al. (2000).

The residue of sugar extraction was washed twice with 1 ml of 80% (v/v) ethanol. Starch was decomposed with 0.4 ml 0.2 N KOH for 16 h at 4°C and neutralized with 70 µl of 1 M acetic acid. Hydrolysis of starch was performed using a 1:1 ratio of sample and a buffer containing 50 mM sodium acetate, pH 5.2 and 7 units mg-1 of amyloglucosidase (Roche, Germany). The cocktail was incubated at 37°C for 16 hours. Determination of produced Glc was performed according to Hajirezaei et al. (2000).

4.9. Measurement of free amino acids

Free amino acids were extracted as described in above section of soluble sugars extraction. To detect primary and secondary amino acids, a fluorescing reagent AQC (6-aminoquinolyl-N-hydroxysuccinimidylcarbamate) was used. ACQ was dissolved in 3 mg ml^{-1} of acetonitrile and incubated at 55°C for 10 min. Twenty ml of sugar extract were derivatized in a cocktail containing 20 µl of the fluorescing reagent ACQ, 160 µl of a 0.2 M boric acid buffer (pH 8.8) in a final volume of 200 µl. The solution was incubated at 55°C for 10 min.

The separation of derivatized samples was carried out with a reversed phase HPLC system (Waters, Germany) consisting of a gradient pump (Alliance 2795 HT, Waters, Germany), a degassing module, an autosampler and a

fluorescence detector (Waters 2475, Germany). A reversed phase column (XBridge; 150 mm, 5 µm) was used for separation and detection of amino acids at an excitation wavelength of 300 nm and an emission wavelength of 400 nm. The gradient was accomplished with buffer A containing 140 mM sodium acetate, pH 5.8 (Suprapur, Merck) and 7 mM triethanolamine (Sigma, Germany). Acetonitrile (Roti C Solv HPLC, Roth) and purest HPLC water (Geyer, Germany) were used as eluents B and C. Chromatograms were recorded using the software program Empower Pro.

4.10. Measurement of anions

Anions were extracted as described in above section of soluble sugars extraction. The separation and detection of anions was carried out using an ion chromatography system connected to a conductivity detector (Dionex, Thermofisher Germany). The control of the complete system, recording of the spectra and data aquisition were performed with the Chromeleon software, release 7.0 (Dionex GmbH, Germany). To separate anions an ICS 5000 system (Dionex, Germany) was used including a gradient pump DC, an autosampler AS-AP and a conductivity detector. Separation of the anions was carried out using a high capacity ion exchange column (AS11-HC, 250 x 2 mm) connected to a guard column of the same material (AG 11-HC, 10 x 2 mm) and an ATC-1 anion trap column which is placed between the eluents and separation columns to remove the anions present in the solutions. The Gradient was accomplished with purest water (buffer A, Millipore) and a concentrated potassium solution EGCIII KOH (Dionex, Germany, buffer B) and the corresponding gradient was produced using an eluent generator EG-SP (Dionex Germany). The column was equilibrated with a mixture of buffer A (96 %) and buffer B (4 %) at a flow rate of 0.32 ml per minute and heated at 35°C during the whole measurement. The gradient was produced by changes of the buffer B as follows: 0-4 min at 4 %, 4-10 min at 15 %, 10-18 min at 80 % and 18-25 min at 4 %. The duration of the run is 25 minutes.

4.11. Primary Metabolites determination

4.11.1. Extraction of primary metabolites

Primary metabolites were extracted from primary leaves of barley plants. Samples were harvested and frozen immediately in liquid nitrogen. Six independent biological replications were used for measurement. Plant tissue was ground in liquid nitrogen and 100 mg of finely powdered fresh material were extracted using 1 ml (v/v)(1:1) ice-cold methanol and chloroform. Subsequently, 0.4 ml of LC-MS water was added to each tube. The mixture was mixed and kept on ice for 20 min. The samples were centrifuged for 10 min at 14000 rpm and 4°C. Thereafter, the upper phase containing methanol/water was transferred to new Eppendorf tubes and concentrated at 45°C for 2 hours in a speed vacuum concentrator (Christ, Germany). The remaining pellet was re-suspended in 0.3 ml of LC-MS water and was kept at -80°C for metabolite analysis.

4.11.2. Targeted metabolite analysis by ion chromatography mass spectrometry (IC-MS-MS)

For quantification of primary metabolites, a targeted metabolite analysis was performed using external standards. 0.1 ml of sample volume extracted in section 3.9.1 was filtered at 2000g for 90 min using a multiscreen filter plate (multiscreen ultracel-10 ultra-filtration membrane 10000 NMWL). The IC-MS-MS instrumentation consisted of a Dionex ICS5000 (Dionex, Idstein, Germany) with a 6490 triple Quad LC-MS-MS (Agilent, USA). Anionic compounds were separated on a 250×2 mm AS11-HC column (Dionex) connected to a 10×2 mm AG 11-HC guard column (Dionex) and an ATC-1 anion trap column. The gradient was produced with H_2O (buffer A; HPLC grade water) and KOH which was generated by an EGCIII KOH eluent generator cartridge. The column was equilibrated with a mixture of buffer A (96%) and 4% KOH at a flow rate of 0.38 ml min^{-1} and heated to 37°C during the measurement. The gradient was produced by changes of KOH concentration as follows: 0–4 min: 4%; 4–15 min: 15%; 15–25 min: 25%; 25–28 min: 50%; 28–31 min: 80% and 31-40 min; 4%. Quantitative analysis of metabolites was performed using an Agilent 6490 triple quadruple mass spectrometer (Agilent, Germany). Electron spray ionization

(ESI)-MS/MS was set as follows: gas temperature 350°C, drying gas flow rate 12 l min-1, nebulizer pressure 35 psi, capillary voltage ± 3.5 kV. The fragmentor voltage and collision energy were optimized for each compound individually by tuning standards with a defined concentration. Primary metabolites were detected in the negative ion mode using multiple reactions monitoring (MRM) (Appendix Table 3). The data were extracted using the MassHunter software version B.03.01 (Agilent Technologies, Germany). Quantification of metabolites was performed by creating a batch for each sample sets using the Quantitative Analysis (QQQ) software (Agilent Germany). ^{13}C-pyruvate was used to normalize the data and was added to each sample as internal standard before analysis.

4.12. Phloem exudate collecting and analysis

The primary leaves were cut from base by sharp blade and washed by distilled water to prevent the commination from damaged cells. The base of leaves were immersed in 5mM Na_2-EDTA, pH 8, to remove the contamination from damaged cells and xylem. After 10 min the leaves were transferred to 1.5 Eppendorf tubes containing fresh 200 µL Na_2-EDTA (2 leaves each), and kept for 2 h to collect the phloem exudate. In order to decrease the evaporation, the phloem exudate collecting was performed in almost humid saturated atmosphere condition (Fig.4.2). Then the leaves were dried at 80°C for two days and its dry weight was weighted. Phloem exudate was stored at -80°C and analyzed for sucrose and amino acids content as described in previous sections.

Figure 4. 2. Phloem exudate collecting system by Na_2-EDTA method. The leaves were put in 1.5 Eppendorf tubes containing fresh 200 µL Na_2-EDTA (2 leaves each), and kept for 2 h to collect the phloem exudate.

4.13. Statistical analyses

The two way ANOVA analysis was performed by InfoStat/Student program that was downloaded from http://www.infostat.com.ar/index.php?mod=page&id=37 and LSD Fisher Method was used for statistical analyses. Letters were used to represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5. Results

5.1. N availability affects progression of leaf senescence

5.1.1. Experimental setup and physiological markers for leaf senescence under different N regimes

To investigate progression of leaf senescence under different N regimes, photosynthesis parameters, transcriptomic, and metabolic rearrangements were analyzed during N starvation and upon N resupply. Barley seeds (*Hordeum vulgare* L. cv Golden Promise) were germinated and grown in 0.5X Hogland solution (See material and method). At 8 day after germination (DAG) control seedlings were transferred to 5 liter pots supplied with complete Hogland nutrients including 2 mM $Ca(NO_3)_2$. While another group of barley plants was treated with N deficiency condition (0.06 mM $Ca(NO_3)_2$), and kept for another 15 d in order to induce leaf senescence syndrome (Fig.5.1). Subsets of N starved plants were fed again with optimum level of N (2 mM $Ca(NO_3)_2$) after 10 d of N limitation (Fig.5.1).

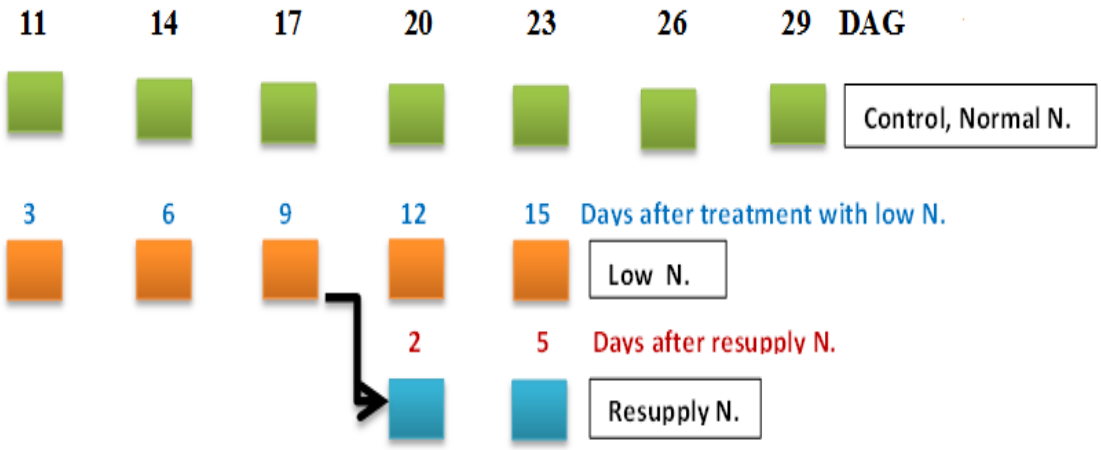

Figure 5. 1. Schematic representation of the sampling time points. DAG: day after germination. Numbers represent the number of DAG in black color, days after N deficiency treatment started in blue color, and days after N resupplied to N starved plants in red color.

To follow the physiological leaf senescence progression, chlorophyll content was determined as senescence marker. The chlorophyll content in primary leaf of control plants kept in N-replete conditions, reflected by SPAD unit, stayed

stable up to 20 DAG. Then after, it started slightly to decrease which reflects onset of developmental senescence. As a consequence of N shortage, the primary leaf kept in N deficiency condition started chlorosis earlier than control plant at 17 DAG (9 days after N shortage), indicating that dismantling of photosynthetic apparatus didn't start immediately after N starvation. The SPAD unit continued to decrease in N deficient leaves and in 23 DAG (15 days after N shortage) the primary leaf was almost yellow (Fig. 5.2A). Notably, when optimum amount of N was resupplied to 10 d N starved plants, the further decrease in chlorophyll content was stopped (Fig. 5.2A). In addition, the photosystem II (PSII) efficiency in N resupplied plants stayed to control level (data not shown), indicating that resupplied N to senescing leaves stopped the further progression of N deficiency-induced leaf senescence program. Same results have been reported for barley flag leaf when plants were additionally fertilized with nitrate and ammonium, but not urea (Schildhauer et al., 2008). However, not much is known about the metabolic rearrangement and how the availability of N is integrated into networks of leaf senescence in barley plants.

It is well documented that plants respond to nitrogen availability by increasing their root/ shoot ratio, this allows the plants to cover more soil area by their roots and to search for more N resources (Shangguan et al., 2004; Agren and Franklin 2003). In parallel, N deficiency inhibits shoot growth and development including stop of tillering and new leaves formation (Fig.5.3). This phenotype can be controlled by sugars and a balance between auxin and cytokinins (CK) (Forde 2002), which makes roots a stronger sink for resources. Limbers et al., (1982) showed that the proportion of N retranslocated from leaves to the roots is higher under N limiting conditions (18%) than at optimal N condition (11%). Moreover, the plants can detect the N availability through highly developed

signaling mechanism, and sustain shoot growth including tillering and new leaves formation, when the optimum N is available (Fig.5.2B; 5.3).

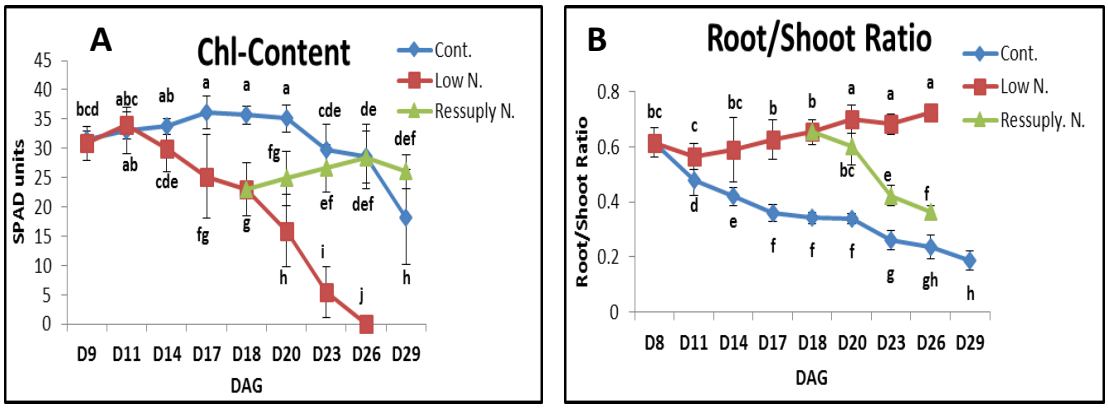

Figure 5. 2. Physiological markers. (A) Chlorophyll content of primary leaves represented by SPAD unit, measurements were performed on the middle of leaves. (B) root/shoot ratio was calculated by divided root to shoot dry weight. DAG: day after germination. Data represent means ±SD (n ≥10 plants). Letters (a-j) represent the significant; Means with a common letter are not significantly different (p > 0.05).

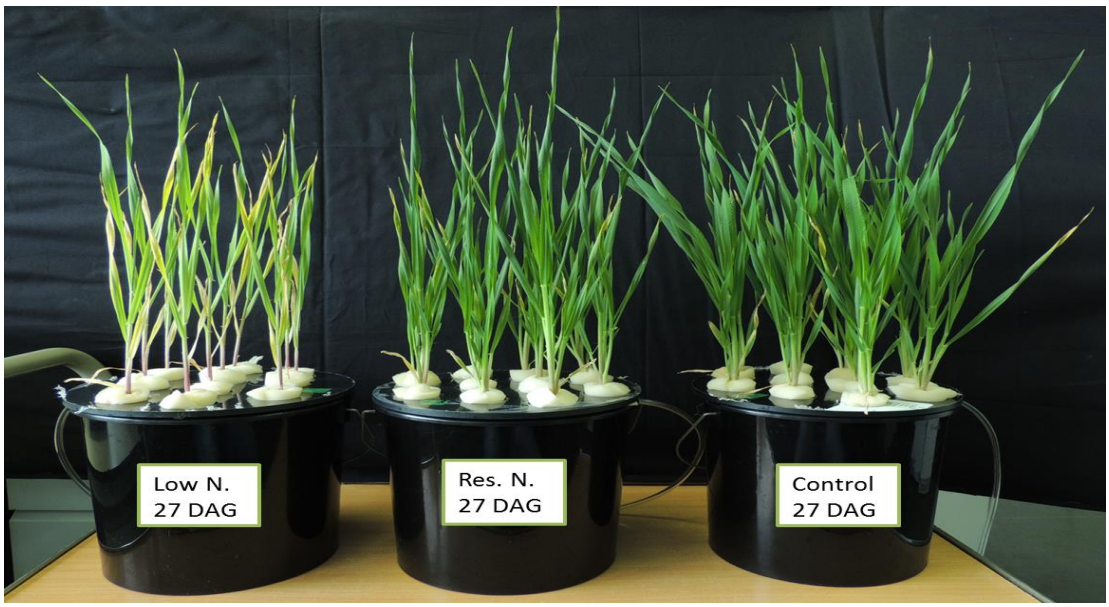

Figure 5. 3. Barley plants phenotype under the three N regimes, Low N., Resupply N, and control from left to right, respectively, at 27 DAG.

5.1.2. Genetic markers for Leaf senescence under different N regimes

To follow the leaf senescence progression at molecular level, total RNA was extracted from primary leaves, harvested in three day interval from at least

three independent biological experiments, and qPCR was performed for a group of chosen genes including two known senescence marker genes (S40, upregulated during leaf senescence (Krupinska et al., 2012), and plastidic glutamine synthtase 2 (GS2), down-regulated during leaf senescence (Schildhauer et al., 2008)). Relative Expression of these genes with leaf age and under different N conditions was compared to that of control plants at 11 DAG. As expected, the expression of S40 was increased during developmental senescence progression, and reached up to 331 folds at 29 DAG. The induction of S40 expression was more pronounced by N deficiency treatment comparing to that in control plant (Fig.5.4). However, a significant induction in N deficient plants was firstly found at 17 DAG coinciding with start of chlorophyll loss. Interestingly, resupply of N to 10 d N starved plants delayed further increase in S40 transcript level upon 2 days, indicating a fast response in delaying leaf senescence. Notably, it wasn't lower than the S40 expression value at 17 DAG of N-deficient plants, this may be explained by the progression age of the leaves during the experimental time course and accumulation of age-determined factors even in control plants. In addition, the expression of another senescence-associated gene (SAG), *HvNAC13*, followed the same pattern as S40 expression (Fig.5.4). Kjaersgaard et al., (2011) showed that *HvNAC13* binds the conserved NAC DNA target sequence and activates genes transcription.

GS2, encoding glutamine synthetase enzyme, plays a major role in primary N assimilation and in the re-assimilation of ammonium produced from photorespiration, which catalyzes the glutamine biosynthesis in plastids. Here, GS2 was downregulated with leaf age in control plant, indicating a decrease in primary N assimilation. N deficiency caused a decrease in GS2 expression comparing to the corresponding control time point starting from 17 DAG (Fig.5.4). It was earlier reported that nitrate induced GS2 level (Cabello et al., 1997). In agreement with these results, N resupplied to senescing leaf induced the GS2 expression. Additionally, the relative expression of GS2 was also higher than in control leaves (Fig.5.4). This higher expression doesn't reflect a higher nitrate level in resupplied N than control leaves (Fig.5.10). However, its

expression regulation might be also under sugar levels and/or systematic signals from roots such as CKs. A putative response regulator (ARR) followed the same expression pattern as GS2 in response to N supply. While no differences (less than two folds change) in control during the experiment time (Fig.5.4).

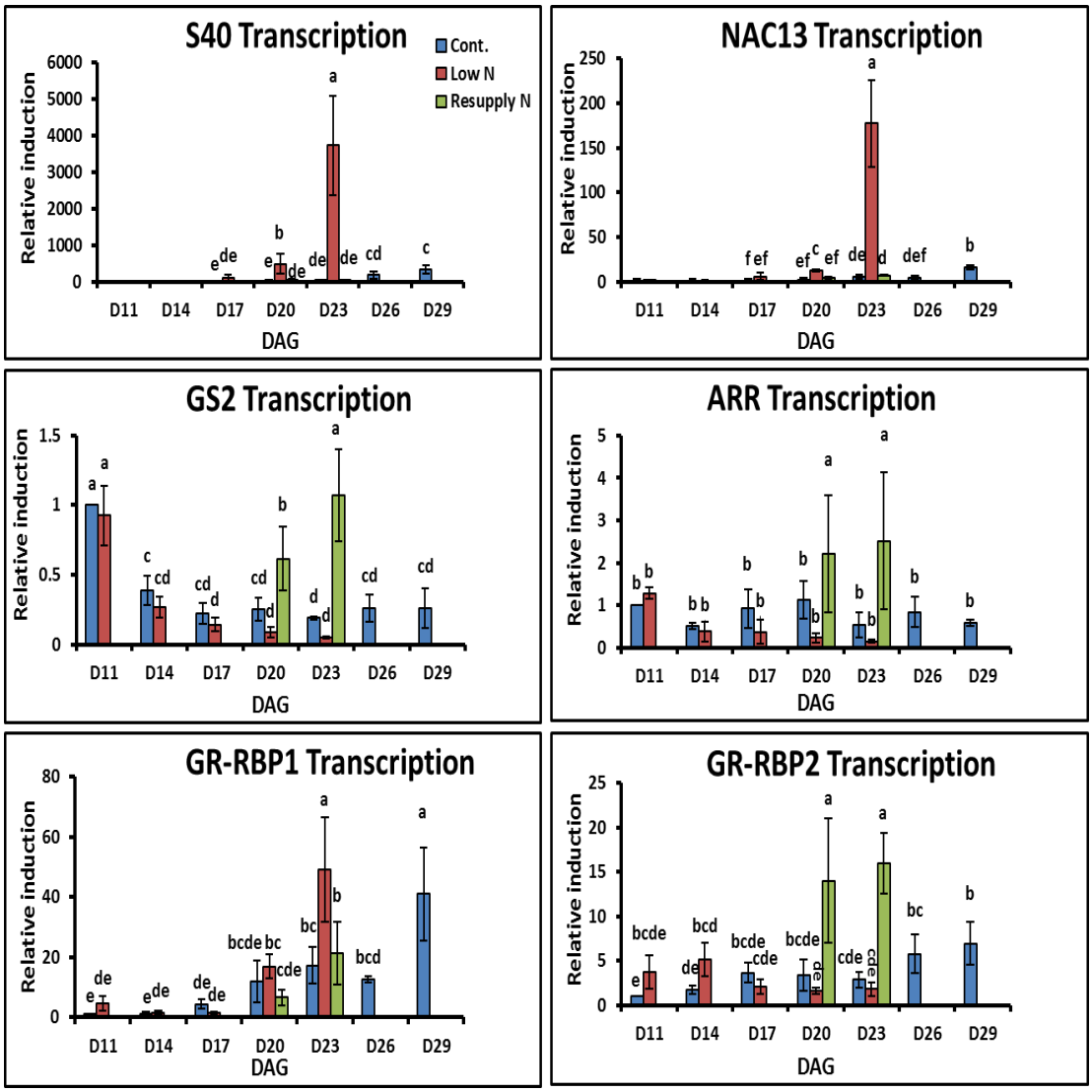

Figure 5. 4. Senescence marker genes transcription was determined by qPCR. Actin was used as a reference, and the relative induction of all results were compared to the expression value of control plants at 11 DAG. GS2: Glutamine synthtase 2, ARR: a response regulator, GR-RBPs: Glycine-rich RNA-binding proteins. Data represent means ±SD (n ≥3). Letters (a-f) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

Posttranscriptional processes are largely regulated by diverse RNA-binding proteins. Glycine-rich RNA-binding proteins (GR-RBPs) are functionally

conserved RNA chaperones and are involved in stresses responses such as cold adaptation process in diverse plant species (Mangeon et al. 2010; Kim et al., 2012). The expression of two putative GR-RBPs in barley, *HvGR-RBP1* and *HvGR-RBP2*, was determined. *HvGR-RBP1* was upregulated with leaf age in control plants reaching 41 fold higher transcript levels at 29 DAG (Fig.5.4). However, it wasn't responding to N deficiency, resupplied N decreased the expression level at 20 DAG (Fig.5.4). Moreover, *HvGR-RBP1* is one of four candidate genes found in grain protein content (GPC) locus that exhibit early flag leaf senescence (Lacerenza et al., 2010). *HvGR-RBP2* was upregulated with leaf age of control plants. Although, it was induced in early N deficiency treatment, it was downregulated with N-induced leaf senescence progression. On other hand, when N resupplied, the expression of *HvGR-RBP2* was upregulated to several folds (Fig.5.4). It is interesting in further studies to characterize if there is a physiological role for *HvGR-RBP2* in N deficiency tolerance and senescence process.

5.2. Transcription profiling of barley primary leaf with designed 60K Agilent microarray

5.2.1. Transcriptome analysis of N deficiency-induced leaf senescence and its reversal by N resupply

Comparative transcriptome profiling was performed in barley primary leaves to identify genes involved in global metabolic rearrangement, hormonal response, remobilization, and transport processes, as well as putative regulatory elements associated with N deficiency-induced leaf senescence and delaying the senescence program by N resupplied. Three independent biological experiments were carried out to harvest samples (10 primary leaves each; three biological replicates for each time point) from barley plants treated with different N availability regimes for transcript profiling. The whole RNA extracted from sufficient N treated-plants (time points: 17, 20 DAG) or in N deficiency treated-plants (17, 20 DAG, corresponding to 9 and 12 d, respectively, of N starvation). In addition, leaves were also harvested from 2 days of N resupplied to 10 days N starved plants (20 DAG; 2 d from N resupplied) as shown in schematic

presentation (Fig.5.1). The transcriptome profiling was performed using barley 60K designed Agilent microarrays (15 microarray hybridizations in total), and ratios of gene expression level were calculated (log_2 fold change (FC)). N deficiency results were compared to corresponding control time points (17, 20 DAG), and N resupply results were compared to corresponding N deficiency time point (20 DAG). After removing outliers and transcripts without significant expression at any time point, ANOVA ($P \leq 0.05$, $FC \geq 2$) and FDR correction (Benjamini- Hochberg) was performed. These stringent parameters were chosen in order to identify important transcripts without unnecessarily expanding the data set. The list of differentially regulated and highly differentially expressed genes can be found in appendix (Table 10.2)

The data set analyses showed that 751 and 885 transcripts were induced by at least two folds at 9 d (17 DAG) and 12 d (20 DAG), respectively, after N starvation including putative genes coding protein and amino acids degradation, ABA biosynthesis and response, mitochondrial electron transport, Calcium regulation, MYB, MYB- related, NIN- like and NAC transcription factors. Moreover, one gene coding histone acetyltransferase indicating epigenetic regulation is involved. While 864 and 1053 transcripts were downregulated after 9 and 12 d, respectively, including putative genes coding photosynthesis apparatus, tetrapyrrole metabolism, glycolysis, TCA cycles, CK response, redox regulation, G2-like, nucleosome assembly, C2C2- Gata transcription factors and most putative genes that might have a role in secondary metabolism pathways. The data also revealed that 4083 genes differentially regulated upon 2 days of N resupply to 10 d N-starved plants, 2220 genes were upregulated including putative genes coding photosynthesis apparatus, photorespiration, glycolysis, TCA cycles, amino acids and nucleotides biosynthesis, CK response, glutaredoxin- and carotenoid- related genes, histones synthesis, heat shock transcription factors. 1863 downregulated including putative genes coding protein and lipid degradation, mitochondrial electron transport, receptor kinases, calcium regulation, WRKY, ARF, Myb, and Myb- related transcription factors.

From 1947 genes that differentially regulated after 12 d of N deficiency, 1208 genes (62%) were recovered after 2 d of N resupply, 665 were re-upregulated and 543 were re-downregulated.

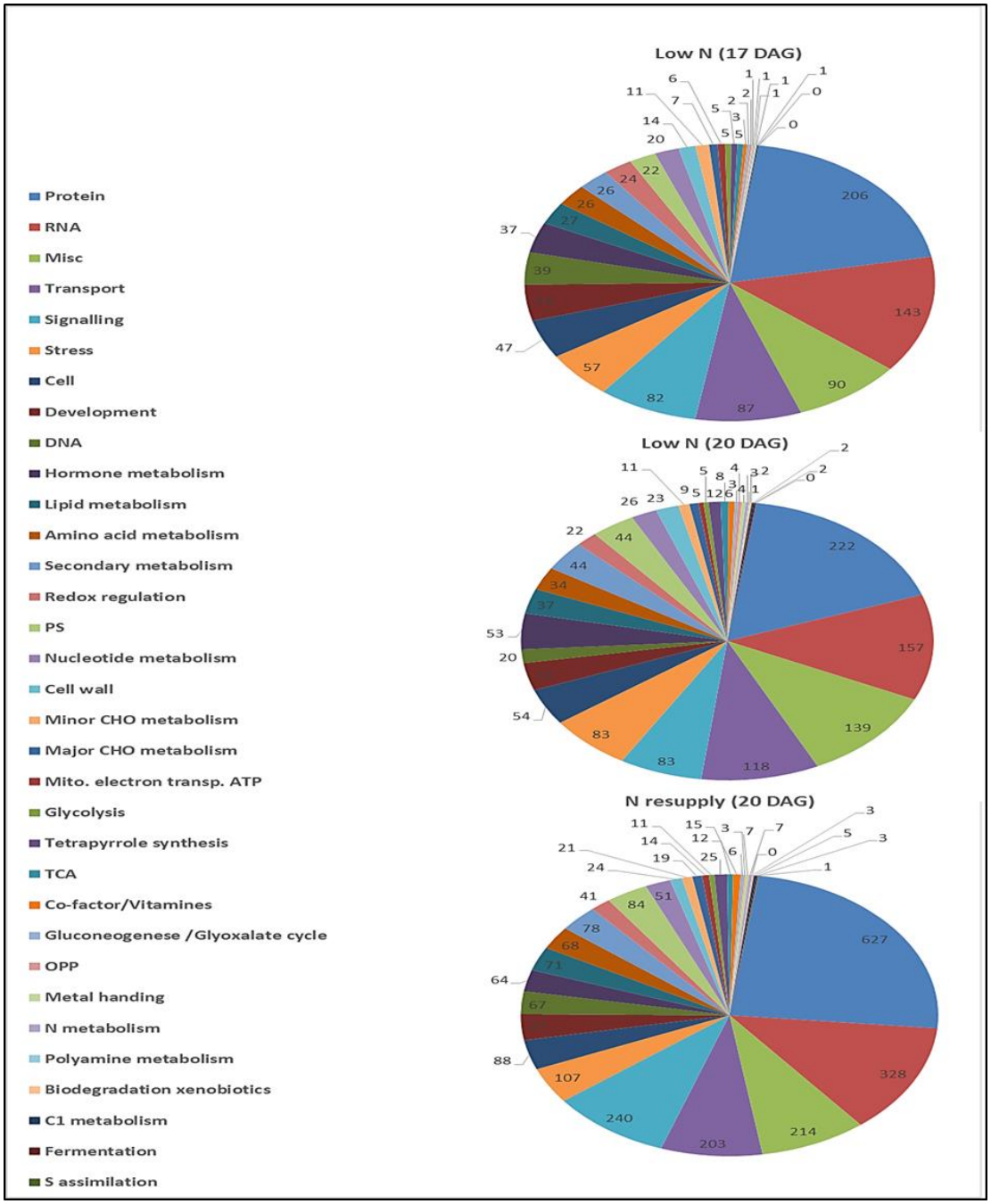

Figure 5. 5. Functional classes of differentially regulated genes classified by Mapman software. Genes were grouped according to predicted function (groups 1-36). The number in slices represents the number of genes that are differentially regulated for every group.

Mapman software classified the differentially regulated genes by N availability to different functional classes. These functional classes were divided into the broad categories "metabolism", "stress", "regulatory processes" and "Other". About 63% of the differentially regulated genes from the records low N (17 and 20 DAG) and N resupply (20 DAG) could at least be assigned to one functional class. The remaining genes could not be assigned and belong to the category "not assigned". These genes were not considered for the analysis in Fig. 5.5. When comparing the proportions of functional categories to be affected by N availability, the most affected groups related to regulatory processes about half of the regulated genes was in the category "regulatory processes" (low N (17 DAG):49%; low N (20 DAG):42%; N resupply: 51%). These include factors of hormone biosynthesis, influencing stability and activity of nucleic acids and proteins as well as components of the signal switching and routing (such as transcription factors, kinases, second messengers). These factors influence the expression of factors of stress response, development and metabolism, as well as degradation process during senescence program or stability upon N resupply. Another important category that was affected by N availability is metabolism (low N (17 DAG):15%; low N (20 DAG):18%; N resupply: 16%). This includes photosynthesis, lipid, amino acid, and nucleotide metabolism, as well as CHO metabolism, cell wall, TCA, and other. Stress Category was also affected by N availability (low N (17 DAG):10%; low N (20 DAG):12%; N resupply: 9%). To the class "secondary metabolism" is one of a plurality of genes encoding enzymes in the biosynthesis of secondary plant substances (such as isoprenoids, flavonoids, and alkaloids). Most of genes belongs to this class was upregulate after 9 d of N deficiency, whereas mostly were suppressed after 12 d of N deficiency and 2 d of N resupply. Transport group was also highly affected by N availability (8% of the total assigned regulated genes). This includes transporters act in transportation process from senescing leaf to sink tissues. For more details about the number genes upregulated or downregulated in every functional group see appendix (Table.10.5). Differentially regulated genes related to the responses at hormonal and

metabolic levels are discussed in more details in later chapters together with the metabolic and hormones data.

5.2.2. Comparison of gene expression patterns of N deficiency-, natural- and drought- induced leaf senescence

Guo and Gan (2012) have shown by analysing microarray expression data from developmental and 27 different treatments in Arabidopsis plants, that are known to induce senescence, that at early induction of senescence initiation showed limited common regulation in the induction of gene expression to that of developmental leaf senescence, but once the senescence is started, the different senesce inducers share a huge proportion of SAGs expression. We looked for overlap between N deficiency-, natural- and drought- induced leaf senescence at transcriptomic level, by comparing my data with those reported for natural- and drought-induced senescence in our group by Christina Mohr (unpublished results). To identify genes with common expression in response to the three senescence inducers, ≥3 fold expression change was taken as threshold for the comparison (as also done for natural- and drought-induced senescence experiments by Christina Mohr). 1181 genes are more than three-folds differentially regulated after 12 d (20 DAG) of N deficiency treatment, 567 genes induced and 614 genes downregulated. This data set was used to look for overlap with transcriptome of natural leaf senescence dataset (Christina Mohr) using the same 60K Agilent microarray design. Focusing on genes differentially regulated in stage 3 of natural senescence (S3; around 43 days after sowing (DAS); 50% of chlorophyll lost at the middle of the leaf). 43% of genes that are differentially regulated in N deficiency were also found to be regulated in S3 natural senescence. A total of 288 were commonly induced in the both senescence regulating process (Fig.5.6A) including putative genes coding calcium regulation, gluconeogenesis, sucrose synthases (Susy), ABA biosynthesis (NCED), auxin response, DNA cleavage, protein and amino acid degradation, as well as MYB-related, NIN-like, NAC, and C2C2-Dof transcription factors.

Figure 5. 6. Number of genes commonly regulated in N deficiency-induced and natural leaf senescence. A, upregulated genes. B, downregulated genes. Green circle: genes involved in N deficiency- induced senescence. Black circle: genes involved in natural senescence at stage 3 (S3; around 43 days after sowing; 50% of chlorophyll lost at the middle of the leaf)**.**

In addition, 218 genes were commonly downregulated (Fig.5.6B) including putative genes coding photosynthesis, amino acids biosynthesis, CKs response, jasmonate biosynthesis (lipoxygenases), glutaredoxin, ascorbate and glutathione, as well as ARF, ARR-B, G2-like, and C2H2 transcription factors.

Moreover, we looked for common overlap between N deficiency- and drought-induced leaf senescence at transcriptomic level. Focusing on genes differentially regulated at drought stage 2 (D2; around 19 DAS subjected to 15 days of drought stress, 25% of chlorophyll lost at the middle of the leaf) that was also determined in our lab using the same 60K Agilent microarray design. In total, 77 genes were commonly induced (Fig.5.7) including putative genes involved in protein degradation, sucrose synthase (Susy; two genes), carotenoid biosynthesis, MYB-related-, C2C2-Dof-, and C2H2- transcription factors (one gene each), as well as ABA biosynthesis 9-cis-epoxycarotenoid dioxygenase (NCED; hv_29667). 116 genes were commonly downregulated (Fig.5.7) including putative genes involved in photosynthesis, receptor kinases (3 genes), peroxidases, and glutaredoxin, as well as bZIP and ARR-B transcription factors. In addition, 38 genes were contrary regulated including putative genes involved in jasmonate biosynthesis (three genes coding lipoxygenases) that downregulated by N deficiency. However, the results of drought-induced leaf senescence at stage 3 (D3, around 23 DAS subjected to 19 days of drought stress, 50% of chlorophyll lost at the middle of the leaf) isn't available. It is expected that a more overlap could be found between the both senescence inducers.

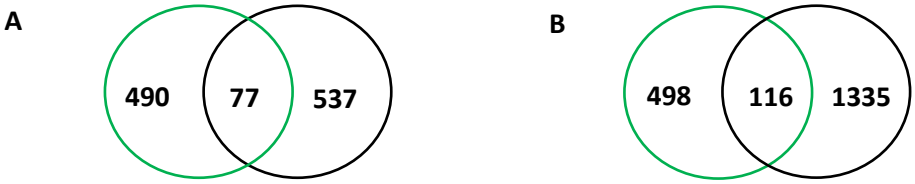

Figure 5. 7. Number of genes commonly regulated in N deficiency- and drought-induced leaf senescence. A, upregulated genes. B, downregulated genes. Green circle: genes involved in N deficiency- induced senescence. Black circle: genes involved in drought-induced leaf senescence at stage 2 (D2; around 19 DAS subjected to 15 days of drought stress, 25% of chlorophyll lost at the middle of the leaf)**.**

5.3. ABAs and CKs profile of primary and 4th leaves under different N regimes

Plant adaption to different environmental stresses is determined by hormonal signaling pathway that allows the co-ordination of shoots and roots growth. The regulation of senescence by cytokinin and ethylene is conserved; however, the action of other hormones varies between plant species (Schippers et al., 2007). Abscisic acid (ABA) and cytokinins (CKs) have been considered as signals to mediate leaf senescence. While ABA was shown to induce the senescence program in natural and stress- induced leaf senescence (Pourtau et al., 2004; Schippers et al., 2007), CKs could delay leaf senescence (Gan and Amasino, 1995). To test the changes in phytohormones level in natural- and N deficiency-induced leaf senescence, the concentration of ABA and CKs were determined, using UPLC-MSMS, in primary of N deficient plants and were compared with control plants and with plants resupplied by the optimum amount of N. Furthermore, the growing leaf (4th leaves) was analysed to determine the differences of changes in phytohormones level between leaves going to senescence and new growing leaves at the same time point upon different N regimes.

5.3.1. Changes in levels of abscisic acid (ABA) and its degradation products and in expression of ABA metabolism genes in response to N availability

5.3.1.1. Changes in levels of abscisic acid (ABA) and its degradation products

It was possible to determine the ABA and its degradation forms (PA and DPA) using established protocol of UPLC-MSMS in barley leaves. Under N sufficient condition, ABA concentration was around 0.5 $pmol.g^{-1}$(FW) in mature leaves (at 11 DAG), and slightly increased with primary leaf age reaching 2 $pmol.g^{-1}$(FW) at 26 DAG (Fig.5.8). DPA level decreased with natural leaf senescence. Accordingly, the increase in ABA level related to leaf age in control plants might result from a decrease in the degradation process rather than the biosynthesis (Fig.5.8). On other hand, ABA concentration was significantly increase by 1.5-, 1.7-, and 3.4-fold at 9, 12, and 15 d, respectively, after N deficiency treatment compared to control plants (Fig.5.8). This is in agreement with what has been found in Arabidopsis plants under N deficiency condition (Balazadeh et al., 2014). There were no significant changes, except 23 DAG, in ABA degradation forms (PA and DPA) between control and N deficient plants (Fig.5.8). When N resupplied to 10 d of N starved plants, the ABA concentration was less by 2.5- and 6- folds after 2 and 5 d of N resupplied, respectively, than N deficient plants (Fig.5.8). Moreover, PA level was also lower in N resupplied plants, but DPA doesn't show any dramatic change.

In order to have a perspective on whole plant level under the different N treatments. Growing leaf number 4 was also harvested (at 17, 20, and 23 DAG) for phytohormone analyses, which reaches its full length at 20 DAG. The results showed that ABA level was only slightly different at 20 DAG, and the degradation process of ABA in 4^{th} leaf –treated by N deficiency was higher than that in primary leaf, assuming that the developmental factors can modulate the level of ABA by induced its degradation process. While the ABA and its degradation forms (PA and DPA) were lower after N resupplied compared to N deficient plants (Fig.5.8).

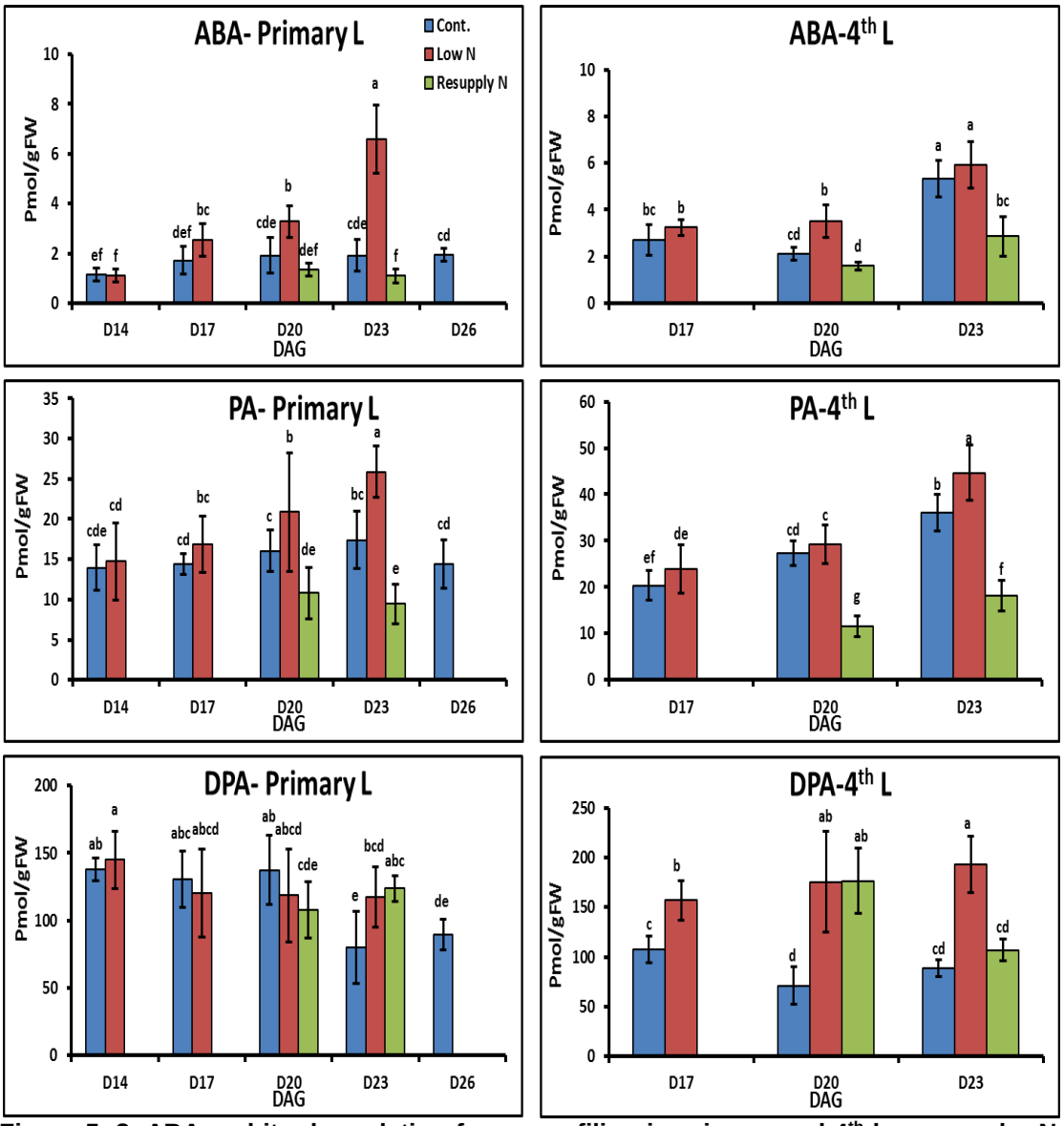

Figure 5. 8. ABA and its degradation forms profiling in primary and 4th leaves under N deficiency-induced leaf senescence and its reversal by N resupply. ABA, abscisic acid; PA, phaseic acid; DPA, diphaseic acid; DAG, day after germination. Left side graphs for primary leaf and right side graphs for 4th leaf (4th L). Bars indicate mean ± SD (n=5-9). Letters (a-g) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5.3.1.2. The regulated genes by N availability that are related to ABA metabolism and signaling

Alterations in ABA levels in response to N availability correlate with differential regulation of genes involved in ABA metabolism and signaling. Our data (see Table 5.1) showed that two genes putatively encoding ABA biosynthesis related enzymes are upregulated under N deficiency conditions. These are a putative

gene coding AO (hv_36314) which was upregulated by 2 folds after 9 d of N deficient treatment, and a putative gene coding 9-cis-epoxycarotenoid dioxygenase (NCED; hv_29667), coding which was induced by 9-11 folds after 9 and 12 d of N deficient treatment. In addition, two genes coding ABA degradation enzymes (xanthine dehydrogenase (XDH); hv_07410 and hv_41833) were induced after 12 d of N deficient treatment. Interestingly, NCED (hv_29667) transcription was downregulated by 6 folds and XDH (hv_07410), as well as genes related to ABA signaling was also downregulated after 2 d of N resupplied (Table 5.1). This indicates that senescence delaying in barley by N resupplied might be also mediated through ABA level and signaling. It will be interesting in the future work to determine the ABA flow through xylem under N deficiency and resupply in barley plants.

Table 5. 1. Relative expression of putative genes related to ABA metabolism and signaling which is related to N-availability. The values represent fold change. ↑: Upregulated, ↓: downregulated. Values of Low N are relative to corresponding time point of control. Whereas Values of resupply are relative to Low N (20 DAG).

ID	Putative coding	Low N (17 DAG)	Low N (20 DAG)	Resupply N (20 DAG)
hv_36314	Aldehyde oxidase (AO)	↑ 2	–	–
hv_29667	NCED	↑9.45	↑10.93	↓ 6.02
hv_07410	xanthine dehydrogenase (XDH)	–	↑ 2.68	↓ 3.48
hv_41833	xanthine dehydrogenase (XDH)	–	↑ 3.18	–
hv_29613	bZIP transcription factor	–	↑ 2.43	–
hv_11821	ABA-responsive elements	↑ 2	–	–
hv_36872	ABA-responsive elements	↑ 2	–	–
hv_12549	GL2 expression modulator	↑ 2.17	–	↓ 3.12
hv_13574	homology to ATHV22E	↑ 3.46	–	↓ 2.93
hv_21071	ABA-responsive protein-related	↓ 2.48	–	–

5.3.2. Cytokinins (CKs)

5.3.2.1. Cytokinins (CKs) levels

It has been reported that N^6-(Δ^2-isopentenyl)-adenine (iP)- and trans-zeatin (tZ)-type CKs accumulate in phloem and xylem, respectively, which play a role

in systemic signals to control roots and shoot growth and development (Corbesier et al., 2003; Lejeune et al., 1994; Takei et al., 2001). Two CK forms, IP (mostly the shoot's CK form) and tZ (the root's CK form), were determined using an established protocol of UPLC-MSMS. In the primary leaves of control plants, the concentration of IP showed an increase and remained at the level obtained, and then after a decrease with leaf age progression (Fig.5.9). However, a different pattern was shown in the primary leaf under N deficiency condition, IP levels stayed stable to the beginning value (at 11 DAG) until 17 DAG, and decreased with the progression of N deficiency-induced leaf senescence (Fig.5.9). When N resupplied, the further decrease in IP level that caused by N deficiency was prevented and its level slightly increased again. However, IP level wasn't completely recovered to the levels of control plants (Fig.5.9). On other hand, tZ, a major CK form, showed a different pattern than IP. Its concentration decreased with leaf age in control and N deficient plants. Although, the significant change between control and N deficient plants was started in 17 DAG, when the N deficiency-induced leaf senescence had started (Fig.5.9; 5.2). Interestingly, when N resupplied, the tZ level was even higher than control plants (Fig.5.9). This might reflect the role of tZ in delaying the senescence progression upon N resupply. While more investigations needed to figure if this increased reflected by a change CK metabolism and/or its portioning.

Additionally, the two CK-forms concentrations were also determined in 4^{th} leaves In order to have a perspective on whole plant level under the different N regimes. The same pattern of results like in primary leaves were also found in the 4^{th} leaf. While tZ concentration decreased with leaf development, the IP form increased, and both of them were lower in N deficient plants than in control and N resupplied plants (Fig.5.9).

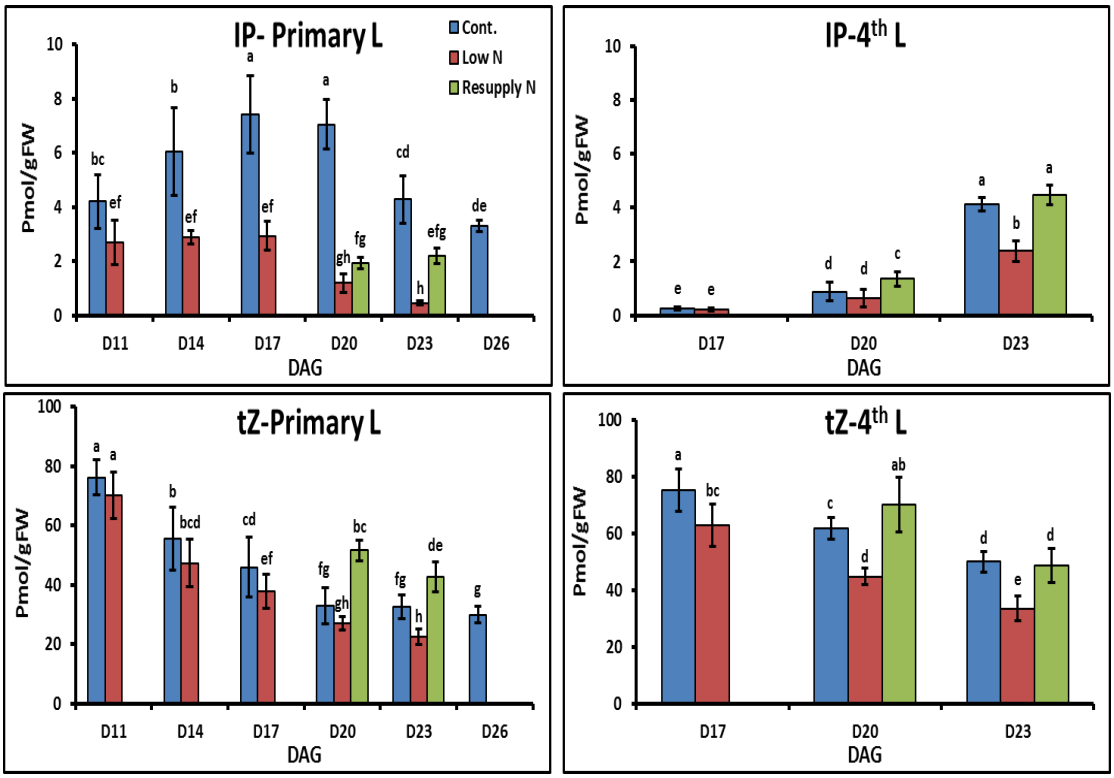

Figure 5. 9. CKs profiling of primary and 4th leaves under N deficiency-induced leaf senescence and its reversal by N resupply. IP, N^6-(⊿^{2}-isopentenyl) adenine; tz, trans-zeatin. Left side graphs for primary leaf and right side graphs for 4th leaf (4th L). Bars indicate mean ± SD (n=5-9). Letters (a-h) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5.3.2.2. The regulated genes by N availability that are related to CKs metabolism and signaling

CKs are irreversibly degraded by cleavage of the side chain, which is catalyzed by CK oxidase (CKX) (Armstrong, 1994). While the active CKs activate response regulators (ARRs) which integrate in complex molecular pathways (reviewed by El-Showk et al., 2013). Microarray analyses showed that one CK Oxidases (CKX; hv_05279) and a response regulator (hv_20179) were downregulated, and one CKX (hv_06430) was induced at 9 d after N deficiency treatment (see table 5.2), indicating an early change in CKs response with onset of N deficiency-induced leaf senescence. While more putative genes related to CKs metabolism and signaling were differentially regulated after 12 d of N deficiency treatment (20 DAG) including other CKX (hv_05278) and a response regulator (hv_21578) genes were also downregulated. It is known that to integrate the actions of the N signal at the whole plant level, one route uses

CK as messenger from root to shoot (Reviewed by Sakakibara et al., 2006). The increasing of tZ level upon N resupplied was also correlated with induction of putative genes coding a response regulator (hv_20179; 5 folds) and CK receptor AHK (hv_24272; 2 folds) (see table 5.2).

Table 5. 2. Relative Expression of putative genes related to CKs metabolism and signaling which are determined by microarray analysis. The values represent fold change. ↑: Upregulated, ↓: downregulated. Values of Low N are relative to corresponding time point of control. Whereas Values of resupply are relative to Low N (20 DAG).

ID	Putative coding	Low N (17 DAG)	Low N (20 DAG)	Resupply N (20 DAG)
hv_05279	CK oxidase (CKX)	↓6.96	↓6.19	↑14.62
hv_06430	CK oxidase (CKX)	↑5.39	↑5.94	–
hv_05278	CK oxidase (CKX)	–	↓2.43	–
hv_42702	CK oxidase (CKX)	–	–	↓2
hv_20179	homology to OsRR1	↓3.27	↓7.57	↑6.43
hv_21578	type- A RR	–	↓2.99	–
hv_24272	CK receptor	–	–	↑2.27

5.4. Metabolism rearrangement in response to N availability and natural senescence

Progress has been achieved concerning the genes controlling senescence. However, the complex metabolome changes during natural or N deficiency-induced senescence in different plant species have not been investigated in detail yet. The transcriptomic profiling revealed high reprogramming of genes for metabolism during N deficiency-induced senescence and/or during its delay when N was resupplied. The data sets were loaded to MapMan program to sort genes to the different metabolic and regulatory categories. Here, we show metabolic category after 15 d of N deficiency treatment (20 DAG) and 2 d of N resupplied (Fig.5.10). Therefore, we conducted a comprehensive profiling of metabolites including anions, C/N ratio, amino acids, sugar, and organic acids in leaves, as well as amino acids and sucrose in phloem exudate of primary leaf. This provided an extensive catalog of metabolites and their spatiotemporal cobehavior with N availability and progressing senescence. In following sections, the metabolites analyses and related transcriptomic will be presented.

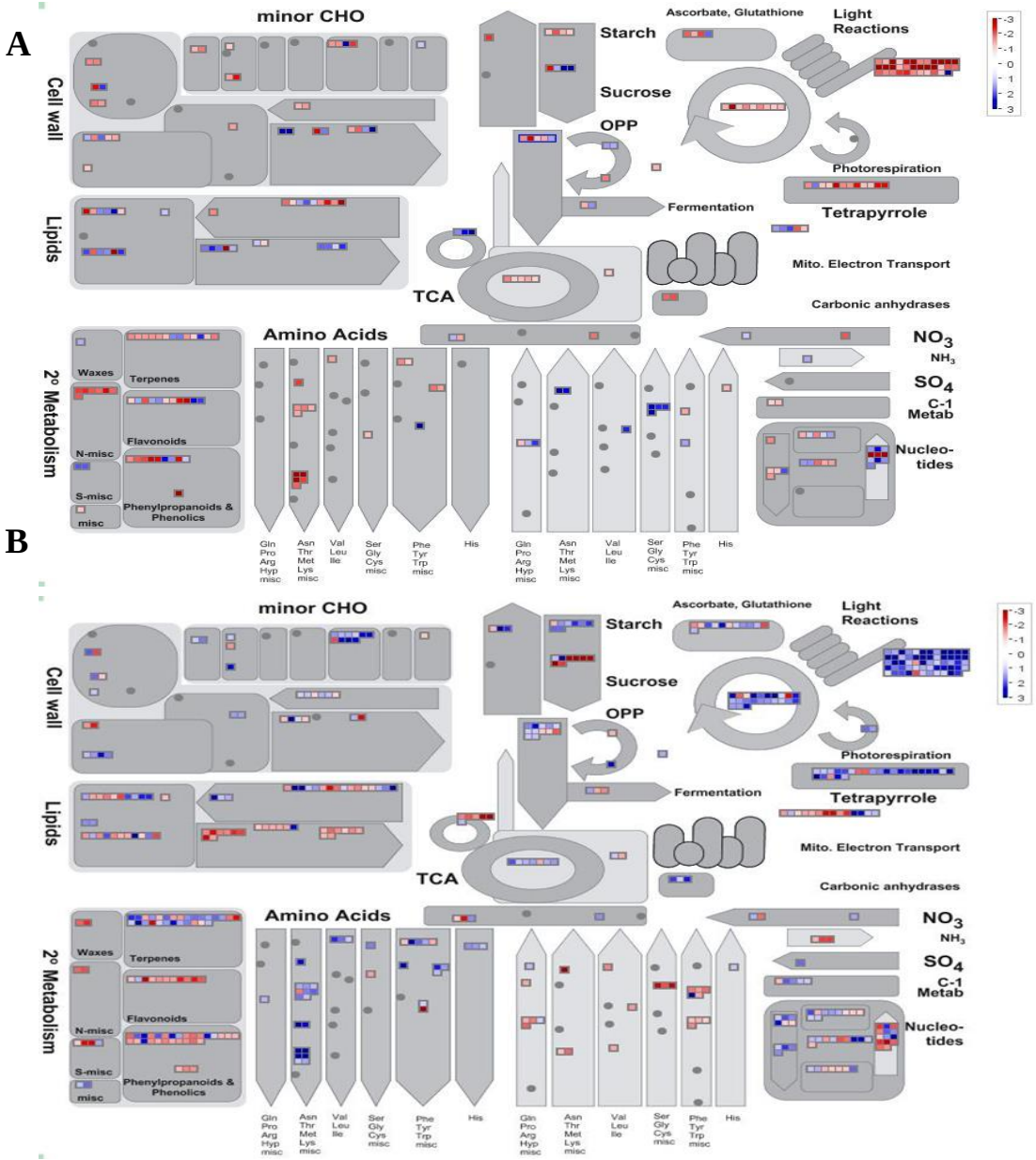

Figure 5. 10. N-responsive expression of genes involved in metabolism. Transcript levels of genes involved in metabolism after 12 d of N deficiency (A), or 2 d of N resupplied (B). The results displayed using the MapMan software downloaded from http://mapman.gabipd.org/web/guest/mapman-download. Genes that upregulated and downregulated are shown by an increasingly intense blue and red, respectively.

5.4.1. Anion levels in primary leaves under different N regimes.

To investigate the changes in nitrate level, and other anions levels in barley plants subjected to different N regimes, anion levels in primary leaves were determined by established protocol of ion chromatography (IC). In control plants, Nitrate (NO_3^-) concentration was 50 µmol.g^{-1}(FW) in mature leaf at 14

DAG, and decreased with leaf age (Fig.5.11). These observation is in agreement with Sicher (2001) who reported results for the primary leaf of barley plants that were grown in soil. The decrease in NO_3^- concentration in primary leaf could result from a decrease in xylem flow to older leaves and/or NO_3^- dilution in whole plant by rapid vegetative growth. Whereas, the NO_3^- concentration in N deficient plants was as expected almost diminished. While, N resupply recovered NO_3^- concentration to control plants after 5 d (Fig.5.11). Chloride (Cl^-) concentration was stable in control plants, and was accumulated in N deficient plants by 5- 6.8 folds higher than control plants. However, it is concentration was again declined by three folds when N was resupplied (Fig.5.11). Phosphate (PO_4^{3-}) concentration was stable in control plants during the time course of the experiment, and was lower in N deficient plants by 3-14 folds than control plants. Although, its concentration wasn't recovered by N resupply (Fig.5.11).

Microarray analyses showed that two putative genes coding phosphate transporters (hv_05148 and hv_08311) were downregulated by three folds after 12 d of N deficiency. While N resupply caused an upregulation of hv_11662 and hv_11664 that coding phosphate transporters by two folds. On other hand, sulphate (SO_4^{2-}) decreased with leaf age in control plants, and accumulated by 2-32 folds higher in N deficient than control plants. Although, when N resupplied the SO_4^{2-} concentration decreased by 2-4 folds compared to N deficient plants (Fig.5.11). In addition, the transcript of a sulphate transporter (hv_05789) was induced by 4 folds after 9 and 12 d of N deficiency treatment, and it was recovered by N resupply.

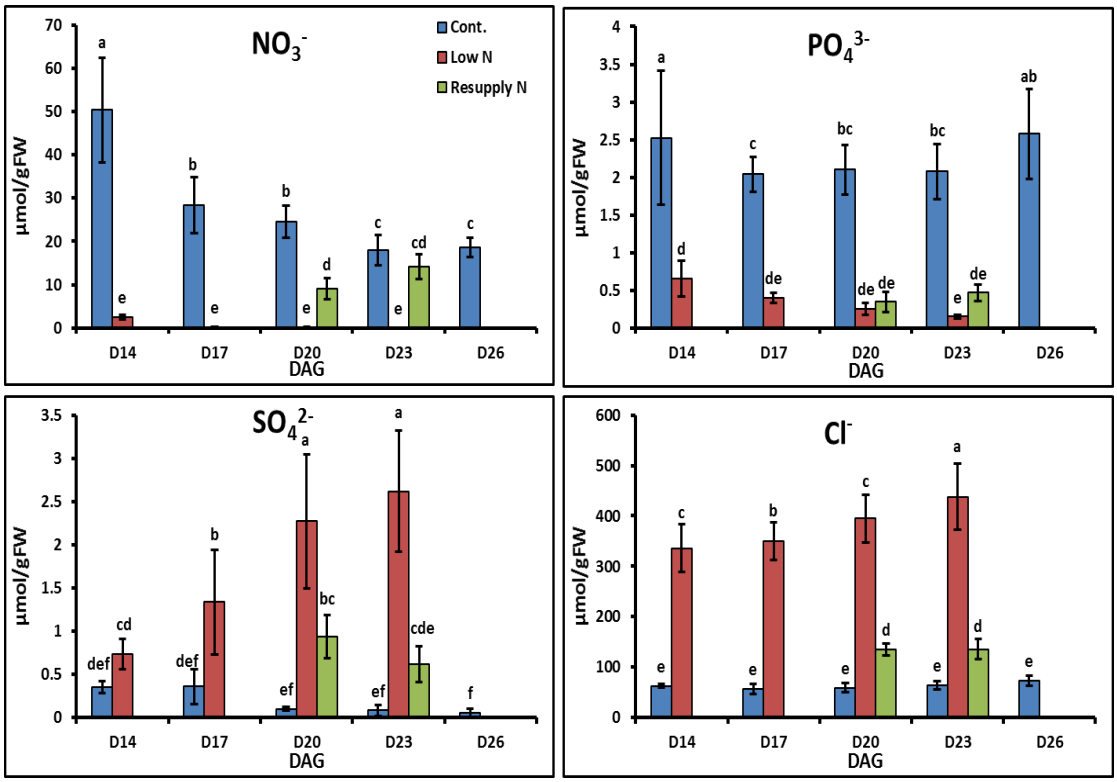

Figure 5. 11. Anions concentration in primary leaves under N deficiency-induced leaf senescence and its reversal by N resupply. Bars indicate mean ± SD (n= 5-6). Letters (a-f) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5.4.2. C/N ratio in primary leaves under different N regimes

Carbon (C) and nitrogen (N) metabolism is tightly coordinated to sustain optimal plants growth and development. In addition, C/N balance is also discussed to be a critical regulator for leaf senescence program (McAllister et al., 2012; O'Hara et al., 2013). The C%, N%, and C/N ratio were determined using elemental analyzer (EA) from frozen dried primary leaf materials. C% and C/N ratio increased with leaf age in control plants. While N% decreased mainly by withdraw the N from the old leaf to sink tissues (Fig.5.12). Hence, C/N ratio was 8.7 in control mature leaves (at 14 DAG). In N deficient plants, it was significantly higher than control plants reaching a value up to 18 at 20 DAG (Fig.5.12). However, it was much lower than a value has been recently reported for barley shoot at 14 DAG (C/N ratio= 55; Comadira et al., 2015) were plants treated with N deficiency condition after germination directly. C content was 39% in mature leaves of control plants, in agreement with Comadira et al., (2015) for barley shoots. Interestingly, C% was significantly higher in 14 DAG

in N deficient plants, and was lowered afterward compared to control plans reflecting a decrease in photosynthetic assimilation (Fig.5.12). As expected N% was also lower under N deficiency condition. N resupplied kept the C/N ratio and N% to control levels. Although there was tendency to increase C% (Fig.5.12).

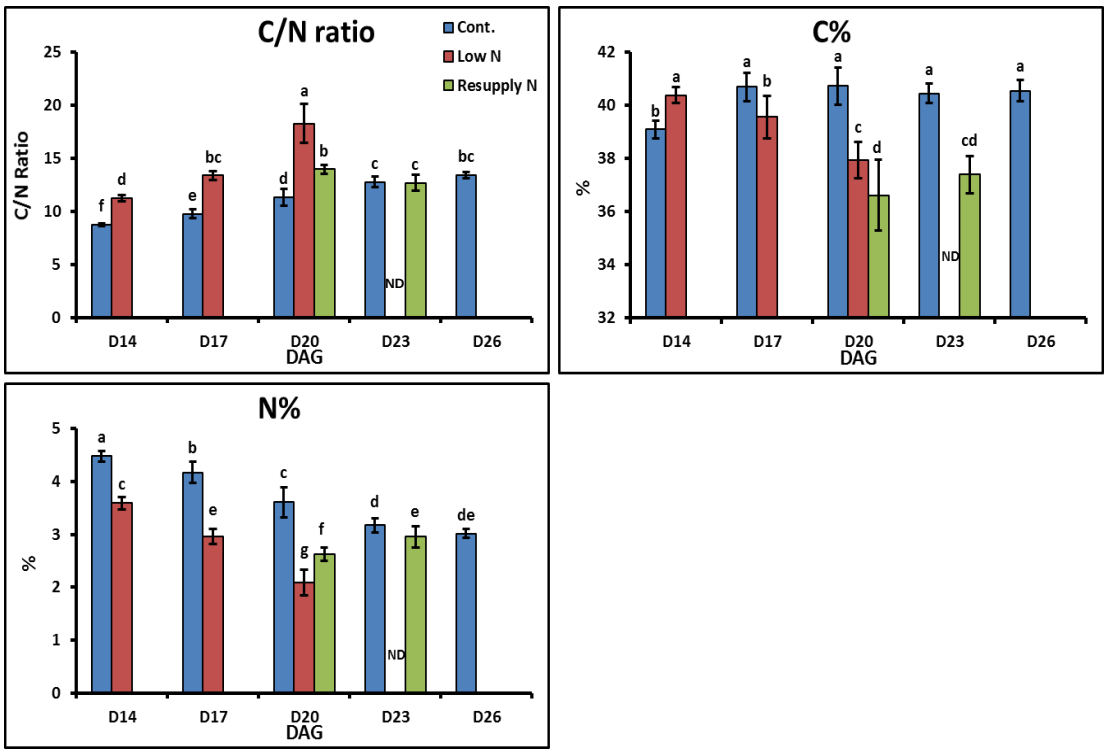

Figure 5. 12. C/N ratio, C%, and N% in primary leaves under N deficiency-induced leaf senescence and its reversal by N resupply. ND: not determined. Bars indicate mean ± SD (n= 5-6). Letters (a-e) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5.4.3. Changes of soluble amino acids in barley primary leaves in response to N deficiency and resupplied N in primary and 4th leaves

5.4.3.1. Total and individual amino acids concentrations

To determine the changes of total amino acids concentrations and its compositions in response to N deficiency and resupply N, total soluble amino acids (AAs) were extracted from primary and 4th leaves and the established protocol of ultra-performance liquid chromatography (UPLC) analysis was performed for the AAs measurement. This established protocol gives the opportunity to detect twenty soluble amino acids; we were able to detect 15 of them in barley leaf tissues, while other five amino acids were below detecting values. Total amino acids (sum of the 15 detected AAs) concentration was 20 $\mu mol.g^{-1}$ (FW) in mature leaves (at 14 DAG), decreased with leaf age in control plants reaching 10 $\mu mol.g^{-1}$ (FW) in the last measured time point (Fig.5.13). To distinguish this decrease in amino acids is a result of leaf age or whole plant development, the soluble amino acids concentration was also determined in the 4th leaf. The total amino acids in 4th leaf was around 10 $\mu mol.g^{-1}$ (FW) at 17 and 20 DAG and slightly increased at the last measured time point (23 DAG; (Fig.5.13)). As expected, the total soluble amino acids concentration was significantly lower in primary and 4th leaves of N deficient plants (Fig.5.13). The significant difference between control and deficient plants was early as 11 DAG (Data not shown). This explained by lower N assimilation in early response stage and higher remobilization of amino acids that produced from degraded proteins during senescence syndrome. Moreover, the total amino acids concentration was recovered to control plants value after 5 d of N resupplied.

Concentrations of individual free amino acids, Glu, Asp, Ser, Gly, Thr, and Ala followed the same patterns as total amino acids in primary leaves (Table 5.4). In the 4th leaf, these AAs (except Gly) and Gln were also lower under N deficiency condition during the time course, although Glu showed only significant difference at 23 DAG (Table 5.5). Interestingly, while the major amino acids concentrations decreased with leaf age in control plants, Gln concertation was constant during the experiment time course, while its concentration was highly decreased by N deficiency treatment (Table 5.4). Asn

concentration increased in control plant with leaf age, while stayed constant in N deficient plants. In addition, Leu, Ile, GABA, Pro, and Lys concentration increased in control and N deficient plants during the experiment time course. Although, Leu, Ile, Lys, and Pro concentrations were higher in primary leaf (at 23 DAG) and 4th leaf of N deficient plants (Table 5.4 and 5.5). When N resupplied, the concentration of amino acids was recovered to control plant levels.

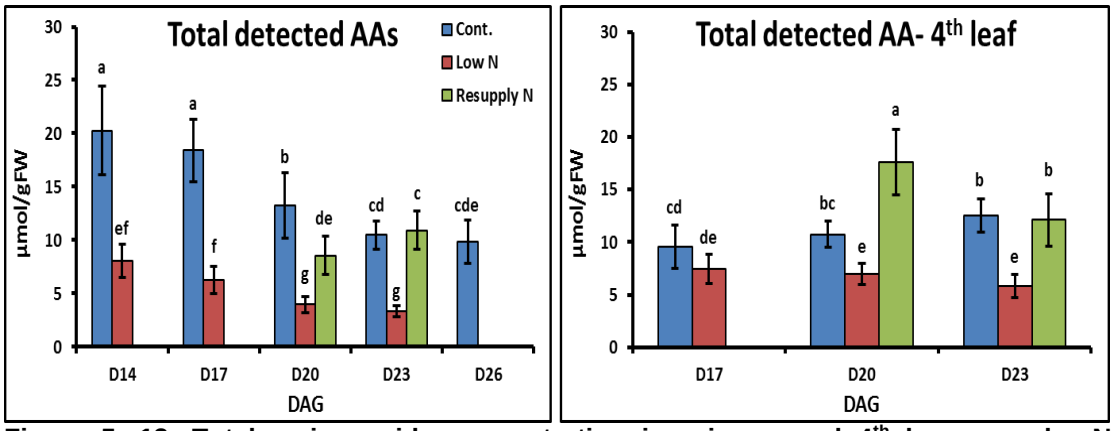

Figure 5. 13. Total amino acids concentration in primary and 4th leaves under N deficiency-induced leaf senescence and its reversal by N resupply. Bars indicate mean ± SD (n= 5-9). Letters (a-g) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5.4.3.2. The regulated genes by N availability that are related to primary N assimilation

The first step of N assimilation is to convert nitrate to nitrite, which is catalyzed in the cytosol by nitrate reductase (NR) enzyme (Meyer and Stitt, 2001). Nitrite is translocated to the chloroplast where it is reduced to ammonium by nitrite reductase (NiR), followed by assimilation of ammonium into glutamate and glutamine in the chloroplast by GS/GOGAT cycle (Lea and Forde, 1994). Microarray analyses revealed that putative genes coding nitrate reductase (hv_02646) and Glu synthase (hv_41958) were upregulated by two folds (Table 5.3) indicating the activation of primary N assimilation upon N resupplied. Three genes coding Glu dehydrogenases (GDH; hv_06558, hv_20741, and hv_41999) were down regulated after 2 d of N resupplied. Glu dehydrogenase plays a role in alternative metabolic pathways which are potentially able to incorporate ammonium into organic molecules, and it is localized in the

mitochondria of the phloem companion cells and in the cytosol of senescing organs (reviewed by Dubois et al., 2003). Comparatively, GDH (hv_41999) was induced by two folds after 12 d of N deficiency (20 DAG) (Table 5.3). This indicates a role for alternative N metabolic pathway in relation to N deficiency and plant development.

Table 5. 3. Relative Expression of putative genes related to primary N metabolism which are determined by microarray analysis. The values are presented fold changes. ↑: Upregulated, ↓: downregulated. Values of Low N are relative to corresponding time point of control. Whereas Values of resupply are relative to Low N (20 DAG).

ID	Putative coding	Low N (17 DAG)	Low N (20 DAG)	Resupply N (20 DAG)
hv_02646	Nitrate reductase (NR)	–	↓4	↑2.71
hv_41958	Glutamate Synthase	–	–	↑2.46
hv_06558	Glutamate Dehydrogenase	–	–	↓2.45
hv_ 20741	Glutamate Dehydrogenase	–	–	↓4.38
hv_41999	Glutamate Dehydrogenase	–	↑2.62	↓ 4.5

Table 5. 4. Amino acid concentrations in primary leaves under different N regimes. DAG: day after germination. Values indicate mean in nmol/g(FW) ± SD (n= 5-9). Letters (a-g) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

	Cont. 14 DAG	Cont. 17 DAG	Cont. 20 DAG	Cont. 23 DAG	Cont. 26 DAG	Low N 14 DAG	Low N 17 DAG	Low N 20 DAG	Low N 23 DAG	Resupply N 20 DAG	Resupply N 23 DAG
Glu	7128.5± 1395 a	6932.8± 797.1 a	4745.8± 1022. b	3691.8± 685.2 c	3189.8± 959.3 cd	3651.1± 915.1 c	3318.6± 824.9 cd	1635.7± 368.4 e	1037.1± 206.2 e	2668.1 ± 782.1 d	3276.1± 750.4 d
Asp	3824± 634.2 a	3023.2± 216.0 b	2487.7± 376.1 c	2299.5± 283.8 cd	2013.6± 472.3 d	1065.4± 195.1 e	432.6± 111.9 f	242.8± 39.9 f	208.9± 34.8 f	1384.2± 362.6 e	1991.4± 454.2 d
Ser	2582.4± 428.9 a	2331.2± 309.3 a	1178.9± 514.2 b	629.1± 79.3 cd	552.2± 126.1 d	1418.7± 362.3 b	717.8± 195.2 cd	228± 34.5 e	182.1± 43.5 e	615± 170.5 cd	792.3± 169.7 c
Gln	1127± 104.4 ab	1153.4± 75.8 ab	1069.6± 173.1 b	1062.3± 141.8 b	1196.3± 191.6 a	319.9± 75.8 c	162.8± 54 d	270.2± 54.9 c	331.8± 101.5 c	1057.1± 149.7 b	1137.1± 68.2 ab
Gly	718.1± 115.5 a	636.0± 86.1 b	368.3± 64.5 c	270.3± 52.7 d	265.9± 47.9 d	277.5± 67.9 d	230.5± 51.7 d	124.5± 24.2 e	82.8± 18.4 e	702.3± 60.7 ab	275.3± 53.2 d
Thr	859.8± 119.0 a	755.4± 94 b	465.9± 76.6 d	392.52± 41.68 d	398.42± 94.05 d	406.3± 116.0 d	241.8± 52.9 e	145.7± 31.5 f	118.2± 22.3 f	428.1± 59.7 d	574.9± 71.4 c
Ala	2175.5± 353.9 a	1387.4± 130.5 c	1306.5± 288.1 c	1135.1± 131 c	1240.9± 196.0 c	424.2± 56.9 d	210.1± 42.6 de	209.4± 39.0 de	203.8± 33.6 e	1234.9± 379.7 c	1856.7± 428.1 b
Asn	132.7± 13.1 bcd	154.5± 23.2 abcd	215.2± 115.4 ab	206.9± 98.6 ab	217.2± 98.4 a	100.5± 34.2 d	118.2± 66.4 cd	114.7± 39.5 cd	111.3± 19.6 cd	229.2± 105.2 a	172.6± 67.4 abc
Leu	28.4± 5.5 e	63.5± 14.1 cd	82.6± 23.7 bc	66± 10.2 c	73.8± 7.1 c	31.7± 7.9 e	97.0± 13.6 b	137.6± 18.6 a	144.4± 37.9 a	44.8± 10.7 de	40.6± 11.5 de
Ile	34.1± 7.7 ef	50.8± 11.3 cde	73.2± 15.1 b	50.8± 8.3 cd	54.7± 7.4 c	26.4± 5.2 f	58.9± 12.2 bc	90.3± 12.0 a	98.3± 24.9 a	37.8± 8.8 def	39.8± 9.1 de
GABA	28.8± 6.1 ef	109± 62.8 a	97.9± 70.3 ab	43.9± 16.8 def	77.4± 17.9 abcd	19.5± 3.5 f	29.8± 7.0 ef	68.0± 27.7 bcde	81.9± 37.5 abc	53.7± 17.2 cdef	74.7± 33.0 abcd
Pro	82.9± 29.7 def	113± 28.2 bcd	116.3± 26.8 bcd	109.8± 14.7 bcd	121.2± 29.0 bc	58.2± 20.4 f	73.8± 12.6 ef	114.5± 36.4 bcd	169.0± 41.6 a	131± 47.1 b	97.9± 21.2 cde
Lys	62.5± 11.8 de	105.4± 24.4 ab	112.2± 34.6 ab	92.6± 10.2 bc	96.1± 16.1 bc	41.7± 9.4 e	78.3± 21.6 cd	110.9± 27.2 ab	119.4± 15.5 a	60.1± 18 de	70.5± 5.9 d
Val	191.8± 26.5 a	191.3± 14.9 ab	189.0± 66 ab	155± 30.2 abcd	156.5± 29.6 abcd	104.6± 24.2 e	132.1± 24.9 de	151.2± 26.7 bcd	165.7± 36.3 abc	139.5± 36.2 cde	172.1± 23.6 abc
Phe	95.5± 17.0 bc	125.6± 18.9 a	116.2± 20.6 ab	95.8± 19.5 bc	88.2± 15.6 cd	61.6± 21.3 f	80.9± 11.4 cde	72.1± 12 def	83.3± 25.6 cde	60.0± 11.5 f	70.3± 14.1 ef

Table 5. 5. Amino acid concentrations in 4th leaves under different N regimes. DAG: day after germination. Values indicate mean in nmol/g(FW) ± SD (n= 5-9). Letters (a-f) represent the significant; Means with a common letter are not significantly different (p > 0.05).

	Cont. 17 DAG	Cont. 20 DAG	Cont. 23 DAG	Low N 17 DAG	Low N 20 DAG	Low N 23 DAG	Resupply N 20 DAG	Resupply N 23 DAG
Glu	3417.1± 696.7 ab	3131.6± 590.2 bc	3888.2± 457.9 a	3371.1± 565.8 ab	3432.8± 417.9 ab	2443.6± 492.9 c	3948.2± 667.9 a	3751.2± 722.9 ab
Asp	2082.6± 417.8 b	2241.8± 443.6 ab	2602.3± 423.8 a	1152.2± 241.0 c	1154.9± 182.9 c	588.7± 107.2 d	2372.3± 438.2 ab	2386.2± 444.8 ab
Ser	1393.0± 212.5 b	1430.5± 138.8 b	1425.3± 210.9 b	648.8± 103.8 c	725.1± 103.3 c	606.0± 111.7 c	1874.9± 388.9 a	1514.0± 316.4 b
Gln	620.6± 110.3 c	666.4± 134.8 c	644.3± 106.6 c	206.9± 39.9 d	101.3± 19.1 d	61.4± 9 d	1487.5± 288.3 a	899.7± 165.1 b
Gly	124.9± 23.1 b	97.6± 11.4 b	100.6± 14.9 b	97.1± 16.1 b	93.5± 17.5 b	58.8± 11.0 b	1837.7± 633.6 a	120.3± 19.5 b
Thr	479.5± 89.2 cd	542.0± 64.7 bc	626.2± 72.1 b	436.4± 76.9 cd	452.3± 51.7 de	286.1± 51.2 e	968.4± 175.0 a	602.1± 87.1 b
Ala	1310.4± 276.3 d	1540.5± 283.1 cd	2377.0± 307.3 a	791.6± 134.9 e	544.2± 90.8 e	809.2± 134.6 e	1706.5± 241.2 bc	1949.6± 392.6 b
Leu	25.9± 4.6 f	28.3± 3.9 ef	61.0± 5.7 c	22.7± 2.7 f	43.7± 6.8 d	102.6± 9.7 a	72.0± 16.2 b	36.5± 5.7 de
Ile	31.3± 6.3 cd	28.4± 3 d	46± 4.4 b	32.5± 5.1 cd	39.5± 4.8 bc	74.4± 5.6 a	75.6± 15.0 a	33.3± 6.0 cd
GABA	23.6± 9.1 d	73.1± 8.5 bc	225.2± 28.1 a	58.7± 7.2 c	54.9± 10.7 c	210.7± 38 a	52.4± 7 c	97.1± 24.4 b
Pro	51.2± 7.5 bc	39.8± 7.8 de	35.7± 3.4 de	62.1± 12.4 a	54.8± 9.3 abc	43.9± 2.5 cd	60.2± 9.0 ab	31.6± 6.1 e
Lys	102.0± 25.4 e	151.0± 33.2 cd	226.4± 28.7 a	161.3± 26.7 bc	118.7± 20.2 de	172.2± 35.4 bc	194.6± 42.2 ab	159.1± 28.5 bc
Val	161.1± 32.2 b	155.4± 18.5 b	165.1± 17.8 b	147.3± 23.2 b	150.0± 15.9 b	173.3± 32.6 b	218.5± 31.0 a	160.4± 33.6 b
Phe	54.8± 10.0 bcd	50.7± 10.3 bcde	60.9± 5.4 b	44.2± 6.2 de	43.1± 6 e	47.7± 10 cde	90.7± 15.8 a	57.0± 6.8 bc

5.4.3.3. Composition of amino acids under N deficiency-induced leaf senescence and its reversal by N resupply.

Tschoep et al., (2009) suggested that the reduced growth normally observed under N limitation is triggered by changes in the regulation of central metabolism than by decreased levels of total amino acids. To show the changes in predominance and importance of amino acids, individual amino acids percentage of the total measured pool were calculated. In primary and 4th leaves of control plants, Glu was the dominant amino acid followed by Asp, Ser, and Ala each generally occurring >10% abundance. While, the abundance of individual amino acids was changing with leaf age and N deficiency treatment (Table.5.6 and 5.7).

Glu which is the first synthesized amino acid in primary nitrogen assimilation and is then metabolized to other N-containing metabolites, obsessed 35% of total detected amino acids, and its percentage was fluctuated in a range of 35-40% with leaf age progression (Table.5.6). In primary leaves which were subjected to N deficiency conditions, the Glu percentage increased up to 54% at 17 DAG (9 d after N deficiency applied). Notably, it was also increased in 4th leaves at 17 DAG (47%), even before significant alterations in concentration of total amino acids between control and N- Deficient plants were noted (Table.5.7; Fig.5.13). This indicates a transient decrease in Glu catabolism under N deficiency. Corresponding changes were also observed on transcript levels of genes related to AA metabolism. For example, the transcription of putative gene coding alanine aminotransferase (AlaAT; hv_39940) that catalyzes the reversible transfer of an amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine was downregulated in microarray analyses by two and three folds after 9 and 12 d, receptively, of N deficiency treatment. However, its percentage decreased with N deficiency-induced senescence reaching 31% at 23 DAG (Table.5.6). When N resupplied, the percentage of Glu decreased to around 31% in primary and 4th leaves (Table.5.6 and 5.7).

Asp concentration was slightly increased with leaf age in primary leaves of control plants reaching 21.7% at 26 DAG, but its percentage decreased by N deficiency to a value around 6%. Whereas, Asp abundance was recovered to

control plants level when N resupplied. Same pattern of results was found in 4th leaves (Table.5.6 and 5.7). Moreover, Ala almost followed the same result patterns as Asp. In contrast, Ser abundance decreased with control leaf age. Although, it was increased at 14 DAG in N deficient plant (18%), and decreased afterward. Its abundance was also lower under N deficiency in 4th leaves (Table.5.6 and 5.7). Gln is a major transported amino acid during leaf senescence and has higher N/C ratio. Its abundance increased from 5.5% (in mature leaves) to 11.5% with control leaf age progression. Notably, Gln concentration in contrast to other AA was stable in control plant in the experiment time course (Table 5.4), resulting in an increased percentage (Table 5.6). Gln percentage transiently decreased in primary and 4th leaves that were subjected to N deficiency condition, but increased again during prolonged N deficiency- induced senescence when it is expected that N remobilization from senescing leaves is high. Leu, Ile, Lys, GABA, Phe, and Pro increased with leaf age in control and N deficient plants. Notably, they became highly abundant in primary and 4th leaves under N deficiency- condition (Table.5.6; 5.7). Possibly, they have a role in stress defense. While their percentages decreased by N resupplied. Gly and Thr percentage almost stable with leaf age and N availability except at 20 DAG, the Gly abundance increased in primary leaf after 2 d of N resupply (8.3%; Table.5.6). Interestingly, it was also higher in 4th leaf (12.72%; Table. 5.7). This increase in Gly abundance probably reflects an early photorespiration induction by N resupplied.

Table 5. 6. Amino acid composition in primary leaves under different N regimes. DAG: day after germination. Values indicate mean of individual AA percentage from the total detected AAs ± SD (n= 5-9). Letters (a-i) represent the significant; Means with a common letter are not significantly different (p > 0.05).

	Cont. 14 DAG	Cont. 17 DAG	Cont. 20 DAG	Cont. 23 DAG	Cont. 26 DAG	Low N 14 DAG	Low N 17 DAG	Low N 20 DAG	Low N 23 DAG	Resupply N 20 DAG	Resupply N 23 DAG
Glu	36.2± 0.6 def	39.4± 2.1 cd	36.4± 2.2 de	36.5± 2.2 d	32.6± 4.0 fgh	45.2± 4.5 b	55.3± 4.8 a	42.7± 2.6 bc	33± 2.1 efg	29.8± 3.5 gh	30.7± 1.9 h
Asp	20.9± 0.7 ab	18.3± 1.5 c	20.7± 1.2 ab	22± 0.7 a	20.4± 1 b	13.4± 2.1 e	7.4± 2 f	6.7± 0.7 f	6.7± 0.6 f	15.5± 1.4 d	18.4± 1.5 c
Ser	14.1± 0.5 b	13.6± 1.5 b	9.9± 2.3 d	6.2± 0.6 ef	5.7± 1 f	17.5± 1.8 a	11.8± 1.4 c	6.3± 0.8 ef	5.8± 0.5 f	6.9± 1 ef	7.4± 0.5 e
Gln	6.3± 1 ef	7± 0.6 de	9± 1.1 cd	10.3± 1.8 bc	12.4± 2.3 a	4.2± 1.8 fg	2.8± 1.3 g	7.7± 2.1 de	10.4± 1.6 bc	12.1± 2.4 ab	11± 2.5 abc
Gly	3.9± 0.3 b	3.8± 0.5 b	2.8± 0.3 bc	2.7± 0.2 c	2.9± 0.7 bc	3.7± 1.7 b	3.9± 0.7 b	3.4± 0.4 bc	2.6± 0.4 c	8.3± 1.6 a	2.6± 0.6 c
Thr	4.7± 0.1 bc	4.3± 0.2 cd	3.8± 0.8 d	3.8± 0.2 d	4.1± 0.5 d	5± 0.8 ab	4.0± 0.2 d	3.8± 0.0 d	3.8± 0.1 d	4.9± 0.6 bc	5.4± 0.6 a
Ala	10.4± 0.3 de	8.4± 0.8 f	9.7± 0.5 e	10.9± 0.7 d	12.8± 1.3 c	5.4± 1 h	3.6± 0.7 i	5.8± 0.6 gh	6.5± 0.5 g	14.2± 1.4 b	17.2± 1.2 a
Asn	0.7± 0.1 g	0.9± 0.1 fg	1.5± 0.8 defg	1.9± 0.9 cde	2.1± 0.7 cd	1.2± 0.4 efg	1.7± 0.9 def	3± 0.9 ab	3.6± 0.8 a	2.5± 0.8 bc	1.5± 0.5 defg
Leu	0.2± 0.0 f	0.4± 0.1 ef	0.7± 0.1 de	0.6± 0.1 de	0.8± 0.1 d	0.4± 0.1 ef	1.7± 0.6 c	3.8± 0.2 b	4.5± 0.5 a	0.5± 0.1 def	0.4± 0.2 ef
Ile	0.2± 0.0 g	0.3± 0.1 fg	0.6± 0.2 d	0.5± 0.1 def	0.6± 0.1 de	0.3± 0.1 fg	1.0± 0.3 c	2.5± 0.1 b	3.1± 0.3 a	0.4± 0.1 def	0.4± 0.1 ef
GABA	0.1± 0.0 d	0.4± 0.2 cd	0.7± 0.6 cd	0.4± 0.1 cd	0.8± 0.2 c	0.2± 0.0 d	0.5± 0.2 cd	1.9± 0.7 b	2.4± 0.7 a	0.6± 0.2 cd	0.8± 0.4 c
Pro	0.4± 0.1 c	0.6± 0.1 c	0.8± 0.1 c	1.0± 0.1 c	1.3± 0.1 c	0.7± 0.2 c	1.2± 0.2 c	3.2± 0.9 b	5.7± 2 a	1.3± 0.5 c	0.9± 0.1 c
Lys	0.4± 0.0 f	0.6± 0.1 def	0.8± 0.2 de	0.9± 0.1 d	1± 0.1 cd	0.5± 0.1 ef	1.3± 0.4 c	3.1± 0.5 b	3.9± 0.8 a	0.7± 0.2 def	0.7± 0.1 def
Val	1.0± 0.0 f	1.2± 0.1 ef	1.5± 0.4 def	1.4± 0.2 def	1.7± 0.2 de	1.3± 0.3 def	2.3± 0.9 c	4.1± 0.5 b	5.2± 0.3 a	1.6± 0.2 d	1.7± 0.2 de
Phe	0.5± 0.0 e	0.7± 0.1 de	0.9± 0.1 de	0.9± 0.3 d	0.9± 0.2 d	0.8± 0.3 de	1.4± 0.5 c	2.0± 0.4 b	2.6± 0.4 a	0.7± 0.2 de	0.7± 0.2 de

Table 5. 7. Amino acid composition in 4th leaves under different N regimes. DAG, day after germination. Values indicate mean of individual AA percentage from the total detected AAs ± SD (n= 5-6). Letters (a-g) represent the significant; Means with a common letter are not significantly different (p > 0.05).

	Cont. 17 DAG	Cont. 20 DAG	Cont. 23 DAG	Low N 17 DAG	Low N 20 DAG	Low N 23 DAG	Resupply N 20 DAG	Resupply N 23 DAG
Glu	36.1± 1.3 d	31.4± 0.6 e	31.2± 0.5 e	45.9± 0.5 b	49.4± 1.2 a	43.0± 2.2 c	26.7± 1 f	31.6± 1.1 e
Asp	21.7± 1.3 ab	22.5± 0.7 a	20.8± 1.2 b	16.4± 1.6 c	16.8± 0.5 c	10.8± 0.8 d	16.1± 0.7 c	21.1± 0.8 ab
Ser	13.1± 0.9 a	13.5± 0.7 a	11.4± 0.6 b	9.3± 0.3 d	10.1± 1.2 cd	10.9± 0.8 bc	12.7± 0.3 a	12.5± 0.5 a
Gln	6.1± 0.3 c	6.5± 0.9 c	5± 0.3 d	3± 0.2 e	1.4± 0.2 f	1.0± 0.1 f	9.5± 1.1 a	7.8± 0.7 b
Gly	1.2± 0.1 b	0.9± 0.1 b	0.8± 0.0 b	1.4± 0.3 b	1.4± 0.1 b	1.1± 0.1 b	12.7± 1.8 a	1.0± 0.1 b
Thr	4.7± 0.3 e	5.1± 0.0 d	5.0± 0.1 de	5.9± 0.2 b	5.6± 0.2 bc	5.2± 0.4 d	6.5± 0.3 a	5.3± 0.3 cd
Ala	12.4± 2 c	15.4± 0.5 b	19.1± 1.0 a	10.8± 1.3 d	7.9± 0.4 e	13.5± 0.9 c	10.9± 0.1 d	15.5± 0.9 b
Leu	0.3± 0.0 d	0.3± 0.0 d	0.5± 0.1 c	0.3± 0.0 d	0.6± 0.1 b	1.7± 0.3 a	0.5± 0.0 c	0.3± 0.1 d
Ile	0.3± 0.0 ef	0.3± 0.0 f	0.4± 0.0 de	0.4± 0.0 cd	0.6± 0.1 b	1.2± 0.2 a	0.5± 0.0 c	0.3± 0.0 ef
GABA	0.2± 0.1 d	0.7± 0.1 c	1.8± 0.3 b	0.9± 0.2 c	0.8± 0.2 c	3.7± 0.3 a	0.3± 0.0 d	0.9± 0.3 c
Pro	0.5± 0.0 c	0.4± 0.0 d	0.3± 0.0 e	0.8± 0.1 a	0.8± 0.1 b	0.7± 0.1 b	0.4± 0.1 d	0.3± 0.1 e
Lys	1.1± 0.2 f	1.3± 0.1 ef	1.8± 0.3 c	2.2± 0.3 b	1.7± 0.1 cd	3.1± 0.4 a	1.2± 0.1 ef	1.4± 0.2 de
Val	1.6± 0.3 c	1.5± 0.0 cd	1.3± 0.1 d	2.0± 0.1 b	2.2± 0.1 b	3.1± 0.2 a	1.4± 0.0 d	1.4± 0.1 cd
Phe	0.5± 0.0 cd	0.4± 0.0 e	0.5± 0.0 de	0.6± 0.0 b	0.6± 0.0 b	0.9± 0.1 a	0.6± 0.0 bc	0.5± 0.1 d

5.4.4. Changes in sugar concentrations and in expression of sugar metabolism genes in response to N availability

5.4.4.1. Changes in sugar concentrations in barley primary and 4th leaves in response to N deficiency and N resupply

Nitrogen and carbon metabolism are closely connected, since carbon forms the skeleton for N-containing metabolites. Additionally, it is widely discussed that sugars accumulation can trigger leaf senescence especially under stress conditions. However, sugar starvation in darkness also induced leaf senescence. Whether or not sugars are involved in regulating senescence response with low N treatment remains unresolved (reviewed by Wingler et al., 2006). To further investigate the changes in sugar concentrations in primary leaves under N deficiency and N resupply conditions, ELISA reader and ion chromatography-mass spectrometry (IC-MSMS) analyses were performed for targeted metabolites profiling. Ten sugar compounds were determined by these two methods. The results were normalized and logarithmically presented in Fig.5.14 using heat map, and for absolute values see appendix (Table 10.4). The sucrose concentrations were slightly increased with control leaf age (Fig.5.14). However, glucose and fructose concentrations were stable from 14 DAG through the experiment time course in control plants that had been grown in N-replete conditions (Fig.5.14). The results also show that sucrose concentration increased under N deficiency conditions compared to control plants, and the sugar metabolism was modulated to synthesize more sucrose for transportation to the sink tissues under N deficiency condition. Moreover, other sugars also showed an increase in their concentrations including glucose-1-P, hexose-6-P, sucrose-6-P, trehalose-6-P, fructose, and glucose in N deficient plants relative to control plants (Fig.5.14). Balazadeh et al., (2014) showed that fructose level was increased by 20 folds in N deficient Arabidopsis plants relative to control plants that had been grown in N-replete conditions. However, here we reported that in barley plants the fructose level was less than two folds changed, and glucose showed minority change under N deficiency condition. In contrast, UDP-glucose concentrations were lower in N deficient plants than control plants. Generally, the differences in sugars concentrations between control and N- deficient plants were small, indicating the ability of

barley leaves to transport it and/or make more investment in structural polysaccharides than other plant species, beside inhibits photosynthesis. When N resupplied, the concentration of sucrose-6-P and sucrose decreased at 20 DAG. As well as fructose concentrations were lowered at 20, and 23 DAG by 1.9- and 4 folds, receptively, relative to N deficient plants.

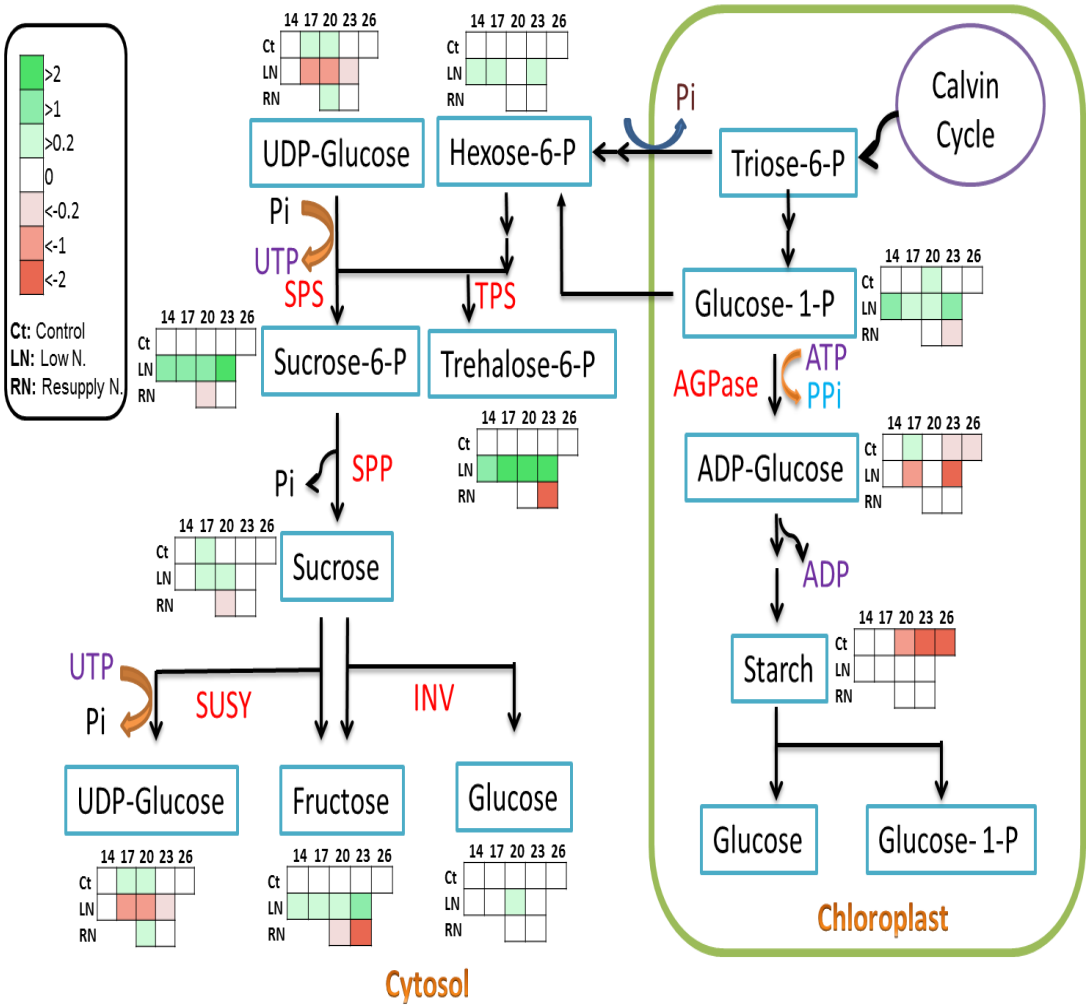

Figure 5. 14. Sugar metabolism in primary leaves under N deficiency-induced leaf senescence and its reversal by N resupply. Control results logarithmic (log_2) normalized to the value of control plant at 14 DAG; Low N results: logarithmic normalized to corresponding control time point, while resupply N results: logarithmic normalized to corresponding low N time point. Ct: control; LN: low N; RN: resupply N; AGPase: ADP-Glc pyrophosphorylase; TPS: Trehalose-6-P synthase; SPS: Sucrose-phosphate synthase; SPP: Sucrose-phosphate phosphatase; SUSY: Sucrose synthase; INV: Invertase. (n= 5-9); P< 0.05.

Interestingly, the starch concentration decreased with leaf age independent on N supply in barley plant (Fig.5.14). This observation is in contrast with the

finding in other plant species such as maize (Sekhon et al., 2012), which accumulates starch with leaf age and senescence syndrome. There was tendency to lower starch concentration after 2 d of N resupply, but it wasn't significant between the different experiments (data not shown). This indicates the demand for sugar molecules after 2 d of N resupplied for energy and amino acids biosynthesis. Moreover, ADP-Glc also showed a decrease during the experiment time course, but it was more pronounced in N deficient plants (Fig.5.14). When N resupplied to N starved plants, the concentrations of starch and ADP-Glc were slightly increased after 5 d of N resupply. It is interesting to check the possibility that barley shifts sugar metabolism toward structural polysaccharides in the older leaf.

Recently, trehalose-6-P (T6P) has received more attention to have an important role in modulating leaf senescence. It has been reported that in otsB-expressing Arabidopsis plants, T6P accumulated less strongly during senescence than in wild-type plants, showing a similar phenotype as described for plants overexpressing the SnRK1 gene, KIN10, including reduced anthocyanin accumulation and delayed senescence program (Wingler et al., 2012). In barley plants, T6P showed different concentration patterns in developmental senescence and N deficiency-induced leaf senescence (Fig.5.14). T6P concentration decreased in old leaf than mature leaf of control plant, but the differences were not significant. However, its concentration increased by 2.7-, 11-, 9-, and 10- folds after 6, 9, 12, 15 d, respectively, of N deficiency treatment (Fig.5.14). N resupply returned the T6P concentration to control plants level after 5 d, but there was no significant change after 2 d. This means that T6P could play a role in delaying senescence in late response to N availability than an early and fast response to N resupply.

Additionally, the sucrose and starch concentrations were also determined in 4th leaves that reached its full length at 20 DAG in order to have a perspective on whole plant level under the different N regimes. Sucrose concentration in 4th leaf of control plants was stable through the experiment time course, and it accumulated under N deficiency conditions (Fig.5.15). The starch concentration started to decrease in the early of leaf development for control plants. But it

accumulated by 1.8-, 2.8-, and 4- folds in 17, 20, and 23 DAG, respectively, in N deficient plants relative to control plants (Fig.5.15). This indicates that there is a different sugar metabolism between young and old leaves of barley plants under N deficiency condition. On other hand, the concentrations of sucrose and starch decreased upon N resupplied (Fig.5.15).

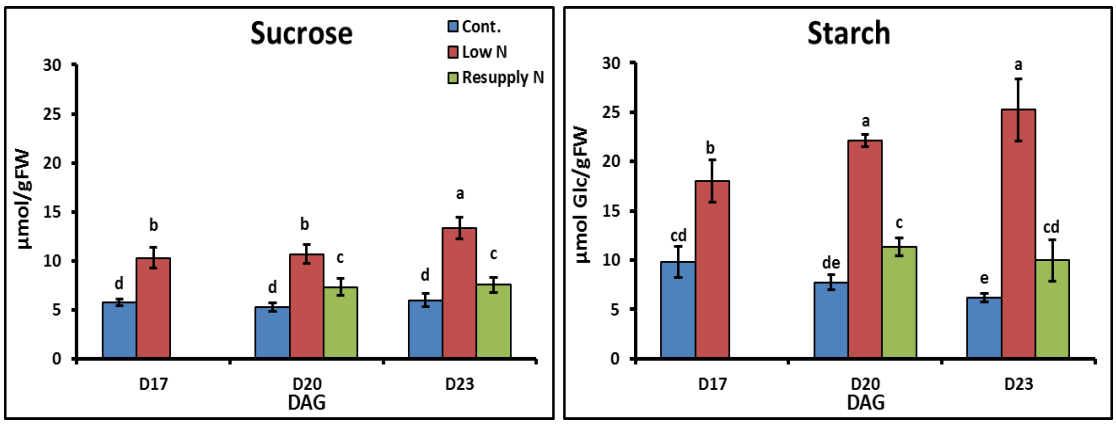

Figure 5. 15. Sucrose and starch level changes in the 4th leaves under different N Regimes. Bars indicate mean ± SD (n= 6). Letters (a-e) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5.4.4.2. Genes regulated by N availability that are related to sugar metabolism

Transcriptomic profiling determined that many genes related to sucrose metabolism were differentially regulated. Such as, two putative genes coding sucrose synthase (SuSy), hv_03530 and hv_03531, were upregulated by 8 folds after 9 and 12 d of N deficiency treatment. As well as, a gene coding cell wall invertase, hv_19372, was upregulated by 2.5 folds after 12 d of N deficiency treatment (Table 5.8). A putative gene coding fructokinase, hv_23265, that converts fructose to fructose-1-P was downregulated by 15- and 5.5 folds after 9 and 12 d of N deficiency treatment, respectively (Table 5.8). When N resupplied, the two putative genes coding sucrose synthase (SuSy), hv_03530 and hv_03531, were recovered besides another gene, hv_20661, was downregulated by 4 folds. Whereas, genes coding cell wall invertase, hv_22536, and vacuolar invertase, hv_03914 and hv_42447, were downregulated by 13-, 60-, and 25 folds, respectively. In addition the

transcription of two genes coding fructokinase, hv_23265 and hv_11681, were upregulated 28- and 2 folds, respectively (Table 5.8). Moreover, many genes related starch metabolism were differentially regulated in response to N supply. Some genes related to starch metabolism were downregulated by N deficiency condition such as beta amylase, isoamylase, maltose transporter, and starch synthase (Table 5.8). Although, the genes coding starch degradation enzymes were mainly upregulated after 2 d of N resupplied, and starch synthase, hv_36595, was downregulated by 2.5 folds (Table 5.8).

Table 5. 8. Relative Expression of putative genes related to sugar metabolism which are determined by microarray analysis. The values are presented fold change. ↑: Upregulated, ↓:downregulated. Values of Low N are relative to corresponding time point of control. Whereas Values of resupply are relative to Low N (20 DAG).

ID	Putative coding	Low N (17 DAG)	Low N (20 DAG)	Resupply N (20 DAG)
hv_03530	Sucrose synthase (SuSy)	↑9.15	↑9.06	↓7.52
hv_03531	Sucrose synthase (SuSy)	↑8.69	↑8.51	↓8.06
hv_20661	Sucrose synthase (SuSy)	–	–	↓4.53
hv_19372	Cell wall invertase	–	↑2.62	–
hv_22536	Cell wall invertase	–	–	↓13.55
hv_03914	Vacuolar invertase	–	–	↓60.55
hv_42447	Vacuolar invertase	–	–	↓25.28
hv_23265	Fructokinase	↓15.14	↓5.46	↑27.86
hv_11681	Fructokinase	–	–	2.35
hv_12878	Starch cleavage-beta amylase	–	–	-2.5
hv_22195	Starch cleavage-beta amylase	–	↓2.16	↑2.97
hv_01022	Degradation starch.D enzyme	–	–	↑2.48
hv_02206	Degradation starch.D enzyme	–	–	↑4.69
hv_02207	Degradation starch.D enzyme	↓3.51	–	↑3.46
hv_02208	Degradation starch.D enzyme	–	–	↑4.56
hv_04176	Degradation starch.D enzyme	–	↓4.08	↑3.73
hv_45799	Degradation starch.transporter	–	↓2.53	↑3.68
hv_08578	Glucan water dikinase	↓4.79		–
hv_03439	Degradation starch.ISA3	–	↓2.13	–
hv_36596	Starch synthase	–	–	↓2.5
hv_05868	Starch branching enzyme	–	–	↑43.41
hv_23288	Starch debranching enzyme	–	↓4.63	↑ 4.72
hv_12166	T6P synthase (TPS)	–	↓2.11	↑ 2.22
hv_10051	T6P phosphatase (TPP)	↓2.93	–	–
hv_41351	T6P phosphatase (TPP)	–	–	↓2.97
hv_04008	Potential TPS/TPP	–	↓2.69	–
hv_04009	Potential TPS/TPP	–	↓5.62	↑6.32

Transcriptomic profiling also determined that putative gene coding T6P synthase (TPS; hv_12166) that converts of UDP-Glc and Glc-6-P to trehalose

6-phosphate (T6P) followed by hydrolysis of T6P to trehalose in a reaction catalyzed by T6P phosphatase (TPP), was downregulated by 2 folds, and two putative genes coding trehalose potential TPS/TPP, hv_04008 and hv_04009, were also downregulated by 2.5- and 5- folds, respectively, after 12 d of N deficiency (Table 5.8). Moreover, a TPP gene (hv_10051) was downregulated by 3 folds after 9 d of N deficient leaf. TPS and trehalose potential TPS/TPP genes, hv_12166 and hv_04009, transcription were recovered, and another TPP gene was also downregulated by 3 folds after 2 d of N resupply (Table 5.8).

Transcriptomic profiling indicates an effect of N availability on expression of genes of cell wall metabolism. The Transcriptomic profiling after 9 d of N deficiency treatment (17 DAG) revealed that three putative genes for cellulose synthases (hv_41784, hv_01787, and hv_13146) were up regulated by 2 folds each. While, a transcription of genes related to hemicellulose, and two genes (hv_13495 and hv_13496) coding cell wall precursor synthesis, ADP-glucose pyrophosphorylase, were down regulated. Moreover, the transcription of putative genes related to cell wall degradation were downregulated (Table 5.9). On other hand, The transcriptomic profiling after 12 d of N deficiency treatment (20 DAG) coincided by senescence progression revealed that the transcription of gene related to cell wall precursor synthesis (ADP-glucose pyrophosphorylase and UDP-glucuronate decarboxylase), cellulose synthase, and cell proteins were downregulated. In contrast, the transcription of genes related to cell wall degradation such as cellulose were upregulated (Table 5.9). When N resupplied, the transcription of genes related to cell wall precursor synthesis, cellulose synthesis, cell wall proteins, and cell wall modification were upregulated. However, two putative genes coding pectinesterase and pectinacetylesterase were downregulated by N resupply (Table 5.9).

Table 5. 9. Expression of putative genes related to cell wall metabolism. The values are presented in fold change. ↑: Upregulated, ↓: downregulated. Values of Low N are relative to corresponding time point of control. Values of resupply are relative to Low N (20 DAG).

ID	Putative coding	Low N (17 DAG)	Low N (20 DAG)	Resupply N (20 DAG)
hv_05738	Cell wall proteins.LRR	↓2.75	↓ 5.5	↑3.65
hv_45236	Cell wall proteins.LRR	↑ 3.18	↑ 4.5	↓ 2.2
hv_00311	Cell wall proteins.AGPs	–	↓3.16	–
hv_18736	Cell wall proteins.AGPs	–	↓3.32	–
hv_16495	Cell wall proteins.AGPs	–	–	↑3.89
hv_21979	Cell wall proteins.AGPs	–	–	↓4.32
hv_18985	Cell wall proteins.RGP	–	↓ 3.43	↑2.17
hv_39520	Cell wall proteins.RGP	–	↓ 2.89	–
hv_05583	Cell wall modification	↓2.53	–	–
hv_24390	Cell wall modification	↑2.57	–	–
hv_02324	Cell wall modification	–	↑2.14	↑ 2.3
hv_06937	Cell wall modification	–	–	↑2.89
hv_15301	Cell wall modification	–	–	↑6.02
hv_24491	Cell wall modification	–	–	↑3.27
hv_14427	Pectin esterases.PME	–	↑2.48	–
hv_27339	Pectin esterases.PME	–	↓3.25	–
hv_09267	Pectin esterases.acetyl esterase	–	↑3.68	↓5.17
hv_11507	Pectin esterases.acetyl esterase	–	↓ 2.27	–
hv_11510	Pectinesterases.acetyl esterase	–	↓ 2.68	–
hv_19529	Pectin esterases.PME	–	–	↓2.97
hv_01787	Cellulose synthesis.cellulose synthase	↑2.08	–	–
hv_15033	Cellulose synthesis.cellulose synthase	–	↓2.81	↑ 2.75
hv_31920	Hemicellulose synthesis	↓2.41	–	–
hv_41784	Cellulose synthesis	↑ 2.08	–	–
hv_24934	Cellulose synthesis	–	–	↑ 2.83
hv_13146	Cellulose synthesis.COBRA	↑2.46	–	–
hv_13495	Cell wall.precursor synthesis	↓2.99	–	↑2.07
hv_13496	Cell wall.precursor synthesis	↓3.27	↓2.44	↑2.28
hv_13458	Cell wall.precursor synthesis.UXS	–	↓2.99	↑2.57
hv_36062	Cell wall.precursor synthesis.UGE	–	–	↓2.08
hv_08900	Cell wall.precursor synthesis.GAE	–	–	↑2.33
hv_36062	Cell wall.precursor synthesis.MUR4	–	–	↓2.08
hv_22710	Cellulases and beta -1,4-glucanases	↓5.24	–	↑44.02
hv_38529	Cellulases and beta -1,4-glucanases	↓5.28	–	–
hv_02928	Cellulases and beta -1,4-glucanases	–	–	↓ 2.33
hv_41054	Cellulases and beta -1,4-glucanases	–	–	↑ 2
hv_41394	Cellulases and beta -1,4-glucanases	–	–	↓ 2.19
hv_13386	Cellulases and beta -1,4-glucanases	–	↑7.89	–
hv_22711	Cellulases and beta -1,4-glucanases	–	↑11.96	–
hv_38796	Mannan-xylose-arabinose-fucose	↓8.11	6.1	–
hv_43038	Mannan-xylose-arabinose-fucose	–	↑3.36	–
hv_21735	Pectate lyases and polygalacturonases	↑ 2.41	↑2.93	↓5.82
hv_13657	Pectate lyases and polygalacturonases	–	↓2.73	–
hv_15447	Pectate lyases and polygalacturonases	–	↓ 3.84	↑2.53
hv_37543	Pectate lyases and polygalacturonases	–	↑9.45	–

5.4.5. Phloem exudate analyses of primary leaf in relation to natural senescence and N supply

5.4.5.1. Sucrose and amino acids export rate through Phloem is related to natural senescence and N supply

Although senescence syndrome is characterized by assimilate remobilization from senescing leaf to sink tissues, not much is known about the level and compositions of sugar and amino acids that are actually transported through phloem under different leaf senescence inducing conditions in large time course scale. To figure this questions, the phloem exudate of primary leaf was collected in 200µl of 5 mM Na_2-EDTA solution for two hours (5-7 biological replicates; 2 primary leaves each), and further was analyzed for sucrose concentration, using ELISA reader, and for amino acids concentration, using UPLC, and then results were normalized to leaf dry weight. The results revealed that no significant changes in sucrose export rate (around 1 $\mu mol.g^{-1}(DW).h^{-1}$) was found from primary leaf of control plants during the experiment time course (Fig.5.16). However, the sucrose export rate was higher in N deficient plants relative to control plants. It was 2, 1.5 and 2 folds higher at 14, 17, and 20 DAG, respectively, (Fig.5.16). Amino acids export rate was more interestingly, the total amino acids (sum of 15 detected amino acids) export rate was stable with 2 $\mu mol.g^{-1}(Leaf\ DW).h^{-1}$ up to 20 DAG in control plant, and it increased afterward, coinciding with the starting of natural leaf senescence process and activating N compounds remobilization. The total amino acids export rate was decreased to 59% and 41% at 14 and 17 DAG, respectively, in N deficient plants compared to control plants. However, it was only significant at 17 DAG. In addition, there was no significant difference between control and N deficient plants at 20 DAG (Fig.5.16). Notably, the total amino acids export rate was recovered to control plants levels after 2 d of N resupplied (Fig.5.16). Similar to pattern of total amino acids export rate, the level of Gln, Glu, Thr, Ala, Asn, Val, Ile, Leu, and Phe in phloem exudate increased with leaf age of control plants. While Asp level showed no changed during the experiment time course. Moreover, Ser, Gly, GABA, and Lys export rate decreased with control leaf age (Fig. 5.16; Table 5.10). Interestingly, Gln that has a higher N/C ratio, export rate was highly decreased in phloem exudate of N deficient leaf compared to control

plants (Fig. 5.16), besides Asp also decreased by N deficiency. While Glu, Ser, and Ala export rate didn't show significant change, except at 17 DAG was less compared to control plants in Ala case, and tendency to decrease in Glu and Ser case (Fig. 5.16; Table 5.10). In addition, Gly, Thr, and GABA export rates didn't show a significant change with progression of N deficiency-induced senescence (17 and 20 DAG) (Table 5.10). The export rates of Asn, Ile, Leu, Lys, Val, and Phe were higher at 20 DAG in N deficient than control plants. Their export rate has tendency to increase earlier, however it wasn't significant (Table 5.10). This indicates that there is a different preferring for the amino acids remobilization during natural and N-induced leaf senescence. The export rates for almost all amino acids were recovered to control plants after 2 d of N resupplied (Fig. 5.16; Table 5.10).

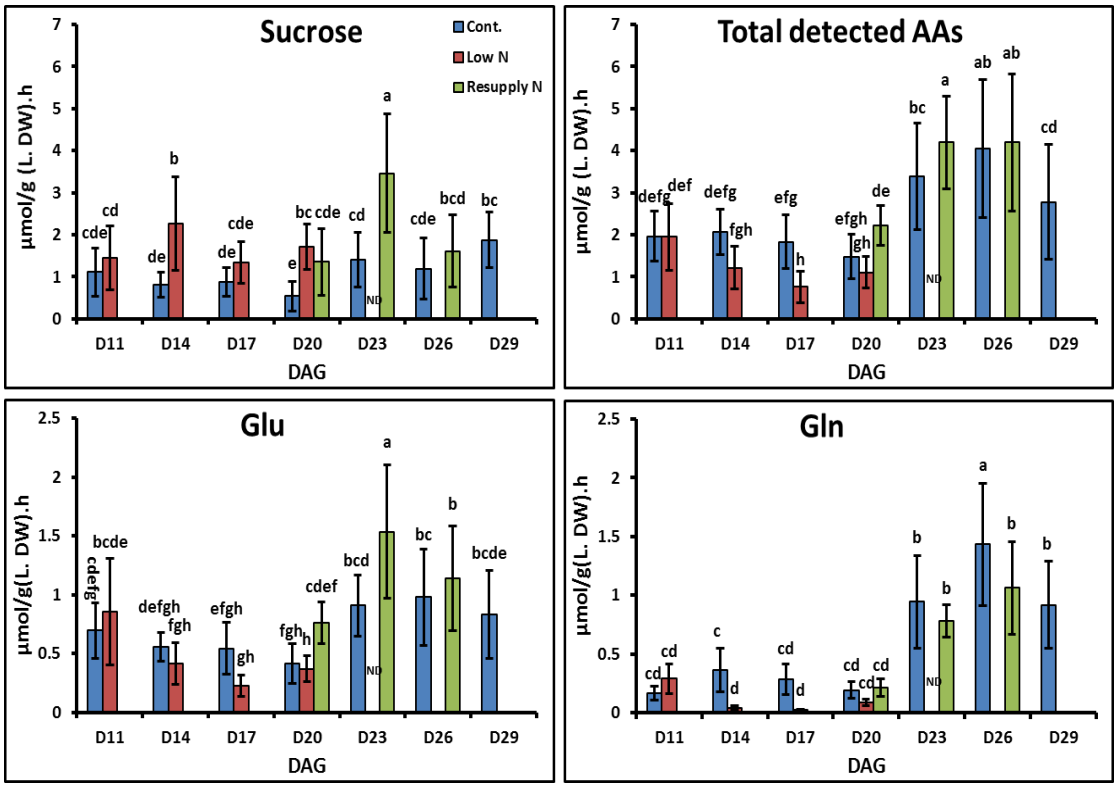

Figure 5. 16. Sucrose and amino acids export rate in the phloem of primary leaves in response to natural senescence and N availability. Bars indicate mean ± SD (n= 5-7), the results were normalized to leaf dry weight (L. DW).ND: not determined. Letters (a-h) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

Table 5. 10. Individual amino acids export rate in the phloem of primary leaves in response to natural senescence and N availability. Values indicate mean of individual AA concentration in nmol.g^{-1}(leaf DW).h^{-1} ± SD (n= 5-6). Letters (a-h) represent the significant; Means with a common letter are not significantly different ($p > 0.05$). DAG: day after germination.

	Glu	Asp	Ser	Gln	Gly	Thr	Ala	Asn
Cont. 11 DAG	697.7± 237.1 cdefg	400.5± 106.9 b	204.7± 51.6 bc	165.2± 62.0 cd	76.1± 21.6 bc	141.3± 53.8 cde	128.0± 38.6 def	5.8± 3.1 ef
Cont. 14 DAG	558± 118.8 defgh	341.3± 62.9 bc	252.0± 71.3 ab	363.0± 188.9 c	87.3± 26.9 b	138.9± 51.1 cde	171.9± 46.2 def	5.0± 2.2 ef
Cont. 17 DAG	544.4± 217.5 efgh	302.2± 88.2 bc	170.5± 49.2 cde	283.1± 128.7 cd	39.9± 6.2 ef	125.2± 45.9 def	201.8± 73.9 cde	6.5± 2.6 ef
Cont. 20 DAG	417.7± 170 fgh	261.3± 119.4 cd	123.3± 56.0 defg	192.4± 68.3 cd	15.2± 4.3 h	66.4± 18.4 ef	130.6± 49.7 def	6.5± 1.9 ef
Cont. 23 DAG	908.8± 261.5 bcd	327.1± 100.6 bc	181.6± 53.3 bcd	943.0± 397.0 b	52.1± 16.3 de	216.2± 73.4 b	345.4± 131.0 b	25.3± 12.1 bcd
Cont. 26 DAG	978.8± 406.7 bc	347.2± 137.8 bc	109.9± 47.1 defg	1432.2± 522.8 a	35.2± 7.2 efg	200.9± 87.2 bc	363.3± 167.8 b	27.2± 9.1 bc
Cont. 29 DAG	832.9± 377 bcde	259.5± 80 cd	94.35± 39.71 fg	918.6± 373.1 b	19.2± 5.0 fgh	183.7± 73.8 bcd	227± 92.1 bcd	28.2± 14.6 bc
Low N 11 DAG	855.0± 452.6 bcde	319.7± 113.3 bc	224.8± 124 bc	288.6± 123.8 cd	127.4± 37.5 a	145.6± 56.5 cd	139± 60.2 def	6.6± 4.6 ef
Low N 14 DAG	413.4± 177.4 fgh	156.1± 62.9 de	186.0± 87.5 bcd	40.2± 16.7 d	67.2± 18.6 cd	118.9± 56.5 def	114.4± 50.8 def	2.7± 0.8 ef
Low N 17 DAG	225.3± 89.1 gh	82.8± 34.5 e	100.6± 39.9 efg	22.6± 5.9 d	39.2± 7.5 ef	61.6± 24.9 ef	58.2± 21.5 f	1.2± 1. f
Low N 20 DAG	371.5± 108.1 h	138.4± 21.7 de	68± 19.0 g	87.5± 28.3 cd	18.8± 6.3 fgh	54.0± 15.0 ef	87.9± 32.5 ef	16.5± 8 de
Resupply N 20 DAG	763.9± 176.3 cdef	305.3± 56.4 bc	162.3± 56.0 cdef	213.5± 75.2 cd	18.1± 5.5 gh	111.4± 26.4 def	309.1± 87.1 bc	54.6± 15.7 a
Resupply N 23 DAG	1535.3± 566.4 a	553.3± 204.5 a	318.4± 78.1 a	781± 136.9 b	76.2± 27.5 bc	375.5± 93.4 a	640.6± 197.4 a	22.3± 3.7 cd
Resupply N 26 DAG	1138.1± 443.4 b	378.1± 123.4 bc	219.7± 70.5 bc	1064.4± 394.8 b	53.6± 19.9 cde	317.2± 113.2 a	565.5± 243.2 a	35.6± 11.2 b

Table.5.10. Continued

	Leu	Ile	GABA	Pro	Lys	Val	Phe
Cont. 11 DAG	7.7± 2.7 g	15.6± 5.8 e	43.3± 11.5 b	8.5± 0.3 cd	36± 17.3 ab	52± 20.9 cdef	10.3± 4.2 d
Cont. 14 DAG	9.7± 3.1 g	14.4± 5.5 e	37.4± 12.8 bc	29.1± 10.6 ab	8.8± 3.4 fg	50.6± 20.2 def	11.4± 5.6 d
Cont. 17 DAG	11.5± 7.2 efg	13.7± 6.6 e	33.3± 8.9 bcd	31± 11.9 ab	15.7± 10.2 defg	48.5± 22.7 ef	15.4± 9.3 bcd
Cont. 20 DAG	11.1± 4.2 fg	11.2± 5.5 e	34.5± 12.4 bcd	21.9± 12.8 bc	7.4± 2.4 g	27.9± 6.9 f	9.2± 4.2 d
Cont. 23 DAG	27.4± 10.5 cd	31.9± 12.5 bcd	25.6± 8.1 de	27.8± 3.3 ab	22.1± 8.8 cde	91.3± 36.8 ab	32.6± 17.0 a
Cont. 26 DAG	34.5± 12.9 bc	33.1± 12.8 bc	20.2± 3.8 e	24.6± 12 ab	22.5± 9.4 cde	90.4± 36.3 bcd	30.4± 10.8 a
Cont. 29 DAG	43.3± 23.3 b	40.6± 18.7 b	26.1± 7.8 cde	21.3± 11.6 bc	27.8± 14.1 bc	85.8± 35.7 abc	22.9± 9.8 abc
Low N 11 DAG	13.8± 5.1 efg	20.1± 7.0 de	58.8± 19.7 a	20.4± 13.7 bc	16.7± 5.3 cdefg	74.9± 26.8 bcde	13.9± 3.4 bcd
Low N 14 DAG	10.4± 5.8 fg	15.0± 8 e	36.1± 9.6 bcd	28.9± 2.8 ab	12± 7.4 efg	74± 36.5 bcde	10.7± 6.3 d
Low N 17 DAG	22.2± 14.2 def	21.3± 13.1 cde	26.7± 7.6 cde	1.3± 0.2 d	20± 12.8 cdef	21.9± 10.6 f	14.6± 10.8 bcd
Low N 20 DAG	76.8± 16.6 a	61± 12.3 a	17.6± 4.7 e	2.7± 1.6 d	43± 12.4 a	68± 27.0 bcde	23.8± 9.5 abc
Resupply N 20 DAG	14.7± 5.8 efg	16.9± 5.5 e	44.4± 13.0 b	32.5± 7.8 ab	24.8± 8.6 bcd	68.4± 27.74 bcde	13.7± 6.0 cd
Resupply N 23 DAG	23.9± 5.7 cde	30.1± 6.8 bcd	39.0± 12.1 b	24.2± 15.5 abc	17.5± 6.1 cdefg	118.3± 30.2 a	24.4± 4.4 ab
Resupply N 26 DAG	32.0± 11.6 bcd	34.4± 12.3 b	24.5± 5.7 de	37.1± 20.6 a	17.7± 6.7 cdefg	96.4± 33.7 ab	28.7± 10.3 a

5.4.5.2. Abundance of amino acids in the phloem is related to natural senescence and N supply

It was important to focus on changes in individual amino acid levels at different time points and N treatment conditions. In phloem exudate of control mature leaf (11 DAG), Glu was the most abundant amino acid, which occupy 34.5% of total amino acids, followed by Asp (20%), Ser (10.4%), Gln (8.1%), Thr (6.9%), Ala (6.4%), and Gly (4.1%) (Table 5.11). Notably, Gln was the most abundant amino acid in phloem exudate during natural leaf senescence, which occupied 38.2 and 32.7 % from the total amino acids at 26 and 29 DAG, respectively. Followed by Glu (29.3%), Asp (9.5%), Ala (7.9%), Ser (3.3%), and Val (3%) at 29 DAG (Table 5.11). Nevertheless, the abundance of Asp, Ser, Gly, and GABA decreased with natural leaf senescence compared to their abundance in mature leaves (Table 5.11).

Glu was the most abundant amino acids upon N deficiency treatment and during N deficiency-induced leaf senescence (around 33%), followed by Asp (12%), Ala (8.4%), Gln (8%), Leu (6.6%), Val (6.6%), Ser (6.5%), Ile (5.3%) at 20 DAG, and other amino acids were below 5% of total amino acids in phloem exudate (Table.5.11). The abundance of Leu, Ile, Lys, Val, Phe, and Asn increased in N deficiency- induced senescence compared to corresponding time points of control plant. While the abundance of Asp and Gln decreased. GABA and Pro increased in early N deficiency treatment, but decreased then after (Table.5.11). The abundance of Asp, Gln, Ala, Asn, and Pro increased after 2 d of N resupplied. However, abundance of Leu, Ile, Lys, Val, and Phe decreased, and other amino acids didn’t show a significant change (Table.5.11).

Table 5. 11. Individual amino acid percentage in phloem exudate of primary leaves in response to natural senescence and N availability. Values indicate mean of individual AA percentages from the total detected amino acids ± SD (n= 5-6). Letters (a-g) represent the significant; Means with a common letter are not significantly different (p > 0.05). DAG, day after germination.

	Glu	Asp	Ser	Gln	Gly	Thr	Ala	Asn
Cont. 11 DAG	34.5± 2.9 abc	20.3± 1.0 a	10.4± 0.7 bc	8.1± 0.9 fg	4.1± 1.4 c	6.9± 0.9 bc	6.4± 0.5 d	0.3± 0.2 e
Cont. 14 DAG	27.2± 2.3 ef	16.8± 2.6 bc	12.1± 1.0 ab	16.6± 4.3 d	4.3± 1.1 bc	6.5± 0.7 cdef	8.3± 0.6 bcd	0.2± 0.1 e
Cont. 17 DAG	27.4± 1.9 ef	17.3± 1.6 bc	9.8± 1.3 bcd	15.8± 3.1 d	2.4± 0.8 d	7.0± 0.7 bc	10.4± 0.9 b	0.4± 0.2 de
Cont. 20 DAG	31.2± 3 bcdef	19.2± 2.5 ab	9.1± 1.0 cde	14.5± 2.2 de	1.1± 0.3 d	5.2± 1.3 ef	9.7± 1.0 bc	0.5± 0.1 cde
Cont. 23 DAG	28.6± 6.7 cdef	10.1± 2.1 fg	5.7± 1.2 fg	28.4± 5.7 bc	1.7± 0.7 de	6.6± 0.8 cdef	9.9± 1.3 bc	0.8± 0.5 c
Cont. 26 DAG	25.6± 3.2 f	9.0± 2.6 g	3.2± 1.3 g	38.2± 3.2 a	1.0± 0.4 d	6.0± 2.4 cdef	9.5± 2.4 bc	0.8± 0.2 cd
Cont. 29 DAG	29.3± 11.0 bcdef	9.5± 3.0 g	3.3± 0.9 g	32.7± 10.4 b	0.7± 0.3 d	6.3± 1.3 cdef	7.9± 1.8 cd	0.9± 0.3 c
Low N 11 DAG	39.2± 7.7 a	13.2± 1.1 de	9.9± 2.4 bcd	7.6± 7.2 fg	5.7± 1.3 a	6.9± 1.4 c	6.4± 1.9 d	0.4± 0.3 de
Low N 14 DAG	32.1± 2.8 bcde	12.5± 3.1 def	14.2± 2.2 a	3.3± 1.2 g	5.3± 1.7 ab	9.1± 2.4 a	9.7± 5.5 bc	0.2± 0.1 e
Low N 17 DAG	33.5± 4.6 abcd	11.2± 1.3 efg	14.5± 7.0 a	3.2± 0.6 g	5.7± 1.2 a	6.7± 0.8 cd	7.9± 0.9 cd	0.2± 02 e
Low N 20 DAG	31.4± 6.1 bcdef	12.0± 1.6 ef	6.5± 0.6 ef	8.0± 3.9 fg	1.6± 0.4 de	4.6± 0.6 f	8.4± 0.8 bcd	1.4± 0.5 b
Resupply N 20 DAG	34.5± 3.1 ab	14.8± 1.8 cd	7.7± 1.0 cdef	10.3± 2.2 ef	0.8± 0.3 d	5.3± 1.6 def	13.9± 1.3 a	2.6± 0.9 a
Resupply N 23 DAG	30.5± 1.8 bcdef	10.7± 1.4 efg	7.5± 1.0 def	18.7± 3.1 d	1.6± 0.7 de	8.8± 0.5 a	14.9± 1.6 a	0.5± 0.1 cde
Resupply N 26 DAG	27.5± 3.3 def	9.3± 0.6 g	5.9± 0.3 fg	25.9± 3.9 c	1.4± 0.4 de	8.5± 0.5 ab	13.5± 1.8 a	0.9± 0.2 c

Table.5.11. Continued

	Leu	Ile	GABA	Pro	Lys	Val	Phe
Cont. 11 DAG	0.4± 0.0 d	0.8± 0.1 d	2.1± 0.5 cd	0.5± 0.2 ef	2.1± 1.4 b	2.5± 0.4 de	0.6± 0.3 b
Cont. 14 DAG	0.5± 0.0 d	0.7± 0.1 d	1.9± 0.9 d	1.5± 0.7 bcd	0.4± 0.1 d	2.4± 0.4 e	0.5± 0.1 b
Cont. 17 DAG	0.7± 0.2 d	0.7± 0.2 d	1.9± 0.3 d	1.8± 0.8 ab	0.8± 0.4 cd	2.7± 0.6 de	0.8± 0.3 b
Cont. 20 DAG	0.8± 0.2 d	0.8± 0.2 d	2.6± 0.6 bc	1.7± 1.1 bc	0.6± 0.2 cd	2.2± 0.6 e	0.7± 0.1 b
Cont. 23 DAG	0.8± 0.1 d	1.1± 0.6 cd	0.8± 0.2 e	1.0± 0.3 de	0.7± 0.1 cd	2.8± 1.0 cde	0.8± 0.2 b
Cont. 26 DAG	0.9± 0.1 d	0.9± 0.1 d	0.5± 0.1 e	0.7± 0.3 ef	0.6± 0.1 cd	2.2± 0.4 e	0.8± 0.1 b
Cont. 29 DAG	1.5± 0.8 c	1.4± 0.4 c	0.9± 0.2 e	0.7± 0.3 ef	1.0± 0.4 cd	3.0± 1.0 cde	0.8± 0.2 b
Low N 11 DAG	0.6± 0.2 d	1.0± 0.4 cd	2.6± 0.8 bc	1.1± 0.9 cde	0.9± 0.4 cd	3.8± 1.4 bc	0.7± 0.2 b
Low N 14 DAG	0.8± 0.3 d	1.1± 0.4 cd	3.0± 0.7 b	2.4± 0.6 a	0.9± 0.4 cd	4.6± 1.0 b	0.7± 0.2 b
Low N 17 DAG	2.8± 1.0 b	2.7± 0.9 b	3.7± 0.7 a	0.2± 0.0 f	2.5± 0.9 b	2.9± 1.0 cde	2.2± 2.3 a
Low N 20 DAG	6.6± 1.1 a	5.3± 0.8 a	1.6± 0.2 d	0.2± 0.1 f	3.7± 0.7 a	6.6± 0.97 a	2.0± 0.7 a
Resupply N 20 DAG	0.7± 0.3 d	0.8± 0.3 d	2.1± 0.7 cd	1.4± 0.4 bcd	1.1± 0.2 c	3.3± 0.5 cd	0.6± 0.1 b
Resupply N 23 DAG	0.6± 0.1 d	0.7± 0.1 d	0.9± 0.2 e	0.8± 0.4 ef	0.4± 0.1 d	2.8± 0.1 de	0.6± 0.1 b
Resupply N 26 DAG	0.8± 0.2 d	0.8± 0.1 d	0.6± 0.2 e	1.1± 0.6 bcde	0.4± 0.1 c	2.6± 0.1 de	0.6± 0.0 b

5.4.5.3. The transcription of putative genes coding sugar, amino acid, and oligopeptide transporters analyzed by microarray

The transcriptomic profiling determined that putative genes encoding sugar transporter were upregulated after 9 and/or 12 d of N deficiency treatments (Table 5.12). Transcriptomic profiling revealed that fifteen putative genes classified to amino acid transporters were induced by at least two folds after 9 d of N deficiency, and two genes were downregulated (Table 5.12). Furthermore, three genes were upregulated and seven genes were downregulated after 12 d of N deficiency treatment (Table 5.12). When the N resupplied, seven putative genes coding amino acid transporter were upregulated, and twenty six genes were downregulated (Table 5.12). This indicates different preference for amino acids transportation under N availability and senescence process.

Moreover, the transcription of eighteen putative genes coding oligopeptide transporters were up regulated after 9 d of N deficiency treatment (at 17 DAG). While the transcription of nineteen genes were differentially regulated after 12 d of N deficiency treatment including eight genes were downregulated, and eleven genes were upregulated. Among them seven genes were also upregulated after 9 d of N deficiency treatment. When the N resupplied, twenty nine genes were differentially regulated. Among them three genes were upregulated, and twenty six were downregulated. Additionally, eleven genes that were regulated after 12 d of N deficiency treatment were recovered after 2 d of N resupplied.

:

Table 5. 12. Expression of putative genes coding sugar and amino acid transporter. The values are presented fold changes. ↑ : Upregulated, ↓ : downregulated.

ID	Putative coding	Low N (17 DAG)	Low N (20 DAG)	Resupply N (20 DAG)
hv_07490	Sugar transporter	↑2.33	–	↓3.05
hv_22086	Sugar transporter	↑2.25	–	–
hv_07489	Sugar transporter	–	↑ 4.5	5.54
hv_18626	Sugar transporter	–	↓2.55	↑3.14
hv_07361	Sugar transporter	–	–	↓3.41
hv_10668	Sugar transporter	–	–	↑2.48
hv_15851	Sugar transporter	–	–	↓2.81
hv_19432	Sugar transporter	–	–	↓3.46
hv_22086	Sugar transporter	–	–	↓2.64
hv_00252	Cationic AA transporter	↑2.58	–	↓2.27
hv_00253	Cationic AA transporter	↑2.43	–	–
hv_00254	Cationic AA transporter	↑2.53	–	–
hv_06902	AA transporter	↑15.14	↑ 9.78	↓18.38
hv_07498	AA transporter	↑2.19	–	↓3.58
hv_07499	AA transporter	↑2.22	–	–
hv_09959	AA permease	↑2.68	–	–
hv_19480	Bidirectional AA transporter	↑3.55	–	↓3.41
hv_21730	AA permease	↑10.34	↑15.45	↓13.93
hv_29535	HvCAT4	↑3.51	–	↓3.55
hv_29537	HvANT16	↑2.27	–	–
hv_38297	HvLAT5	↑3.12	–	–
hv_38299	HvANT7	↓20.91	↓17.75	↑ 274.37
hv_38302	HvAAP1	↑3.84	–	–
hv_38304	HvAAP11	↑3.14	–	4.47
hv_40420	HvANT17	↓4.66	↓6.41	↑ 9.06
hv_42335	AA permease	↑2.51	–	↓2.55
hv_07770	Aromatic and neutral AA transporter	–	↓ 9.0	↑3.63
hv_16703	AA transporter	–	↓3.34	–
hv_36352	HvAAP5	–	↓2.87	↑3.01
hv_36356	HvANT9	–	↓5.58	↑4.26
hv_36502	HvAAP13	–	↑2.43	↓3.43
hv_38305	HvAAP4	–	↓3.07	–
hv_08284	AA transporter	–	–	↓3.71
hv_10976	AA permease	–	–	↓ 2.2
hv_11480	Cationic AA transporter	–	–	↓2.53
hv_11481	Cationic AA transporter	–	–	↓2.51
hv_11482	Cationic AA transporter	–	–	↓2.13
hv_11483	Cationic AA transporter	–	–	↓2.55
hv_11631	Lys and His transporter	–	–	↓5.24
hv_15745	HvAAP10	–	–	↓3.07
hv_19876	Lys and His transporter	–	–	↓4.82
hv_20341	AA transporter	–	–	↓2.36
hv_29528	HvGAT1	–	–	↓2.69
hv_29529	HvANT5	–	–	↑2.57
hv_29531	HvLAT3	–	–	↓2.08
hv_29532	HvBAT2	–	–	↓2.58
hv_29533	HvLHT5	–	–	↓3.97
hv_36349	HvAAP2	–	–	↓ 2.1
hv_37316	HvANT14	–	–	↓2.16
hv_38319	HvCAT1	–	–	↓2.04
hv_43602	Trp/Tyr permease	–	–	↑2.68

5.4.6. Primary metabolic rearrangement in response to N availability

*5.*4.6.1. Metabolic profiling related to glycolysis and TCA upon different N regimes

To further investigate the effect of N deficiency- induced leaf senescence and its reversal by N resupply on primary carbon metabolism, targeted metabolites profiling of primary leaves were determined using an established IC-MSMS protocol, to address especially glycolysis and TCA cycle. These metabolites covered the most important metabolic routes connecting primary carbon and nitrogen metabolism. The results were logarithmic normalized and presented in Fig.5.17 using heat map, and for absolute values see appendix (Table 10.4). The results revealed considerable changes in the level of 10 metabolites (Fig.5.17). The concentration of TCA cycle-metabolites slightly increased compared to their concentration at 14 DAG in control plants and stayed stable afterward with developmental progression, except 2-oxoglutarate which its concentrations decreased with leaf age (Fig.5.17).

The 3-Phosphoglycerat (3PGA), also involved in photosynthesis, and phosphoenolpyruvate (PEP) concentrations were not affected at 11 DAG (Data not shown), but highly accumulated in N deficient leaf afterward. Their concentrations were 34- and 150- folds, respectively, higher after 6 d of N deficiency treatment relative to control plant. Although, their concentration decreased afterwards with experiment time course, it was always higher during experimental time course than control plants (Fig.5.17). When the N resupplied, the concentrations of 3PGA and PEP didn't dramatically change, while the concentration of PEP was lower after 2 d of N resupplied (Fig.5.17). On other hand, pyruvate concentration was significantly lowered in N deficient plants compared to control plants through the measured time points. A strong decrease in pyruvate level under N limitation has been also reported in Arabidopsis plants (Balazadeh et al., 2014). Furthermore, no significant change in pyruvate concentration was found in 2 d of N resupply, but it was recovered to control level upon 5 d (Fig.5.17). These results indicate the importance of pyruvate metabolism step in controlling the flow from glycolysis to TCA cycle under N deficiency conditions, which deserves more investigation.

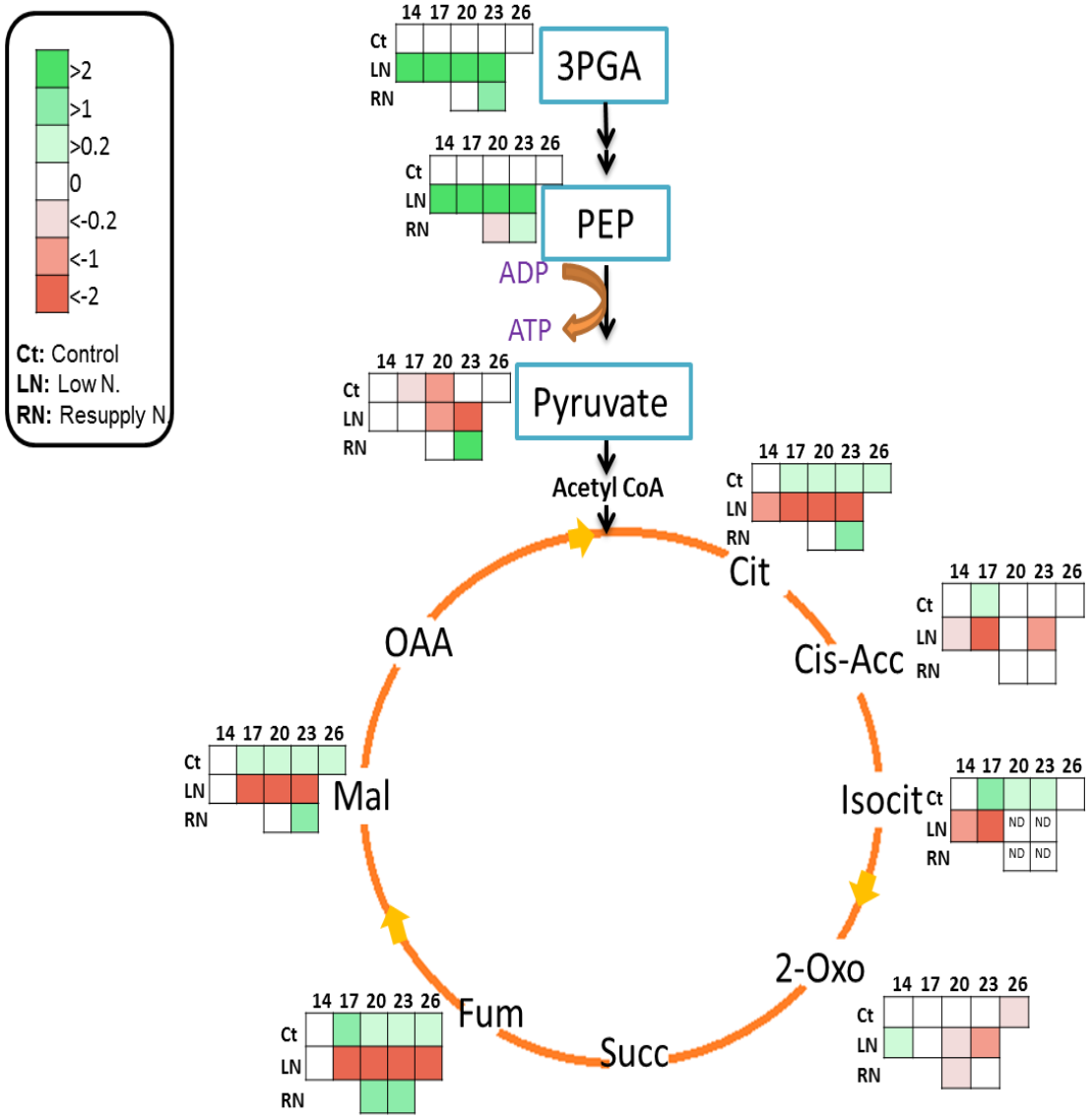

Figure 5. 17. Glycolysis and TCA cycle metabolism in primary leaves under N deficiency-induced leaf senescence and its reversal by N resupply. Control results logarithmic normalized to 14 DAG of control plant; Low N. results logarithmic normalized to corresponding control time point, while resupply N. results normalized to corresponding low N. time point. Abbreviations are as follows: Ct: Control, LN: Low N, RN: resupply N, ND: not detected, 3PGA: 3-phosphoglycerate, PEP: phosphoenolpyruvate, Cit: citrate, Cis-Acc: cis-aconitate, Isocit: isocitrate, 2-Oxo: 2-oxoglutate, Succ: succinate, Fum: furmarte, Mal: malate, OAA: oxaloacetate. (n= 5-6); P< 0.05.

Under N deficiency, there was a strong decrease in all measured metabolites of TCA cycle including citrate, cis-aconitate, isocitrate, 2-oxoglutarate, furmarate, and malate. This decrease was early as 14 DAG in some of these metabolites. Such as, citrate, cis-aconitate, and isocitrate (Fig.5.17), as well as,

after 3 days of N deficiency treatment (11 DAG; data not shown). 2-oxoglutarte concentration wasn't significantly changed before 20 DAG. A large decrease in malate and citrate levels has already been reported when wild tobacco plants were grown on low nitrate condition (Scheible et al., 1997). When N resupplied, almost all TCA metabolites were recovered, completely or partially, to the levels of control plants (Fig.5.17). It is worth to mention that the results were confirmed by another completely independent experiment with 6 biological replicates.

The concentration of NADPH was higher in N deficient plants than control plants at 20 and 23 DAG. Whereas AMP concentration decreased in N deficient plants relative to control plants. This may indicate higher energy status in N deficient plants. N resupplied didn't show an effect on NADPH concentration. Whereas AMP concentration was slightly higher than N deficient plants, in a matter of fact their concentration weren't higher than the value after 9 d of N deficiency treatment (17 DAG) (Fig.5.18).

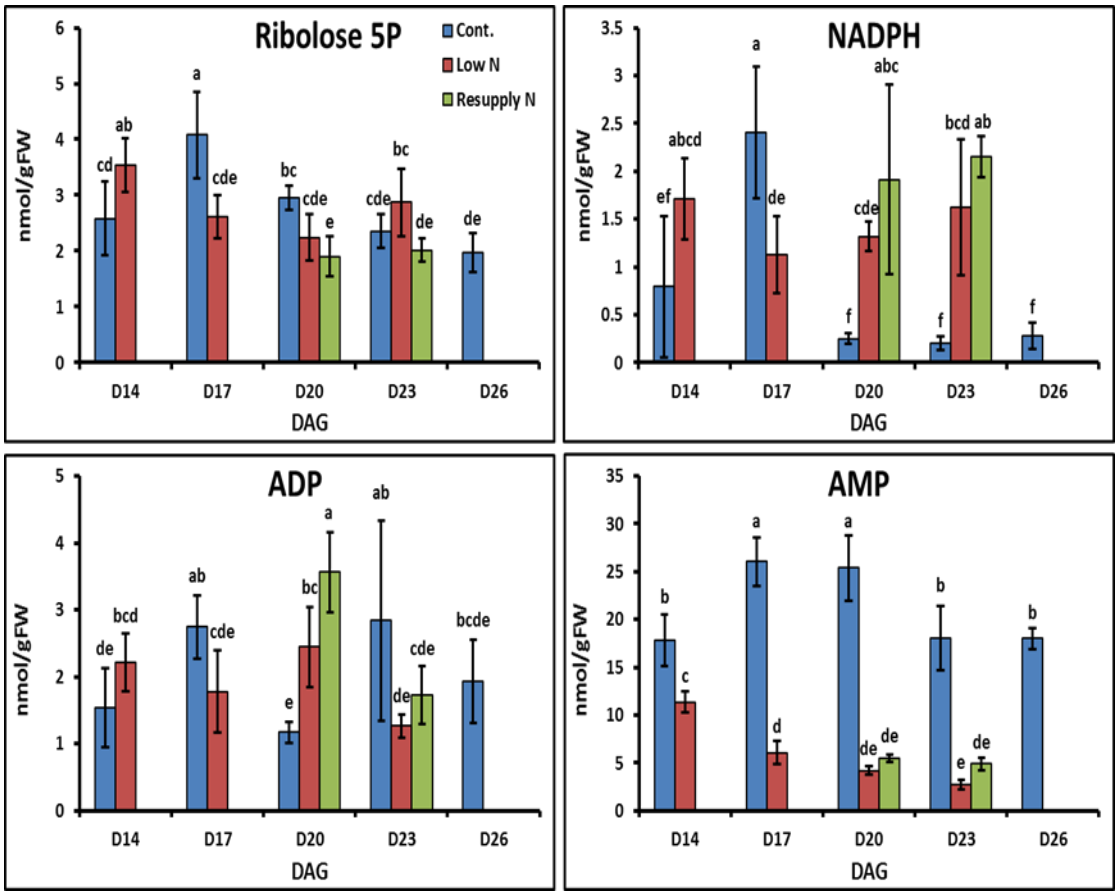

Figure 5. 18. The level of ribolose 5P, NADPH, ADP, and AMP in response to N supply and leaf age. Bars indicate mean ± SD (n= 5-6). Letters (a-f) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

5.4.6.2. The transcription of putative genes coding enzymes for glycolysis, TCA cycle, and gluconeogenesis which are determined via microarray and qPCR

The transcriptomic profiling revealed that the expression of transcripts related to glycolysis, gluconeogenesis, and TCA cycle including cytosolic glyceraldehyde 3-phosphate dehydrogenase (DH), pyruvate kinase, malate DH, isocitrate DH was decreased after 9 d of N deficiency treatment (17 DAG; Table 5.13). Whereas, the expression of genes coding plastidic phosphofructokinase, phosphoglycerate mutase, and pyruvate dikinase were upregulated. Also, the expression of genes coding cytosolic glyceraldehyde 3-phosphate DH, cytosolic enolase, plastidic phosphoglucomutase, pyruvate DH, malate DH, and ATP citrate lyase decreased 12 d of N deficiency treatment (20 DAG; Table 5.13). While the expression of genes coding phosphofructokinase,

malate synthase, and pyruvate dikinase were upregulated. In contrast, N resupplied induced the expression of transcripts related to main glycolysis and TCA cycle pathways such as cytosolic glyceraldehyde 3-phosphate DH, enolase, cytosolic/plastidic pyruvate kinase, cytosolic phospho-enol-pyruvate carboxylase (PEPC), pyruvate DH, malate DH, isocitrate DH, and isocitrate layase. Comparatively, the expression of genes coding plastidic phosphofructokinase, aconitase, citrate synthase, and pyruvate dikinase were downregulated upon N resupplied (Table 5.13).

The results of metabolites profiling revealed that the flux of metabolites from glycolysis to TCA cycle under N deficiency condition was disrupted through decreased pyruvate concentration (Fig.5.17), indicating that this could be the key to control glycolysis and TCA interconnection under N deficiency condition. So we looked in microarray data set for the genes involved in pyruvate metabolism that were a differentially regulated under N deficiency condition and/or when the N resupplied. Ten genes that encode pyruvate kinase (PK), pyruvate dehydrogenase (PDH), and pyruvate orthophosphate dikinase (PPDK) were differentially regulated by at least two-folds in response to N deficiency and/or N resupplied. In microarray results, two PKs genes were induced after 2 d of N resupplied, and we mentioned to them hv_21109 as PK1, and hv_40111 as PK2, but no significant change was found for the both genes in 9 and 12 d after N deficiency treatment (Table 5.13). Four genes encode PDHs were also induced by at least two–folds after 2 d of N resupplied, and we mentioned to them hv_11270 as PDH1, hv_16354 as PDH2, hv_16402 as PDH3, and hv_41416 as PDH4. Furthermore, the PDH1, PDH2, and PDH3 were also downregulated after 12 d of N deficiency treatment. Moreover, four genes encode PPDKs were downregulated after 2 d of N resupplied, and we mentioned to them hv_00102 as PPDK1, hv_00103 as PPDK2, hv_22459 as PPDK3, and hv_43181 as PPDK4. Whereas, PPDK3, and PPDK4 were induced after 12 d of N deficiency treatment (Table 5.13).

In order to have a wider kinetic on transcription pattern of genes coding enzymes for pyruvate metabolism and to validate the array data, primers were

designed and qPCR was performed. There was mainly agreement between microarray and qPCR results. PK2 was induced by more than 6 folds after N resupplied (Fig.5.19), which could play role to recover pyruvate concentration to control plants level (Fig.5.17). The expression of PDH2 was downregulated with continuous N deficiency condition. While, the transcription of PDHs (PDH1, PDH2, and PDH4) were induced upon N resupply (Fig.5.19). Assuming that this induction activate the flux to TCA cycle upon N resupplied. Interestingly, PPDKs, bidirectional enzyme interconverts pyruvate and phosphoenolpyruvate, transcription was highly induced by N deficiency-induced senescence conditions. PPDK3 was induced by 77 folds after 15 d of N deficiency treatment (Fig.5.19). Moreover, PPDKs were also up-regulated in naturally senescing leaves. Taylor et al., (2010) reported that ^{13}C-labelled pyruvate feeding indicated that PPDK functions in a pathway that generates the transport amino acid glutamine during Arabidopsis leaf senescence. Accordingly, the nitrogen remobilization was accelerated from leaves during senescence when PPDK was overexpressed. It is not clear if PPDK has a role in the PEP and 3PGA accumulation in N deficient plants (Fig.5.17), but their accumulation preceded the PPDKs induction.

Furthermore, to test if these genes are also regulated in other leaf senescence inducers such as drought-induced and natural leaf senescence, samples were harvested in our group from primary leaf of *Hordeum vulgare* L. cv Golden Promise grown in soil exposed to drought stress or control conditions (Paramon et al., unpublished). The whole RNA was extracted from three independent biological experiments, and qPCR was performed to determine the transcription change of genes coding enzymes involved in pyruvate metabolism. Differently from N deficiency-induced leaf senescence, PK1 was induced by four folds in drought stressed plants at 20 DAS (Fig.5.19). Although, PDH1 and PDH4 was downregulated with leaf age in control plants, this change wasn't detected in plants grown hydroponically (Fig.5.19). Even though they almost have the same age. The transcription of the four PPDKs was interestingly induced in drought-induced and natural leaf senescence. Their transcription seemed to be

commonly regulated in the different leaf senescence inducer. PPDK3 was highly induced, and its transcription was induced by 903- and 228-folds in drought-induced and natural senescence, respectively, (Fig.5.19).

Table 5. 13. Expression of putative genes coding enzyme for glycolysis, TCA cycle, and gluconeogenesis. The values are presented fold changes. ↑: Upregulated, ↓: downregulated. Values of Low N are relative to corresponding time point of control. Whereas Values of resupply are relative to Low N (20 DAG).

ID	Putative coding	Low N (17 DAG)	Low N (20 DAG)	Resupply N (20 DAG)
hv_13593	Glyceraldehyde 3-P DH	↓2.35	↓3.16	↑3.16
hv_40636	Glyceraldehyde 3-P DH C subunit	↓9.92	↓6.41	↑5.43
hv_21406	Phosphofruktokinase	↑3.68	–	↓3.92
hv_09087	Phosphofruktokinase	–	↑2.69	↓2.19
hv_09091	Phosphofruktokinase	–	–	↓2.14
hv_39004	Plastidic pyruvate kinase beta subunit	↓2.79	–	–
hv_08013	Phosphoglycerate/bisphosphoglycerate mutase	↑ 2.5	–	–
hv_23968	Copper ion binding / phosphopyruvate hydratase	–	↓2.28	↑2.95
hv_42359	Glucose phosphomutase	–	↓2.66	–
hv_18314	Enolase	–	–	↑ 2.1
hv_23969	Enolase	–	–	↑ 2
hv_21109	Pyruvate kinase 1 (PK1)	–	–	↑2.14
hv_40111	Pyruvate kinase 2 (PK2)	–	–	↑2.27
hv_11270	Pyruvate DH E1 subunit 1 (PDH1)	–	↓ 3.25	↑ 4
hv_16354	Pyruvate DH 2 (PDH2)	–	↓ 2.13	↑2.14
hv_16402	Pyruvate DH 3 (PDH3)	–	↓2.31	↑ 2.5
hv_41416	Pyruvate DH 4 (PDH4)	–	–	↑2.79
hv_36512	Isocitrate dehydrogenas	↓2.07		↑ 2.6
hv_10029	Malate DH	–	↓2.69	↑2.89
hv_41621	Malate DH	–	↓ 2	–
hv_41416	Dihydrolipoyllysine-residue acetyltransferas	–	–	↑2.79
hv_42774	Citrate hydro-lyase/aconitase	–	–	↓2.69
hv_00102	Pyruvate orthophosphate dikinase 1 (PPDK1)	–	–	↓ 4
hv_00103	Pyruvate orthophosphate dikinase 2 (PPDK2)	–	–	↓3.03
hv_22459	Pyruvate orthophosphate dikinase 3 (PPDK3)	↑4.92	↑5.43	↓7.21
hv_43181	Pyruvate orthophosphate dikinase 4 (PPDK4)	↑10.78	↑10.85	↓23.26
hv_19873	Malate synthase	–	↑3.12	–
hv_17622	Citrate synthase	–	–	↓3.29
hv_36529	Carboxyvinyl-carboxyphosphonate phosphorylmutase	–	–	↑2.44

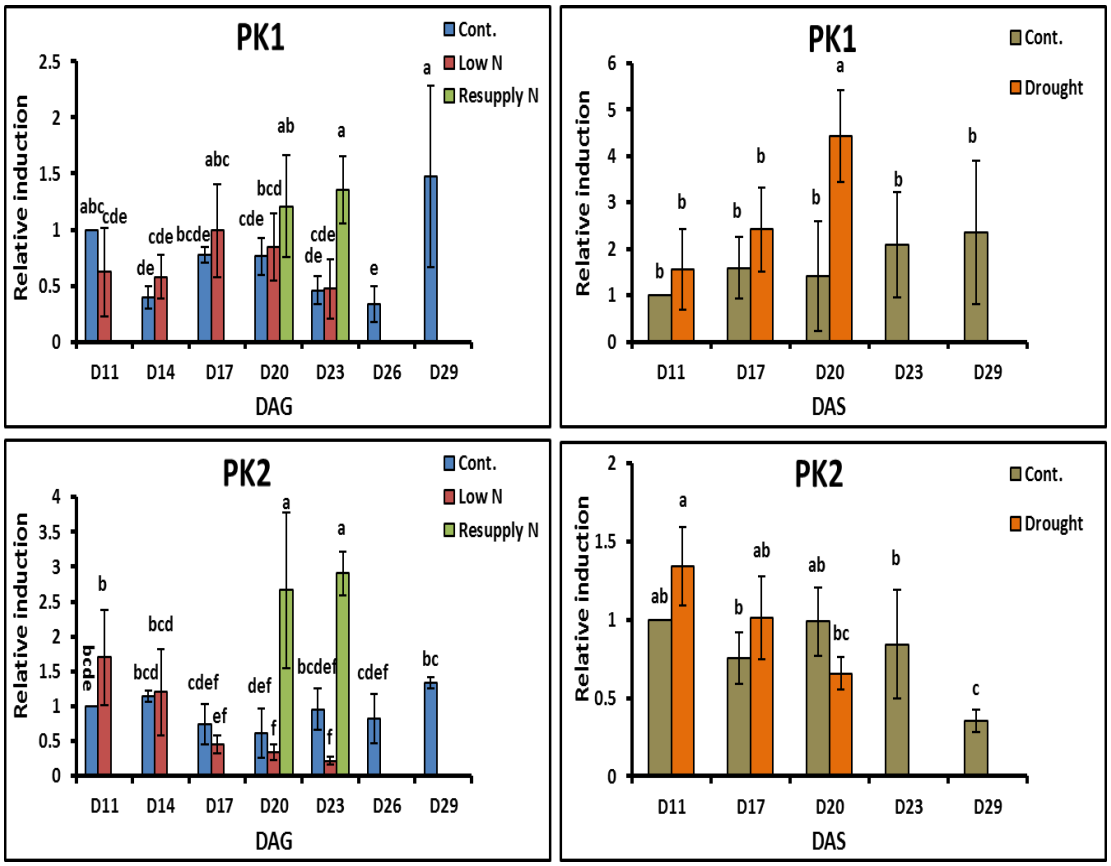

Figure 5. 19. PKs, PDHs, and PPDKs transcription under N deficiency-induced, drought-induced, and natural leaf senescence. Right side, samples from Paramon et al experiment. PK: Pyruvate kinase, PDH: pyruvate dehydrogenase, and PPDK: pyruvate, orthophosphate dikinase, DAG: day after germination, DAS: day after sowing. Bars indicate mean ± SD (n ≥3). Letters (a-g) represent the significant; Means with a common letter are not significantly different ($p > 0.05$).

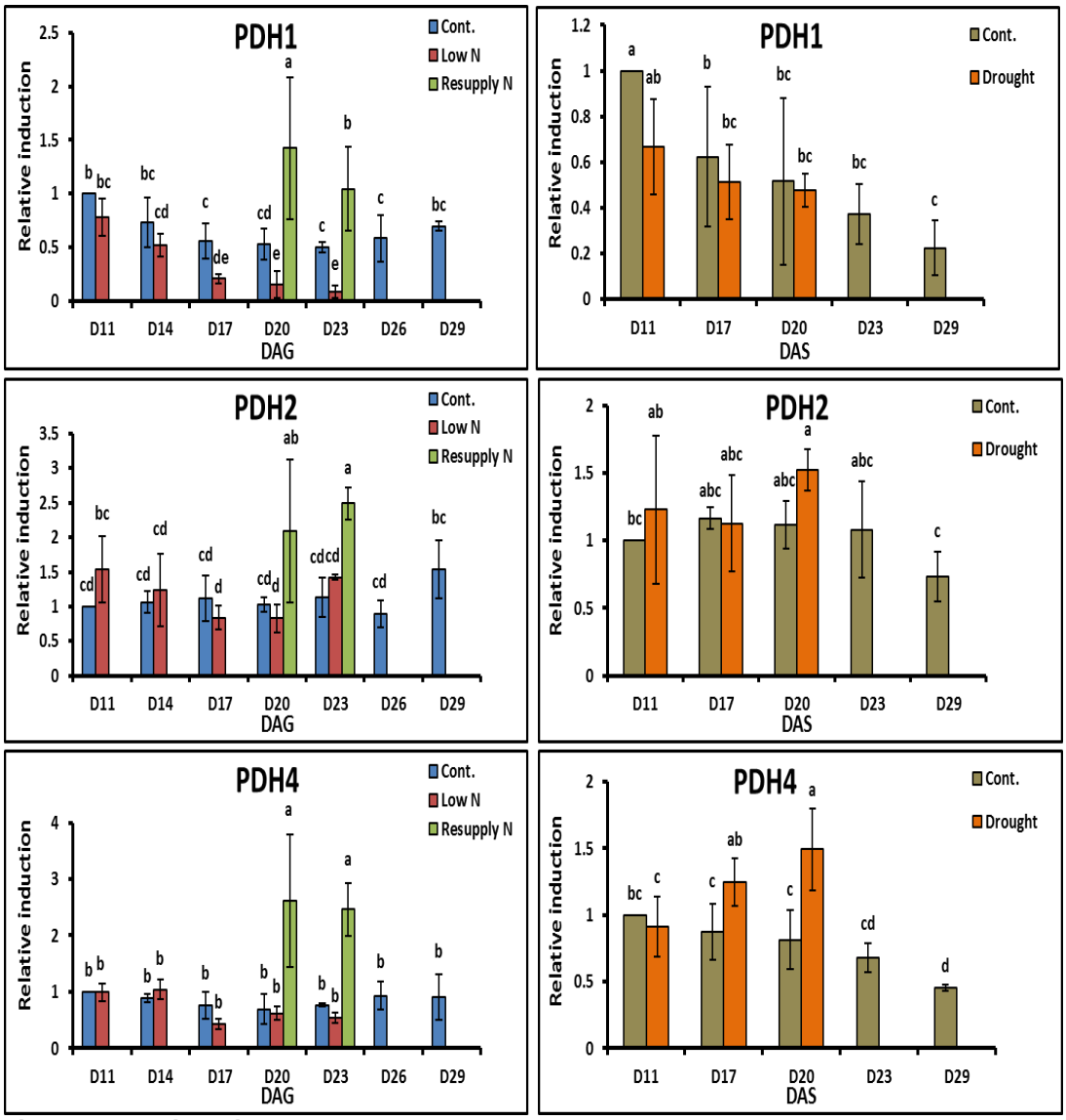

Fig.5.19. Continued

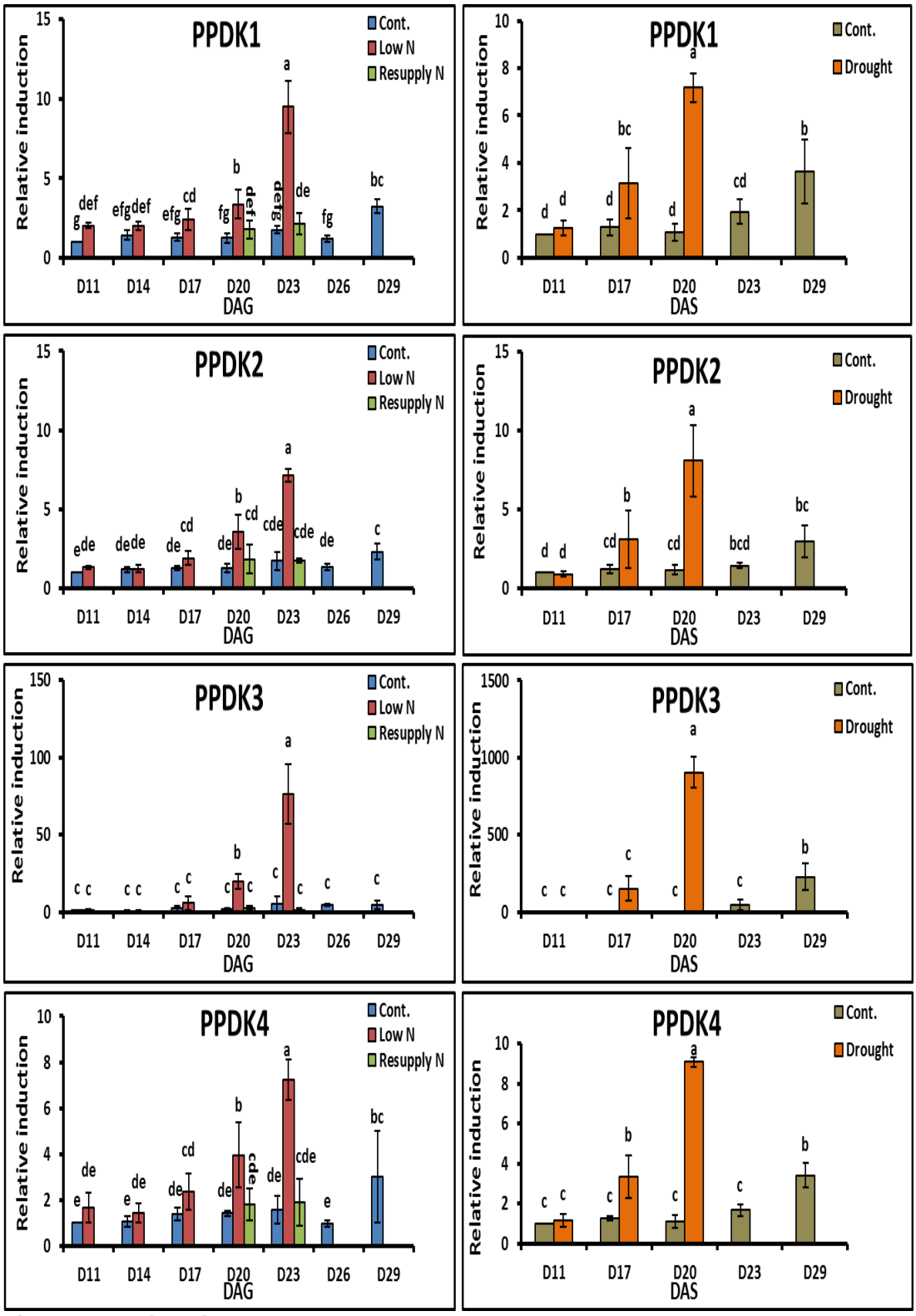

Fig.5.19. Continued

6. Discussion

6.1. N-availability affects the course of leaf senescence and root/shoot relations

Leaf senescence can be defined as a developmentally controlled process, ended by leaf death, and characterized by loss of chlorophyll (yellowing) and photosynthetic capacity. During the senescence syndrome, macro/ micromolecules are remobilized to sink tissues such as seeds and roots. Therefore, orderly running senescence is a prerequisite for high yield in crop plants. Although many research was done over decades on leaf senescence, most of these approaches lack the information about sink/source relations and their role in senescence signaling. Thus, understanding how the sink/source relation controls the senescence process and how this is connected to remobilization might give opportunity to increase crop yield quantity and/or quality.

Leaf senescence is intimately connected to developmental age. However, adverse environmental conditions such as N deficiency or drought prematurely induce the senescence program and cause massive loss in yield worldwide. Nevertheless, it has been reported that the premature senescence process can be stopped and even reverted, e.g. after resupply of N to starved plants (Schildhauer et al., 2008; Balazadeh et al., 2014). As a matter of fact, the molecular and metabolic mechanisms underlying this process have not been studied in detail, particularly in crop plants. In this approach, barley plants (*Hordeum vulgare* L. cv Golden Promise) were used to establish an experimental set-up that allows to induce leaf senescence by N starvation, and then to delay the senescence progression by N resupply.

It is shown that N starvation triggers a decrease in chlorophyll content (Fig.5.2). When N was resupplied on 10 d of starvation, the chlorophyll content did not decrease any more up to the last time point of the experiment (29 DAG; Fig.5.2). Similarly to physiological markers, the expression of senescence marker gene, S40, was strongly induced by natural and N deficiency- induced senescence.

Its expression was strongly induced at 17 DAG which coincided with chlorophyll loss (Fig. 5.4). Interestingly, the induction of S40 expression was delayed upon N resupply, and transcript stayed at level of plants kept under N-replete condition (Fig.5.4). In addition, the primary N assimilation was activated upon N resupply reflected by induction of the GS2 expression (Fig.5.4). Interestingly, GS2 expression was also higher in N-resupplied plants compared to control plants, but this induction wasn't reflected by higher nitrate concentration upon N resupplied than in control plants (Fig.5.10).

Under N deficiency condition, plants respond by increasing their root/ shoot ratio. This allows the plants to cover more soil area by their roots and search for more N resources (Shangguan et al., 2004; Agren and Franklin 2003). Here, we reported the same upon N deficiency treatment, while, the root/shoot ratio was decreased upon N resupplied. This was reflected by lower roots and increased shoot dry weight compared to N deficient plants. Likewise, Comadira et al., (2015) has reported that root/ shoot ratio was increased in barley plants which after N deficiency condition (0.1 mM KNO_3) compared to other plant group that were kept in N-replete condition (5 mM KNO_3) during the experiment time course. In contrast to our findings, in this report the dry weight of roots wasn't significantly different between N-deficient and control plants during the experiment time course. Comadira et al., (2015) has reported that roots biomass was less by 8 folds in N-deficient plants compared to control plants at one restricted time point for measurement (14 DAG). This differences might be explained by the different experimental set up in the both approaches. While, in our approach, the seedlings had been germinated for 8 d in 1 mM $Ca(NO_3)_2$ and then after the plants were kept in N deficiency condition (0.06 mM $Ca(NO_3)_2$), Comadira et al., (2015) had germinated seedlings for 7 d in the absence of added N and then the plants were grown for 7 d under N deficiency condition (0.1 mM KNO_3). These results indicate that roots in our experiment were stronger sink tissues for remobilized assimilates from leaves, and might explain the differences in metabolites levels that found between the two approaches, which it will be discussed in the following sections.

6.2. Transcription profiling of barley primary leaf with designed 60K Agilent microarray

It is now clear that the senescence program is a highly complicated process that is controlled at multiple layers of regulation, including chromatin structuring (epigenetic), and transcription, as well as by post-transcriptional, translational and post-translational regulation (Woo et al., 2013). Senescence involves reprogramming of a plethora of genes including senescence associated genes (SAGs) that are upregulated during leaf senescence, and senescence downregulated genes (SDGs), which include many photosynthesis related genes and also repressors of the senescence process. This reprogramming is under control of signaling pathways triggered by internal and external factors, e.g. hormones or stress conditions. The structure of the complex regulatory pathways inducing leaf senescence and their connection to other developmental and stress responsive regulatory processes is still not known exactly and in focus of research worldwide.

In the present thesis, a systemic approach bringing together physiological, transcriptomic, hormonic and metabolomic data was chosen to unravel mechanisms underlying regulation of leaf senescence in response to changing N availability. Transcriptomic profiling revealed specific down-regulation of genes of photosynthesis, glycolysis, TCA, CK signaling, redox controlling, RNA transcription, RNA processing, protein synthesis initiation, nucleotides and amino acids biosynthesis, as well as chromatin remodeling under N deficiency-induced leaf senescence condition, but specific induction genes of calcium regulation, ABA biosynthesis, protein and amino acids degradation, as well as MYB, MYB-related NAC, and NIN-like transcription factors. In addition, 43% of genes that are differentially regulated in N deficiency (3 folds threshold) were also found to be regulated in natural senescence. It has been reported that once the senescence program is started, the different senesce inducers share a huge proportion of SAGs expression (Guo and Gan, 2012). More than 50% of genes induced after 12 d of N deficiency treatment were also found to be induced in natural senescence (Fig.5.6).

Transcript profiling also demonstrated that N resupply particularly induced the genes related to photosynthesis, glycolysis, TCA cycle, amino acids biosynthesis, CK response, redox regulation, chromatin structure, protein synthesis, RNA transcription and processing, as well as heat shock and ARR-B transcription factors. On other hand, N resupply suppressed genes for calcium regulation, kinase receptors, ABA biosynthesis and response, protein, and amino acids degradation. The results demonstrated that 62% of genes that are differentially regulated after 12 d of N deficiency treatment were recovered after 2 d of N resupply, 665 re-upregulated and 543 re-downregulated. Lim et al., (2007) have reported that 96 transcription factor genes were upregulated in Arabidopsis senescing leaves. Theses belong to transcription factor families include WRKY, NAC, MYB, C2H2-type zinc finger, and AP2/EREBP, which play a major regulatory network to regulate senescence program. Accordingly, N resupply downregulated the expression of genes belonging to MYB, MYB-related, C2H2, GRAS, NAC, WRKY, and ARF transcription factor families. Notably, MYB-related transcription factor was the largest family that was affected by N resupplied followed by WRKY family. In addition, recently that NIN-like protein (NLP) transcription factor has been described to act as a master regulator of nitrate signaling (Marchive et al., 2013). The expression of four putative genes belonging to NIN-like transcription factor family were downregulated upon N resupply.

It appears likely that the further computational analyses and characterization of our candidate genes will shed new light on the mechanism of N-responsive regulation of leaf senescence process. Furthermore, the corresponding expression of genes related to metabolism and hormonal regulation will be discussed together with hormonic and metabolic data in the following sections.

6.3. Phytohormones as upstream regulators in response to N availability

Plants in their life time are subjected to a variety of abiotic stresses and exhibit complex responses to these stresses depending on their degree of plasticity. Phytohormones are known to play major roles in the ability of plants to acclimatize to different environmental stresses, by mediating growth, development, source/sink transitions and nutrient allocation mainly by modulating senescence process. Leaf senescence as a result of developmental process is modulated by different developmental regulators including phytohormones. Understanding the signaling pathways of phytohormones in controlling senescence process will shed a light on mechanism of development dependent and environment sensitive inducing and delaying of the leaf senescence process. While the regulation of senescence by cytokinin and ethylene is more conserved, the action of other hormones shows wider variations between plant species and environmental conditions (reviewed by Schippers et al., 2007). Generally, phytohormones regulate the developmental process through a balance and crosstalk, synergistically or antagonistically, between various phytohormone signaling pathways and other regulators, such as CK/auxin balance in controlling shoot and root growth (reviewed by Ying-Hua et al., 2011).

6.3.1. ABA regulation in response to the different N regimes

As expected, the concentration of ABA slightly increased with primary leaf age of control plants up to two folds higher than mature leaf. In early response to N deficiency condition, there was no differences between control and N deficient plants. But it was significantly higher with N deficiency-induced senescence starting from 17 DAG, and reaching three folds higher at 23 DAG. The results of ABA concentration in primary and 4th leaves indicate that ABA changes in barley plants are mostly related to senescence process than N deficiency response alone (Fig.5.8). But this excludes the early response to N deficiency for example during the first hours. Moreover, it has also been reported that ABA concentration increased in Arabidopsis plants exposed to N deficiency

condition (Balazadeh et al., 2014). Previous studies demonstrated that ABA modulates a balance between inducing senescence program and protection activity such as inducing expression of antioxidant genes and inhibiting ethylene production under drought stress (reviewed by Lim et al., 2007; Sharp, 2002). ABA signaling mutant abi5 showed delayed leaf senescence under low N and high sugar conditions. In addition, ABI5 is transcriptionally induced during senescence syndrome (Buchanan-Wollaston et al., 2005). When N was resupplied, the ABA concentration was lowered by 2.5- and 6- fold after 2 and 5 d of N resupply, respectively, compared to the corresponding N deficiency time point (Fig.5.8). Notably, the decrease in ABA level upon N resupply wasn't found in Arabidopsis plants (Balazadeh et al., 2014), indicating that there could be differences in delaying senescence pathways by N resupply between plant species.

Transcriptomic profiling revealed that genes related to ABA biosynthesis and response were upregulated after 9 d of N deficiency treatment (17 DAG) including NCED, aldehyde oxidase (AO), and genes homologous to Arabidopsis ABA responsive elements-binding factor 2; 3 (ABF2;3) and HVA22E (is an ABA- and stress-inducible gene (Chen et al., 2002a)). NCED and the gene encoding Xanthine DH (XDH) were also upregulated after 12 d of N deficiency treatment (20 DAG) (Table 5.1). It was argued that hydraulic signals reflecting changes in water potential due to soil and root drying can induce local biosynthesis of ABA in leaves before transport of ABA from roots is observed (Christmann et al., 2005; Christmann et al., 2007). Moreover, the ABA level and AAO activity increased especially in old leaves of pea plants, exposed to ammonium, or low N stress, in the absence of both, increased xylem loading and enhanced AAO activity in roots (Zdunek and Lips, 2001). Under salinity stress, an increase in ABA-conjugate concentrations in barley xylem sap could be shown, indicating systemic signaling (Dietz et al., 2000). Nevertheless, Omarov et al., (1998) reported that the activity of aldehyde oxidase (AO) in roots was enhanced in barley plants grown with ammonium, but the leaf AO was not significantly affected by the nitrogen source, indicating

that ABA could be a systemic signal from barley roots to shoot in response to N sources or availability. These observations revealed that it isn't clear if the increase of ABA concentration in barley leafs under N deficiency-induced senescence condition resulted from local ABA biosynthesis in the leaf or from transport of ABA from roots. To clarify this point, in future experiments the analysis of ABA flow through xylem under N deficiency condition is needed. On other hand, when N was resupplied, in the leaves transcription of genes encoding enzymes of ABA metabolism (NCED and XDH), HVA22E, and GL2 expression modulator was downregulated. Although, other genes involved in ABA response including putative genes homologous to Arabidopsis ABA insensitive 3 and HVA22J were upregulated when N was resupplied. The clear transcriptional control of these ABA related genes in the leaf in response to N availability, indicates that at least in part ABA levels are regulated via ABA metabolism in the leaf.

The active ABA levels is modulated by catabolic process, where ABA is hydroxylated at the 8' position by ABA-8'-hydroxylases to produce unstable intermediate compound 8'-OH-ABA that is isomerized to phaseic acid (PA). Then, PA is further reduced to form diphaseic acid (DPA) (reviewed by Finkelstein, 2013). Here, it was observed that PA and DPA accumulated to several- fold higher than ABA level. DPA concentration decreased with leaf age in control plants. This indicates slowdown of ABA turnover rather than activation of biosynthesis alone with leaf age. But there was no significant difference in ABA degraded forms between control and N deficient plants during the experiment time course except at 23 DAG (Fig.5.8). In addition, no change in transcription of genes coding ABA-8'-hydroxylases upon N deficiency or resupplied conditions was found.

6.3.2. CKs regulation in response to the different N regimes

Cytokinins (CKs) could be found in plants naturally as isoprenoid and aromatic forms. Isoprenoid CKs are naturally available in plant as N^6-(Δ^2-isopentenyl)-adenine (iP), trans-zeatin (tZ), cis-zeatin (cZ), and dihydrozeatin (DZ). The most abundant among them generally are tZ and iP as well as their sugar conjugates,

but there is a lot of variation depending on plant species, tissue, and developmental stage (reviewed by Sakaibara, 2006). CK is a major phytohormone that has been reported to regulate senescence process. It was shown by many studies that CKs could delay leaf senescence (e.g. Gan and Amasino, 1995). The application of CKs and increasing its production could delay leaf senescence (Richmond and Lang, 1957; Ori el al., 1999), as well as reducing CKs levels accelerated leaf senescence (Masferrer et al., 2002). The most striking evidence for the direct role of CKs in delaying leaf senescence was by inducing IPT expression under SAG12 promotor using SAG12-IPT construct in transgenic tobacco plant, where the CKs biosynthesis was increased only when SAG12 was induced after initiation of senescence (Ori el al., 1999; Gan and Amasino, 1995). However, CKs no longer can suppress leaf senescence in SAG12-IPT line, when extracellular invertase activity was inhibited (Balibrea Lara et al., 2004). This means that one way of delaying leaf senescence by CKs could be through regulation of invertase enzymes activity. But the whole picture of CKs signaling and its complex pathways is still unclear and needs more studies to be elucidated. Hoth et al., (2003) showed that 823 up- and 917 genes were downregulated in Arabidopsis seedling stage after 24h of endogenous IPT induction.

It was reported that iP-type and tZ-type CKs accumulate in phloem and xylem, respectively, which play a role in systemic signals to control roots and shoot growth and development (Corbesier et al., 2003; Lejeune et al., 1994; Takei et al., 2001). Here, we demonstrate that tZ is a major CK form in barley plants, and its concentration decreases with leaf age (Fig.5.9). But significant changes in tZ concentration between control and N deficient plants was only found after senescence initiated. This means that the decrease of tZ level could be a consequence rather than a trigger for N deficiency-induced leaf senescence in barley plants. This finding is opposite to the fact that Zdunek and Lips (2001) showed that the xylem delivery rate of trans-zeatin ryboside (tZR) decreased by 55% in low N *Pisum sativum* L.-treated plants, but the level of CKs turn over in leaves shouldn't be excluded in analyses. Although, Samuelson and Larsson

(1993) showed that the root zeatin riboside (ZR) level of N-limited barley is largely non-responsive to nitrate dose in the long term, but positively responded to increased nitrate supply. The authors also showed that pretreatment the barely plant with cycloheximide inhibited nitrate-induced ZR response, but the level of ZR wasn't affected. This means N metabolites are required for this response. In the same manner, the ZR concentration wasn't significantly affected by N deficiency at long term in leaves of Arabidopsis plants (Balazadeh et al., 2014). On other hand, resupplied N increased the tZ concentration. This increase was even higher than in control plants. The putative ionotropic glutamate receptor, 1.1 (AtGLR1.1), and isopentenyl transferase 3 (AtIPT3) of Arabidopsis thaliana, which is a key enzyme of cytokinin biosynthesis, are two factors that are thought to link nitrogen nutrition to hormonal signaling (Weaver et al. 1998; Kang & Turano 2003; Takei et al. 2004). But the exact mechanisms of this link are still unknown.

While the IP concentration slightly increased and then after decreased with natural leaf age, its concentration was stable and then decreased in N deficient plants (Fig.5.9). The significant differences between control and N deficient plant was early as 14 DAG. Moreover, IP concentration wasn't completely recovered to the levels of control plants upon N resupplied. These results suggest that N resupplied delay of senescence was mainly modulated by tZ induction from the roots to shoot, than by IP-CK form.

The transcriptomic profiling demonstrated that genes related to CKs metabolism and response were differentially regulated by N deficiency treatment early as 17 DAG. A response regulator (ARR) homologous to rice type-A response regulator 1 (OsRR1) was downregulated after 9 and 12 d of N deficiency treatment (Table 5.2). However ARR expression was recovered by N resupply, beside CK receptor (AHK) was upregulated. Furthermore, 2 genes coding CK oxidase (CKX) were upregulated by N resupply. It has been reported that ABA and CKs itself upregulate genes for CKX in maize (Brugiere et al., 2003).

6.4. Changes of amino acids levels in response to different N regimes

In barley plants, Glu was the dominant amino acid in control mature leaves in agreement with a previous study (Winter et al., 1992), followed by Asp, Ser, and Ala each generally occurring >10% abundance (Table 5.6). In our experiment, the total amino acids (sum the 15 detected AAs) concentration was decreased under N deficiency condition (Fig. 5.13) in agreement what was found in Arabidopsis plants (Balazadeh et al., 2014). It is worth to mention that amino acids were found almost absent from the vacuole and that amino acids patterns in the stroma and cytosol of barley plants are similar (Winter et al., 1992). Here, although glutamate concentration was decreased under N deficiency condition, its percentage from total amino acids increased up to 54% at 17 DAG and decreased then after. This would imply that the reduced growth normally observed under N deficiency condition is triggered by changes in the regulation of central amino acid metabolism, rather only by decreased levels of all amino acids (Tschoep et al., 2009). Interestingly, the decrease in total amino acids under N deficiency condition seems to be not always the case. Comadira et al., (2015) has reported that levels of most detected amino acids except for Asp, Ala, Oxo-pro, Glu, Pro, Try and Lys were significantly increased in barley plants after 7 d of N deficiency treatment. This might be explained by the different experimental design. Moreover, Comadira et al., (2015) in their experiment have reported that roots biomass was less by 8 folds in N-deficient plants compared to control plants after 7 d of N deficiency treatment. But in our experiment there were no significant differences in dry weight of roots between N deficient and control plants. Furthermore, there was no obvious change in shoot phenotype up to 17 DAG in our experiment. But in Comadira et al., (2015) experiment, the shoot and leaves were smaller at 14 DAG under N deficiency condition than under optimum N condition indicating more severed N deficiency. Nevertheless, previous studies in maize indicated almost universal reduction in amino acids concentrations (Amiour et al., 2012). Altogether, the present data suggest that the remobilization of assimilates mainly to strong sink

roots under N deficiency condition was more efficient in our approach. In addition, the differences between the two approaches gives opportunity in further understanding the remobilization regulation upon N deficiency treatment.

6.5. Changes of sugars levels in response to the different N regimes

N and C metabolism are closely connected, since carbon acts as skeleton for amino acid and other N containing compounds. When N is limited, high levels of sugars accumulate. In this condition the C/N ratio appears to be a major signal affecting plant development and leaf senescence (McAllister et al., 2012; O'Hara et al., 2013). In addition, the sugar levels in leaves are affected by many environmental factors including CO_2 concentration, light, and biotic stress, as well as abiotic stress, such as nutrient supply and drought. Plants have mechanisms to regulate photosynthetic rate in response to environmental factors and sink demand. Therefore, photosynthesis genes are sugar-repressible (Jang and sheen, 1994). High CO_2 concentration suppressed photosynthesis mostly when combined with low N condition. Depending on these observations, Paul and Driscoll (1997) suggested that sugar repressed photosynthesis may depend more crucially on C/N ratio of leaves, rather than sugar status alone. It has been reported that steam girdling of barley leaves lead to carbohydrates accumulation and induced leaf senescence (Parrott et al., 2007). Here, the sugars metabolism showed a shift to produce more sucrose for transportation rather than starch biosynthesis in primary leaf (Fig.5.14). Though, the starch concentration was higher under N deficiency condition in 4^{th} leaf compared to control plants (Fig.5.15). This indicates different sugar metabolism in mature and old leaves in response to N supply. Also, the amount of starch was much higher in the leaves of maize plants grown under low N input, but fructose and glucose concertation was lower (Amiour et al., 2012). It was shown that developmental and external supplied glucose induced- leaf senescence of Arabidopsis was delayed in hexokinase-1 (hxk1) mutant. This indicates that HXK1 is involved in sugar-induced leaf senescence (Moore et al., 2003). However, there is crosstalk between sugar and ABA

signaling in controlling many developmental processes. It has been shown that ABA is not required for sugar signaling in the regulation of leaf senescence (Pourtau et al., 2004). Notably, fructose and glucose concentration was slightly decreased, but this wasn't significant, with barley leaf age rather than increased as expected like in other plant species (Fig.5.14). Leaf senescence can be triggered by two conditions related to sugar levels, higher carbohydrate accumulation or sugars starvation by dark (Buchanan Wollaston et al., 2005; Wingler et al., 2006). To figure out if developmental senescence is related to sugar accumulation or starvation, a comparison of the global transcriptomic data of Arabidopsis plants revealed differences between developmental and sugar starvation-induced senescence (Buchanan Wollaston et al., 2005). But there could be difference in this regulation between plant species or even between cultivars. For examples, sugar levels would fall during late senescence as shown in tobacco (Masclaux et al., 2000), while hexoses accumulate until late senescence stages in Arabidopsis leaves (Stessman et al., 2002). Moreover, it has been argued that early SAGs are sugar-inducible, whereas late SAGs are sugar-repressible (Paul and Pellny, 2003). Correspondingly, this comparison is needed for barley plants to determine the role of sugars in developmental senescence. Here, we reported that many sugars compounds such as glucose, fructose, T6P, and starch decreased with leaf age in barley (Fig.5.14) which differed to a reported increase in other plant species such as Arabidopsis (Stessman et al., 2002).

However, the fructose concentration was increased by only two folds under N deficiency relative to control plants, and glucose concentration showed only minor changes (Fig.5.14). Comadira et al., (2015) have reported that sucrose, fructose, and glucose concentration increased by 2.6-, 33- and 19-folds, respectively, in N deficiency-treated barley plants. This again suggests that the plants in their experiment were subjected to more severe N deficiency condition, and that the remobilization of assimilates mainly to strong sink roots under N deficiency condition was more efficient in our experiment. Interestingly, resupplied N to starved plants reduced the concentration of sucrose, fructose,

and glucose (Fig.5.14). Beside, four from five putative genes involved in sugar signaling that coding glutamate receptor downregulated upon N resupplied. Balibrea Lara et al., (2004) have reported that the overexpression of an extracellular invertase gene under control of the senescence-induced SAG12 promoter delayed leaf senescence in transgenic tobacco (Nicotiana tabacum) plants. Whereas, genes coding cell wall invertase, hv_22536, and vacuolar invertase, hv_03914 and hv_42447, were downregulated upon N resupplied by 13-, 60-, and 25 folds, respectively. This indicates that delaying leaf senescence by N resupply doesn't work through invertase activity, at least on long term of N resupply.

Interestingly, T6P concentration decreased with leaf age in control plants. However, it was highly accumulated in N deficient plants (Fig.5.14). T6P has been considered to be a signal for high carbohydrate availability in plant cells. In T6P phosphatase gene (otsB)-expressing Arabidopsis plants, T6P accumulated less strongly during senescence than in wild-type plants, showing a similar phenotype as described for plants overexpressing the SnRK1 gene, KIN10, including reduced anthocyanin accumulation and delayed senescence independent of higher glucose, fructose and sucrose levels compared to wild type plants (Cho et al., 2012; Wingler et al., 2012). But T6P doesn't inhibit SnRK1 activity to the same extent in mature and senescing leaves as in young leaves (Zhang et al., 2009; Wingler et al., 2012). More investigation is needed to understand the role of T6P in modulating developmental and N deficiency-induced leaf senescence in barley plants. Nevertheless, T6P could play a role in delaying senescence in late response to N availability than an early and fast response to N resupply (Fig.5.14).

6.6. Sucrose and amino acids export rate through Phloem in response to N deficiency and N resupply

There are two main methods to collect phloem exudate using aphids (Fisher and Frame, 1984) or EDTA solution (Urquhart and Joy 1981). Using aphids technique gives a pure phloem exudate, but it is time consuming and not suitable for large scale of experimental design because when working with

barley leaf the chance that phloem sap actually exuded from a severed aphid stylet was only 1 in 200 (Winter et al., 1992). On other hand, collecting phloem exudate using EDTA solution is faster and more useful for big experiments including different time points and treatments. For this reason we chose EDTA solution technique to collect phloem exudate from primary leaves in 3 days interval and monitoring transportation rate of sucrose and amino acids under different N regimes. To avoid contamination from broken cells, area of cut was carefully washed by distilled water, and the leaves were kept in EDTA solution for 10 min, and then after transferred to fresh EDTA solution to collect exudate for 2 hours in saturated humid condition.

6.6.1. Sucrose export rates in response to developmental age and N supply

Sucrose transportation rate was slightly higher in N deficient than in control plants reflecting higher sugar available in primary leaf under N deficiency condition (Fig.5.16). Caputo et al., (1997) reported that the exudation rate of sugars from the phloem of wheat plants was unaffected by N supply, but sugars accumulated in the leaf tissue when the N supply was limiting for growth. In this context, phloem loading could be regulated by sucrose pool sizes responding to changes in source: sink ratio through modulating sucrose transporters (SUTs) expression. However, SUTs differ in their response to sucrose signals (reviewed by Lalonde et al., 2003). CKs are the strongest candidates for sink signals transmitted to source leaves to integrate sink demand (Roitsch and Ehneβ 2000). It is also believed that change in source supply or sink demand causes a local change in hydrostatic pressure inside sieve element which alters flow and phloem loading (Smith and Milburn et al., 1980). The transcriptomic profiling determined that putative genes coding sugar transporter were upregulated after N deficiency treatments (Table 5.12). Although, resupply of N downregulated the expression of sugar transporters (Table 5.12), there was no change observed in sucrose transport rate at 20 DAG (Fig.5.16).

6.6.2. Amino acids export rates in response to developmental age and N supply

Interestingly, total amino acids (sum of 15 detected amino acids) export rate was stable with 2 µmol. g leaf $DW^{-1}.h^{-1}$ up to 20 DAG in control plants, and it increased afterward, coinciding with the starting of natural leaf senescence process and activating N compounds remobilization (Fig.5.16). Although, the total amino acid was lower in leaves of N deficient plants, total amino acids export rate did not show a faster response to N deficiency treatment than control plants. But it was lower at 14 and 17 DAG. With progression of N deficiency-induced senescence there was no significant difference in export rate between N deficient and control plants (Fig.5.16). In agreement with these results, Caputo et al., (1997) concluded that the rate of amino acid export from the leaf to the phloem is dependent on the N available to the wheat plant, and the rate of exudation of amino acids to the phloem was independent of the concentration of free amino acids in the leaves. N resupply increased amino acids export compared to N deficient plants (Fig.5.16). This increase upon N resupply was also reported in wheat plants (Caputo et al., 1997).

The results also indicate some discrimination for transportation of individual amino acids with leaf age or N supply. Glu was the most abundant amino acid in the phloem exudate of mature leaf (Table.5.11). Glu was also the one most abundant in the phloem exudate of wheat leaves, although, Asp was the most abundant amino acid in the wheat leaf tissue (Caputo et al., 1997). In addition, Asp and Ala were exported to the phloem at a rate lower than expected from their leaf tissue concentrations, indicating some discrimination for amino acid transportation (Caputo et al., 1997). Notably, Gln became the most abundant in phloem exudate with leaf aging (Table.5.11), reflecting that the cytosolic GS (GS1) is involved in ammonium recycling during leaf senescence (Bernard and Habash, 2009). However, Glu stayed the most abundant in leaves during the experimental time course (Table.5.6). This indicates some discrimination for amino acid transportation during senescence process. By dividing the percentage of individual amino acids in phloem exudate to its percentage in leaf

tissues, the results demonstrated some discrimination of special amino acid transportation at different developmental age and N supply (Table.6.1). Some amino acids were more abundant in phloem exudate. Therefore, it is concluded that specificity of amino acids transporters might mediate phloem loading in barley plant. The transcriptomic data showed different expression patterns of genes encoding amino acids transporter depending on N supply. Fifteen putative genes classified to amino acid transporters were induced by at least two folds after 9 d of N deficiency, and two genes were downregulated (Table 5.12). Furthermore, three genes were upregulated and seven genes were downregulated after 12 d of N deficiency treatment (Table 5.12). When the N resupplied, seven putative genes coding amino acid transporter were upregulated, and twenty six genes were downregulated (Table 5.12). Further characterization is needed to understand the patterns of amino acids transportation at different conditions and age manner. Interestingly, GABA content in phloem exudate was much higher than in leaf tissues at mature stage, but decreased afterward (Table.6.1). These information might be useful for amino acid engineering to achieve higher remobilization under specific conditions of N availability. On the contrary, Glu showed a preferential export at low N supply and during N deficiency-induced leaf senescence (Table.5.11). The same was reported in wheat plants subjected to different N levels (Caputo et al., 1997). On other hand, the abundance of Gln in phloem exudate during N deficiency-induced senescence was much lower than control plants, and its abundance increased after N resupply, but stayed lower than control level (Table.5.11).

The preference of amino acids loading into phloem over sucrose loading is illustrated by total amino acids to sucrose ratios amounting 2-1 in leaves and > 2 in phloem exudate. This ratio also depends on light intensity (Caputo and Barneix, 1999). It has been shown that phloem blockage by callose deposition could lead to an age-dependent sugar accumulation (Jongebloed et al., 2004). However, it is not clear how the export of amino acids is achieved during leaf senescence.

Table 6. 1. The ratio of individual amino acid abundance in phloem exudate to its abundance in leaf tissues. DAG: Day after germination.

	Glu	Asp	Ser	Ala	Gln	Thr	Gly	Val	Asn	Phe	Pro	Lys	Ile	Leu	GABA
Cont. 14 DAG	0.76	0.87	0.95	0.77	3.15	1.57	1.19	2.58	0.37	1.17	4.24	1.43	4.17	3.36	12.86
Cont. 17 DAG	0.78	1.0	0.73	1.45	2.45	1.66	0.63	2.55	0.42	1.22	2.8	1.63	2.74	1.85	3.07
Cont. 20 DAG	0.78	0.99	0.82	0.85	1.63	1.43	0.38	1.22	0.22	0.76	1.55	0.62	1.22	1.24	2.1
Cont. 23 DAG	0.77	0.44	0.93	0.93	3.13	1.78	0.61	1.73	0.3	1.28	0.79	0.76	1.96	1.42	1.42
Cont. 26 DAG	0.75	0.43	0.55	0.76	3.31	1.31	0.4	1.5	0.29	1.05	0.5	0.63	1.76	1.3	0.68
Low N 14 DAG	0.67	0.97	0.8	1.88	1.0	1.73	1.79	5.04	0.16	1.48	2.74	1.79	3.66	2.45	13.33
Low N 17 DAG	0.58	1.85	1.14	2.65	1.42	2.21	1.49	1.66	0.07	1.93	0.16	2.11	3.44	2.34	9.05
Low N 20 DAG	0.81	1.99	1.06	1.47	1.3	1.3	0.52	1.65	0.45	1.31	0.07	1.29	2.42	1.99	1.24
Resupply N 20 DAG	1.06	0.83	0.98	0.87	0.86	1.04	0.11	1.89	0.8	0.95	0.91	1.49	1.81	1.33	3.32
Resupply N 23 DAG	1.07	0.63	0.92	0.77	1.81	1.52	0.69	1.73	0.28	0.98	0.56	0.61	2.28	1.86	2.93
Resupply N 26 DAG	0.84	0.52	0.79	0.86	2.74	1.5	0.59	1.66	0.39	1.27	0.93	0.73	2.83	2.26	1.27

6.7. Metabolic rearrangement in response to N supply

6.7.1. Metabolic profiling related to glycolysis and TCA upon N deficiency- induced leaf senescence and its reversal by N resupply

Glycolysis and TCA metabolites play an important role in connecting N and C metabolism. Thus, these metabolites can be precursors to synthesize amino acids and N containing metabolites. The transcriptomic and metabolic analyses suggest that N deficiency resulted a major reorganization of plant primary metabolism. Notably, the concentration of PEP and 3PGA (involved also in photosynthesis) highly accumulated upon N deficiency treatment (Fig.5.17). This finding hasn't been reported in other plant species such as maize (Amiour et al., 2012) where PEP concentration decreased in plants subjected to N deficiency. Nitrate reduction implies the formation of toxic alkaline ions (1mol of hydroxide for every mol of nitrate reduced). In this context, organic acids (principally citrate and malate) are involved to maintain pH homeostasis where nitrate reduction is occurring (Imsande and Touraine 1994; Touraine et al., 1988). Here, we report besides a decline in total amino acids upon N deficiency

treatment, a reduction in the majority of the organic acids including pyruvate and TCA (Fig.5.17). This decline in organic acids level under N deficiency condition has been also reported in Arabidopsis (Balazadeh et al., 2014) and maize (Amiour et al., 2012). However, the mechanism and physiological explanations behind these changes aren't yet clear. A decline in AMP level under N deficiency condition (Fig.5.18) might have a role in this regulation. On other hand, Comadira et al., (2015) have reported in their experiment on barley plant subjected to 7 d of N deficiency condition that organic acids of TCA increased. This implied that different experimental designs might cause different metabolism regulation. In addition, malate moves down to the roots through phloem, where it accumulates and stimulates nitrate uptake (Touraine et al., 1992). It is possible that this translocation could change its and other organic acids pool in the leaves.

When N resupplied to N starved plants, the concentration of pyruvate and a number of organic acids involved in the TCA cycle was completely or partially recovered to control plant levels. But there were no changes in 3PGA and PEP except the concentration of PEP was lowered at 20 DAG compared to N starved plants (Fig.5.17). It is worth to mention that a completely independent experiment with 6 biological replicates was carried out to verify these results (data not shown). The metabolic analyses demonstrated that pyruvate metabolism is a crucial step in controlling the metabolites flow from glycolysis to TCA cycle (Fig.5.17). The expression of genes involved in pyruvate metabolism will be discussed in the following section.

6.7.2. Correlations between glycolysis and TCA metabolite levels and expression of putative genes

The transcriptomic profiling revealed that N deficiency results in a major reprogramming of plant glycolysis and TCA metabolism. N deficiency reduced the expression of genes related to glycolysis and TCA cycle including cytosolic glyceraldehyde 3-phosphate dehydrogenase (DH), pyruvate kinase, malate DH, isocitrate DH that were decreased after 9 d of N deficiency treatment (17 DAG) whereas, the expression of genes coding plastidic phosphofructokinase,

phosphoglycerate mutase, and pyruvate dikinase were upregulated. In addition, the expression of genes coding cytosolic glyceraldehyde 3-phosphate DH, cytosolic enolase, plastidic phosphoglucomutase, pyruvate DH, malate DH, and ATP citrate lyase decreased after 12 d of N deficiency treatment (20 DAG). While the expression of genes coding phosphofructokinase, malate synthase, and pyruvate dikinase were upregulated. These changes in the genes expression coincided with decrease in concentration of pyruvate and organic acids of TCA cycle when N is limited for plant growth. In contrast, N resupplied activates the glycolysis and TCA cycle, which induced the expression of transcripts related to main glycolysis and TCA cycle pathways such as cytosolic glyceraldehyde 3-phosphate DH, enolase, cytosolic/plastidic pyruvate kinase, cytosolic phospho-enol-pyruvate carboxylase (PEPC), pyruvate DH, malate DH, isocitrate DH, and isocitrate layase. Comparatively, the expression of genes coding plastidic phosphofructokinase, aconitase, citrate synthase, and pyruvate dikinase were downregulated upon N resupply. The results of transcriptomic and metabolites profiling demonstrated that pyruvate metabolism is a crucial step in controlling the flow from glycolysis to TCA cycle dependent on N supply. The decrease in pyruvate concentration under N deficiency condition coincided with decreased expression of genes encoding enzymes for pyruvate synthesis (pyruvate kinase) and catabolism (pyruvate DH). Furthermore, to get more information about these genes regulation at more time points and other senescence inducers including drought-induced and natural senescence. The expression of these genes was found to be differentially regulated using both methods, microarray analysis and qPCR (Fig.5.19). PK2 and PDH1 were slightly down regulated under N deficiency indicating their role in controlling the late glycolysis and the flow to TCA cycle related to N supply. In Arabidopsis roots but not in shoot, K deficiency has been shown to strongly deplete pyruvate and organic acids, which was mainly independent of pyruvate kinase activity. In addition, nitrate level was also lower in roots under this condition, and K was proposed to maintain carbon flux into amino acids (Armengaud et al., 2009). In consequence, it is possible that K and N share the same mechanism to control

pyruvate metabolism and carbon flow. Interestingly, N resupply upregulated the checked genes for enzymes of pyruvate kinase and pyruvate dehydrogenase. The expression of genes coding these enzyme was also found to be upregulated after 3h of nitrate, supplied to N starved Arabidopsis seedlings (Scheible et al., 2004). The expression of genes encoding the bidirectional enzyme, pyruvate dikinase (PPDK), was up regulated under three different senescence inducing conditions, including natural-, N deficiency-, and drought-induced leaf senescence (Fig.5.19). Taylor et al., (2010) have reported that nitrogen remobilization was accelerated from Arabidopsis leaves during senescence when PPDK was overexpressed. However, N resupply delayed the upregulation of the four genes encoding PPDK enzyme. Nevertheless, the higher accumulation of 3PGA and PEP under N deficiency condition preceded the changes in expression of pyruvate kinase and pyruvate dikinase, and can't be explained by changes in their expression, but the changes in enzymes activity cannot be excluded. The obvious question is what causes this decrease and is there a physiological reason for this in glycolytic carbon flux and organic acids of TCA cycle.

7. Conclusion

To summarize, this work showed that N deficiency induced senescence program in leaves. N deficiency caused huge transcriptomic rearrangement, decreased CK levels (IP and tZ), and many alterations in metabolic products including amino acids, late glycolysis, and TCA cycle. However, sugar contents such as sucrose, fructose, and glucose were slightly increased in plant that was subjected to N deficiency condition, but this increase was much lower than reported for other plant species such as Arabidopsis. Moreover, ABA and T6P concentration was increased, which could serve as a senescence signal under N deficiency condition. On other hand, N resupply to N starved plant delayed senesce process. N resupply increased tZ (as systemic signal from root), amino acids biosynthesis, late glycolysis, and TCA cycle as shown in the model

(Fig.7.1). N resupply decreased the concentration of ABA and T6P (Fig.7.1). Nevertheless, sugars have tendency to decrease with leaf progression of control plant. Phloem exudate analyses demonstrated that there is different preference to individual amino acids in response to leaf age and N status.

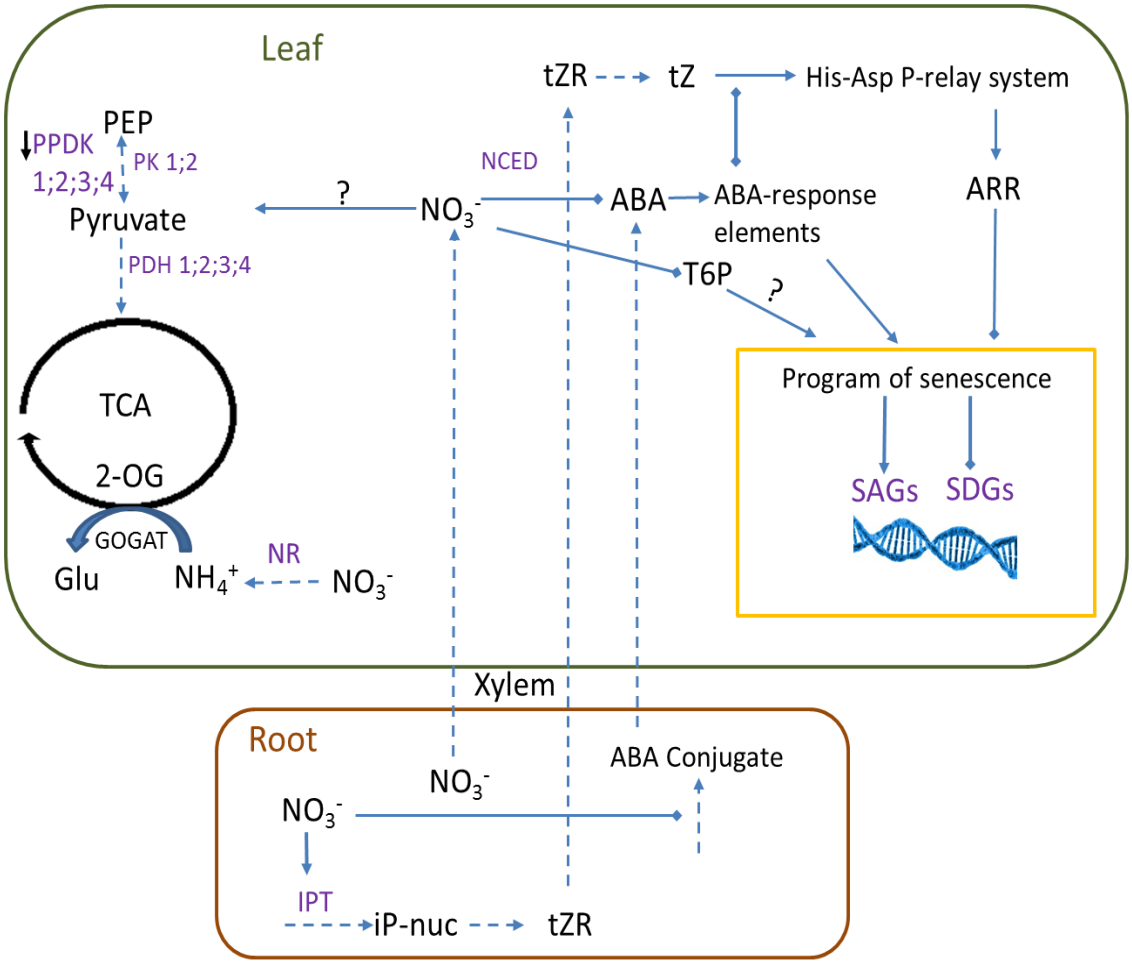

Figure 7. 1. A model of N resupply effect on leaf senescence and primary metabolism. . → :induced; —◆: repressed;↓: downregulated. NR: nitrate reductase, tZ: trans zeatin, IPT: adenine isopentyl transferase, ARR: a response regulator, 2-OG: 2-oxoglutarate, T6P: trehalose 6 phosphate, SAGs: senescence associated genes, SDGs: senescence downregulated genes, PK: Pyruvate kinase, PDH: pyruvate dehydrogenase, and PPDK: pyruvate, orthophosphate dikinase.

8. References

Agren GI, Franklin O. 2003. Root: Shoot Ratios, Optimization and Nitrogen Productivity. *Annals of Botany* **92**, 795-800.

Ahkami AH, Lischewski S, Haensch KT, Porfirova S, et al. 2008. Molecular physiology of adventitious root formation in *Petunia hybrida* cuttings: involvement of wound response and primary metabolism. *New Phytologist* **181,** 613-625

Amiour Y, Imbaud S, Clément G, *et al*. 2012. The use of metabolomics integrated with transcriptomic and proteomic studies for identifying key steps involved in the control of nitrogen metabolism in crops such as maize. *Journal of Experimental Botany* **63,** 5017-5033.

Armengaud P, Sulpice R, Miller AJ, Stitt M, et al. 2009. Multilevel Analysis of Primary Metabolism Provides New Insights into the Role of Potassium Nutrition for Glycolysis and Nitrogen Assimilation in Arabidopsis Roots. *Plant Physiology* **150,** 772-785.

Armstrong DJ. 1994. Cytokinin oxidase and the regulation of cytokinin degradation. In *Cytokinins: Chemistry, Activity, and Function*, ed. DWS Mok, MC Mok, pp. 139-154. Boca Raton, Florida: CRC Press.

Åstot C, Dolezal K, Nordström A, Wang Q,Kunkel T, et al. 2000.An alternative cytokinin biosynthesis pathway. *Proc. Natl. Acad. Sci. USA* **97,** 14778-14783.

Balazadeh S, Schildhauer J, Araújo WL, Munné-Bosch S, et al. 2014. Reversal of senescence by N resupply to N-starved *Arabidopsis thaliana*: transcriptomic and metabolomics consequences. *Journal of Experimental Botany*, **65** (14), 3975–3992.

Balazadeh S, Wu A, Mueller-Roeber B. 2010. Salt-triggered expression of the ANAC092-dependent senescence regulon in Arabidopsis thaliana. *Plant Signal. Behav.* **5**, 733-735.

Balibrea Lara ME, Garcia MCG, et al. 2004. Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. *Plant Cell* **16,** 1276-1287.

Bernard SM, Habash DZ. 2009. The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. *New Phytologist* **182,** 608-620.

Bleecker AB, Patterson SE. 1997. Last exit: senescence, abscission, and meristem arrest in Arabidopsis. *Plant Cell* **9**, 1169-1179.

Brugiere N, Jiao S, Hantke S, Zinselmeier C, Roessler JA, et al. 2003. Cytokinin oxidase gene expression in maize is localized to the vasculature, and is induced by cytokinins, abscisic acid, and abiotic stress. *Plant Physiology* **132,** 1228-1240.

Brusslan JA, Rus Alvarez-Canterbury AM, Nair NU, et al. 2012. Genome-wide evaluation of histone methylation changes associated with leaf senescence in Arabidopsis. *PLoS ONE* **7**, e33151.

Brzobohaty B, Moore I, et al. 1993. Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. *Science* **262,**1051-1054.

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, et al. 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. *Plant Journal* **42,** 567–585.

Burkle L, Cedzich A, Dopke C, Stransky H, Okumoto S, et al. 2003. Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of *Arabidopsis*. *Plant Journal* **34,**13-26.

Cabello P, de la Haba P, Gonzfilez-Fontes A, Maldonado JM. 1998. Induction of nitrate reductase, nitrite reductase, and glutamine synthetase isoforms in sunflower cotyledons as affected by nitrate, light, and plastid integrity**.** *Protoplasma* **201,** 1-7.

Caputo C, Barneix AJ. 1997. Export of amino acids to the phloem in relation to N supply in wheat. *Physiology Plantrum* **101,** 853-860.

Caputo C, Barneix AJ. 1999. The Relationship between Sugar and Amino Acid Export to the Phloem in Young Wheat Plants. *Annals of Botany* **84**, 33-38.

Chen CN, Chu CC, Zentella R, Pan SM, Ho TH. 2002a. AtHVA22 gene family in Arabidopsis: phylogenetic relationship, ABA and stress regulation, and tissue-specific expression. *Plant Mol Biol.* **49,** 633-644.

Chen WQ., Provart NJ, Glazebrook J, et al. 2002b Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *The Plant Cell* **14,** 559-574.

Chiou TJ, Bush DR. 1998. Sucrose is a signal molecule in assimilate partitioning. *Proceeding of national academy of science of USA.* **95,** 4784-4788.

Cho YH, Hong JW, Kim EC, Yoo SD. 2012. Regulatory functions of SnRK1 in stress-responsive gene expression and in plant growth and development. *Plant Physiology* **158**, 1955-1964.

Chomczynski P, Mackey K. 1995. Modification of the TRI reagent procedure for isolation of RNA from polysaccharideand proteoglycan-rich sources. *BioTechniques* **19,** 942-945.

Choudhury A, Lahiri A. 2010. Comparative analysis of abscisic acid-regulated transcriptomes in Arabidopsis. *Plant Biology* **13,** 28-35.

Christiansen MW, Holm PB, Gregersen PL. 2011. Characterization of barley (Hordeum vulgare L.) NAC transcription factors suggests conserved functions compared to both monocots and dicots. *BMC Research Notes* **4**, 302.

Christmann A, Hoffmann T, Teplova I, Grill E, Muller A. 2005. Generation of active pools of abscisic acid revealed by *in vivo* imaging of water-stressed Arabidopsis. *Plant Physiology* **137,** 209-219.

Christmann A, Weiler EW, Steudle E, Grill E. 2007. A hydraulic signal in root-to-shoot signalling of water shortage. *Plant Journal* **52,** 167-174.

Comadira G, Rasool B, Karpinska B, Morris J. 2015. Nitrogen deficiency in barley (*Hordeum vulgare*) seedlings induces molecular and metabolic adjustments that trigger aphid resistance. *Journal of Experimental Botany* **66,** 3639-3655.

Corbesier L, Prinsen E, et al. 2003. Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thaliana* during floral transition. *J. Exp. Bot.* **54,** 2511-17.

Coudert Y, Perin C, Courtois B, Khong NG, Gantet P. 2010. Genetic control of root development in rice, the model cereal. *Trends Plant Science* **15**, 219-226.

D'Agostino IB, Deruere J, Kieber JJ. 2000. Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiology* **124**, 1706-1717.

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

Davies W, Zhang J. 1991. Root signals and the regulation of growth and development of plants in drying soil. *Ann. Rev. Plant Phys. Plant Mol. Biol.* **42,** 55-76.

Dietz KJ, Sauter A, Wichert K, Messdaghi D, Hartung W. 2000. Extracellular beta-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *Journal of Experimental Botany* **51,** 937-944.

Distelfeld A, Avni R, Fisher AM. 2014. Senescence, nutrient remobilization, and yield in wheat and barley. *Journal of Experimental Botany* **65** (14), 3783-3798.

Distelfeld A, Korol A, Dubcovsky J, Uauy C, Blake T, Fahima T. 2008. Colinearity between the barley grain protein content (GPC) QTL on chromosome arm 6HS and the wheat Gpc-B1 region. *Molecular Breeding* **22**, 25-38.

Domagalska MA, Leyser O. 2011. Signal integration in the control of shoot branching. *Nat. Rev. Mol. Cell Biol.* **12**, 211-221.

Dun EA, Brewer PB, Beveridge CA. 2009. Strigolactones: discovery of the elusive shoot branching hormone. *Trends Plant Science* **14**, 364-372.

Egli DB, Leggett JE, Duncan WG. 1976. Influence of N stress on leaf senescence and N redistribution in soybeans. *Agronomy Journal* **70**, 43-47.

El-Showk S, Ruonala R, Helariutta Y. 2013. Crossing paths: cytokinin signalling and crosstalk**.** *Development* **140,** 1373-1383.

Feller U, Anders I, Mae T. 2008. Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. *Journal of Experimental Botany* **59**, 1615-1624.

Fisher DB, Frame JM. 1984. A guide to the use of the exuding stylet technique in phloem physiology. *Planta,* **161,** 385-93.

Finkelstein R. 2013. Abscisic Acid Synthesis and Response. *The Arabidopsis Book* e0166. doi: 10.1199/tab.0166.

Foo E, Morris SE, Parmenter K, Young N. 2007. Feedback Regulation of Xylem Cytokinin Content Is Conserved in Pea and Arabidopsis. *Plant Physiology* **143,** 1418-1428.

Forde BG. 2002. Local and long-range signaling pathways regulating plant responses to nitrate. *Annu. Rev. Plant Biol.* **53,** 203–224.

Fragoso S, Espíndola L, Páez-Valencia J, Gamboa A, et al. 2009. SnRK1 isoforms AKIN10 and AKIN11 are differentially regulated in Arabidopsis plants under phosphate starvation. *Plant Physiology* **149**, 1906-1916.

Gan S, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270**, 1986-1988.

Gan S, Amasino RM. 1997. Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). *Plant Physiology* **113,** 313–319.

Gazzarrini S, Lejay L, Gojon A, Ninnemann O, Frommer WB, von Wiren N. 1999. Three functional transporters for constitutive, diurnally regulated, and starvation induced uptake of ammonium into Arabidopsis roots. *The Plant Cell* **11**, 937-947

Gregersen PL. 2011. Senescence and nutrient remobilization in crop plants. In: Hawkesford MJ, Barraclough PB, editors, *The molecular and physiological basis of nutrient use efficiency in crops*. Oxford, UK: John Wiley & Sons, pp 83-102

Gregersen PL, Culetic A, Boschian L, Krupinska K. 2013. Plant senescence and crop productivity. *Plant Molecular Biology* **82**, 603-622.

Good AG, Shrawat AK, Muench DG. 2004. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production?. *Trends in Plant Science* **9**, 597-605.

Guo Y, Gan S. 2006. AtNAP, a NAC family transcription factor, has an important role in leaf senescence. *Plant J*. **46**, 601-612.

Guo Y, Gan SH. 2012. Convergence and divergence in gene expression profiles induced by leaf senescence and 27 senescence-promoting hormonal, pathological and environmental stress treatments. *Plant, Cell & Environment* **35**, 644-655.

Ha CM, Kim GT, et al. 2003. The *BLADE-ON-PETIOLE 1* gene controls leaf pattern formation through the modulation of meristematic activity in *Arabidopsis*. *Development* **130**,161-172.

He Y,Tang W, Swain JD, Green AL, Jack TP, Gan S. 2001. Networking senescence regulating pathways by using *Arabidopsis* enhancer trap lines. *Plant Physiol*.**126,** 707-716.

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

Hill-Cottingham DG, Lloyd-Jones CP. 1968. Relative mobility of some organic nitrogenous compounds in the xylem of apple shoots. *Nature* **220,** 389-390.

Hirel B, Le Gouis J, Ney B, Gallais A. 2007. The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *Journal of Experimental Botany* **58**, 2369-2387.

Hirose N, Makita N, Yamaya T, Sakakibara H. 2005. Functional characterization and expression analysis of a gene, *OsENT2*, encoding an equilibrative nucleoside transporter in rice suggest a function in cytokinin transport. *Plant Physiology* **138,** 196-206.

Hoth S, Ikeda Y, Morgante M, Wang X, et al. 2003. Monitoring genome-wide changes in gene expression in response to endogenous cytokinin reveals targets in *Arabidopsis thaliana*. *FEBS Lett.* **554,** 373-380.

Imsande J, Touraine B. 1994. N demand and the regulation of Nitrate uptake, *Plant Physiology* **105**, 3–7.

Jang JC, Sheen J. 1994. Sugar sensing in higher plant. *Plant cell* **6**, 1665-1679.

Jeannette E, Rona JP, Bardat F, Cornel D, Sotta B, Miginiac E. 1999. Induction of RAB18 gene expression and activation of K+ outward rectifying channels depend on extracellular perception of ABA in *Arabidopsis thaliana* suspension cells. *Plant Journal* **18,** 13-22.

Jongebloed U, Szederke´nyi J, Hartig K, Schobert C, Komor E. 2004. Sequence of morphological and physiological events during natural ageing of a castor bean leaf: sieve tube occlusions and carbohydrate back-up precede chlorophyll degradation. *Physiologia Plantarum* **120**, 338-346.

Kang J, Turano FJ. 2003. The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 6872-6877.

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 MK, Jung HJ, Kim DH, Kang H. 2012. Characterization of glycine-rich RNA-binding proteins in *Brassica napus* under stress conditions**.** *Physiologia Plantarum* **146,** 297-307.

Kjaersgaard T, Jensen MK, Christiansen MW, Gregersen P, et al. 2011. Senescence-associated Barley NAC (NAM, ATAF1,2, CUC) Transcription Factor Interacts with Radical-induced Cell Death 1 through a Disordered Regulatory Domain. *The journal of biological chemistry* **286,** 35418-35429.

Kohl S, Hollmann J, Blattner FR, *et al.* 2012. A putative role for amino acid permeases in sink-source communication of barley tissues uncovered by RNA-seq. *BMC Plant Biology* **12**, 154.

Krapp A, David LC, et al. 2014. Nitrate transport and signalling in *Arabidopsis. Journal of Experimental Botany* **65**, 789-798.

Krouk G, et al. 2010a. Nitrate-regulated auxin transport by *NRT1.1* defines a mechanism for nutrient sensing in plants. *Development Cell* **18**, 927-937.

Krouk G, Mirowski P, LeCun Y, Shasha DE, Coruzzi GM. 2010b. Predictive network modeling of the high-resolution dynamic plant transcriptome in response to nitrate. *Genome Biology* **11**, R123.

Krupinska K, Mulisch M, Hollmann J, et al. 2012. An alternative strategy of dismantling of the chloroplasts during leaf senescence observed in a high yield variety of barley. *Physiologia Plantarum* **144**, 189-200.

Lacerenza JA, Parrott DL, Fischer AM. 2010. A major grain protein content locus on barley (*Hordeum vulgare L.*) chromosome 6 influences flowering time and sequential leaf senescence. *Journal of Experimental Botany* **61,** 3137-3149.

Lalonde S, Tegeder M, Throne-Holst M, Frommer WB, Patrick JW. 2003. Phloem loading and unloading of sugars and amino acids. *Plant, Cell and Environment* **26,** 37–56.

Lambers H, Simpson RJ, Beiiharz VC, Dailing MJ. 1982. Growth and translocation of C and N in wheat *{Triticum aestivum* L.) grown with a split root system. *Physiologia Plantrum* **56,** 421-429.

Lea PJ, Forde BG. 1994. The use of mutants and transgenic plants to study amino acid metabolism. *Plant, Cell and Environment* **17,** 541-556.

Lee IC, Hong SW, Whang SS, Lim PO, Nam HG, Koo JC. 2011. Age-dependent action of an ABA-inducible receptor kinase, RPK1, as a positive regulator of senescence in Arabidopsis leaves. *Plant Cell Physiology* **52,** 651-662.

Lee KH, Piao HL, Kim HY, Choi SM, et al. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell* **126,** 1109-1120.

Lejay L, Gansel X, et al. 2003. Regulation of root ion transporters by photosynthesis: functional importance and relation with hexokinase. *Plant Cell* **15,** 2218-2232.

Lejeune P, Bernier G, Requier M-C, Kinet J-M. 1994. Cytokinins in phloem and xylem saps of *Sinapis alba* during floral induction. *Physiologia Plantrum* **90,** 522-528.

Lillo C. 2008. Signalling cascades integrating light-enhanced nitrate metabolism. *Biochemical Journal* **415**, 11-19.

Lim PO, Kim HJ, Nam HG. 2007. Leaf senescence**.** *Annu. Rev. Plant Biol.* **58**,115-36.

Maathuis F. 2009. Physiological functions of mineral nutrients. *Current Opinion in Plant Biology* **12**, 250-258.

Mangeon A, Junqueira RM, Sachetto-Martins G. 2010. Functional diversity of the plant glycine-rich proteins superfamily. *Plant Signal Behav*. **5,** 99-104.

Marchive C, Roudier F, Castaings L, Bréhaut V, Blondet E, et al. 2013. Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. *Nature Communications* **4**, 1713.

Masclaux C, Valadier MH, Brugie`re N, Morot-Gaudry JF, Hirel B. 2000. Characterization of the sink/source transition in tobacco (Nicotiana tabacum L.) shoots in relation to nitrogen management and leaf senescence. *Planta* **211**, 510-518.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, et al. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Annals of Botany* **105,** 1141-1157.

Masferrer A, Arro M, Manzano D, Schaller H, et al. 2002. Overexpression of *Arabidopsis thaliana* farnesyl diphosphate synthase (FPS1S) in transgenic *Arabidopsis* induces a cell death/senescence-like response and reduced cytokinin levels. *Plant Journal* **30,** 123–132.

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

Meyer C, Stitt M. 2001. Nitrate reductase and signalling. In: Lea PJ, Morot-Gaudry J-F, eds. Plant nitrogen. *New York: Springer*, 37–59.

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.

Miller AJ,Fan X, Orsel M, Smith SJ, Wells DM. 2007. Nitrate transport and signalling. *Journal of Experimental Botany* **58,** 2297-2306.

Miyawaki K, Matsumoto-Kitano M, Kakimoto T. 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant Journal* **37,**128-138.

Moore B, Zhou L, et al. 2003. Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* **300**, 332-336.

Müller AH, Hansson M. 2009.The barley magnesium chelatase 150- kD subunit is not an abscisic acid receptor. *Plant Physiology* **150,** 157-166.

Munier-Jolain NG, Salon C. 2005. Are the carbon costs of seed production related to the quantitative and qualitative performance? An appraisal for legumes and other crops. *Plant, Cell and Environment* **28**, 1388-1395.

O'Hara LE, Paul MJ, Wingler A. 2013. How do sugars regulate plant growth and development? New insight into the role of trehalose-6-phosphate. *Molecular Plant* **6**, 261-274.

Omarov RT, Sagi M, Lips SH. 1998. Regulation of aldehyde oxidase and nitrate reductase in roots of barley (Hordeum vulgare L.) by nitrogen source and salinity. *Journal of Experimental Botany* **49,** 897–902.

Ongaro V, Leyser O. 2008. Hormonal control of shoot branching. *Journal of Experimental Botany* **59**, 67-74.

Ori N, Juarez MT, Jackson D, Yamaguchi J, Banowetz GM, Hake S. 1999. Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene *knotted1* under the control of a senescence-activated promoter. *Plant Cell* **11,** 1073-80.

Park SY, Fung P, Nishimura N, Jensen DR, et al. 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324,** 1068-1071.

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

Paul MJ, Driscoll SP. 1997. Sugar repression of photosynthesis: the role of carbohydrates in signalling nitrogen deficiency through source:sink imbalance. *Plant, Cell & Environment* **20,** 110-116.

Paul MJ, Foyer CH. 2001. Sink regulation of photosynthesis. *Journal of Experimental Botany* **52,** 1383-1400.

Paul MJ, Pellny TK. 2003. Carbon metabolite feedback regulation of leaf photosynthesis and development. *Journal of Experimental Botany* **54**, 539-547.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* **29,** e45.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (RESTa) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30,** e36.

Pourtau N, Mares M, Purdy S, Quentin N, Ruel A, Wingler A. 2004. Interactions of abscisic acid and sugar signalling in the regulation of leaf senescence. *Planta* **219**, 765–772.

Price J, Laxmi A, St Martin SK, Jang JC. 2004. Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. *The Plant Cell* **16**, 2128-2150.

Puig J, Pauluzzi G, Guiderdoni E, Gantet P. 2012. Regulation of Shoot and Root Development through Mutual Signaling. *Molecular Biology* **5**, 974-983.

Richmond AE, Lang A. 1957. Effect of kinetin on protein content and survival of detached *Xanthium* leaves. *Science* **125,** 650-651.

Rivero RM, Kojimat M, et al. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant**.** *PNAS* **104** (49), 19631-19636.

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

Roitsch T, Ehne R. 2000. Regualtion of source/sink relations by cytokinins. *Plant Growth Regulation* **32,** 359-367.

Romanov GA, Lomin SN, Schmülling T. 2006. Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. *Journal of Experimental Botany* **57**, 4051-4058.

Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR. 2009. Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. *The Plant Cell* **21**, 3567-3584.

Sakakibara H, Takei K, Hirose N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. *TRENDS in Plant Science* **11** (9).

Samuelson ME, Larsson CM. 1993. Nitrate regulation of zeatin riboside levels in barley roots: effects of inhibitors of N assimilation and comparison with ammonium. *Plant Science* **93,** 77-84.

Scheible WR, Gonzalez-Fontes A, Lauerer M, Muller-Rober B, et al.1997. Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco, *Plant Cell* **9**, 783– 798.

Scheible WR, Morcuende R, Czechowski T, Fritz C, et al. 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.

Schippers HM, Jing HC, Hille J, Dijkwel PP. 2007. Developmental and hormonal control of leaf senescence. *Oxford: Blackwell Publishing*. 145–170.

Schlutera T, Leideb J, Conrad K. 2011. Light promotes an increase of cytokinin oxidase/dehydrogenase activity during senescence of barley leaf segments. *Plant Physiology* **168,** 694-698.

Schmitz RY, Skoog F. 1972. Cytokinins: synthesis and biological activity of geometric and position isomers of zeatin. *Plant Physiology* **50,** 702-705.

Schommer C, Palatnik JF, et al. 2008. Control of jasmonate biosynthesis and senescence by miR319 targets. *PLoS Biol.* **6**, e230.

Sekhon R, Childs KL, Santoro N, Foster CE, Buell CR, de Leon N, Kaeppler SM. 2012. Transcriptional and Metabolic Analysis of Senescence Induced by Preventing Pollination in Maize. *Plant Physiology* **159**, 1730–1744.

Seo M, Peeters A, Koiwai H, Oritani T, et al. 2000. The Arabidopsis aldehyde oxidase 3 (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. *Proc. Natl. Acad. Sci. USA* **97,** 12908-12913

Shangguan ZP, Shao MA, Ren SJ, Zhang LM, Xue Q. 2004. Effect of nitrogen on root and shoot relations and gas exchange in winter wheat. *Bot..Bull.Acad.Sin.* **45**, 49-54.

Sharp RE. 2002. Interaction with ethylene: changing views on the role of abscisic acid in root and shoot growth responses to water stress. *Plant Cell Environ*. **25,** 211-222.

Shen YY, Wang XF, Wu FQ, Du SY, et al. 2006. The Mg-chelatase H subunit is an abscisic acid receptor. *Nature* **443,** 823-826.

Shu-Qing C, Rong-Xian Z, Wei L, Zhi-Rui D, Qi-Ming Z. 2004. The Involvement of Cytokinin and Abscisic Acid Levels in Roots in the Regulation of Photosynthesis Function in Flag Leaves during Grain Filling in Super High-yielding Rice (Oryza sativa). *J. Agronomy & CropScience* **190,** 73-80.

Sicher RC. 2001. Responses of nitrogen metabolism in N-sufficient barley primary leaves to plant growth in elevated atmospheric carbon dioxide. *Photosynthesis Research* **68**, 193–201.

Smith JA, Milburn JA. 1980. Phloem turgor and the regulation of sucrose loading in *Ricinus communis L*. *Planta* **148,** 42-48.

Sonoda Y, Ikeda A, Saiki S, Yamaya T, Yamaguchi J. 2003. Feedback regulation of the ammonium transporter gene family AMT1 by glutamine in rice. *Plant Cell and Physiology* **44,** 1396-1402.

Stessman D, Miller A, Spalding M, Rodermel S. 2002. Regulation of photosynthesis during Arabidopsis leaf development in continuous light. *Photosynthesis Research* **72**, 27-37.

Takei K, Sakakibara H, Taniguchi M, Sugiyama T. 2001. Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin

species that induces gene expression of maize response regulator. *Plant Cell Physiology* **42,** 85-93.

Takei K, Ueda N, Aoski K, Kuromori T, Hirayama T, et al. 2004. AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. *Plant and Cell Physiology* **45**, 1053-1063.

Takei K, Yamaya T, Sakakibara H. 2004. *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. *J. Biol. Chem.* **279,** 41866-41872.

Taylor L, Nunes-Nesi A, Parsley K, Leiss A, et al. 2010. Cytosolic pyruvate,orthophosphate dikinase functions in nitrogen remobilization during leaf senescence and limits individual seed growth and nitrogen content. *The Plant Journal* **62**, 641-652.

Thompson A, Jackson A, Parker R, Morpeth D, Burbidge A, Taylor I. 2000. Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase mRNAs by light/ dark cycles, water stress and abscisic acid. *Plant Mol. Biol.* **42,** 833-845.

Touraine B, Grignon N, Gringnon C. 1988. Charge balance in NO3- fed soybean. Estimation of K_ and carboxylate recirculation, *Plant Physiology* **88**, 605-612.

Touraine B, Muller B, Grignon C. 1992. Effect of phloem translocated malate on NO^{3-} uptake by roots of intact soybean plants, *Plant Physiology* **93**, 1118-1123.

Tsay YF, Chiu CC, Tsai CB, Ho CH, Hsu PK. 2007. Nitrate transporters and peptide transporters. *FEBS Letters* **581**, 2290-2300.

Tschoep H, Gibon Y, Carillo P, Armengaud P, et al. 2009. Adjustment of growth and central metabolism to a mild but sustained nitrogen-limitation in *Arabidopsis*. *Plant, Cell and Environment* **32**, 300-318.

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.

Urquhart AA, Joy KW. 1981. Use of phloem exudate technique in the study of amino acid transport in pea plants. *Plant Physiology* **68,** 750-754.

Van Norman JM, Frederick RL, Sieburth LE. 2004. *BYPASS1* negatively regulates a root-derived signal that controls plant architecture. *Current Biology* **14**, 1739-1746.

Veach YK, Martin RC, Mok DW, Malbeck J, Vankova R, Mok MC. 2003.*O*-Glucosylation of *cis*-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. *Plant Physiology* **131,** 1374-1380.

Walch-Liu P, Ivanov II, Filleur S, Gan Y, Remans T, Forde BG. 2006. Nitrogen regulation of root branching. *Annual Botany* **97**, 875-881.

Wang R, Tischner R, et al. 2004. Genomic analysis of the nitrate response using a nitrate reductase-null mutant of Arabidopsis. *Plant Physiology* **136**, 2512-2522.

Wang RS, Pandey S, Li S, Gookin TE, et al. 2011. Common and unique elements of the ABA-regulated transcriptome of Arabidopsis guard cells. *BMC Genomics* **12,** 216.

Wang R, Xing X, Wang Y, Tran A, Crawford NM. 2009. A genetic screen for nitrate-regulatory mutants captures the nitrate transporter gene NRT1.1. *Plant Physiology* **151**, 472-478.

Wang X, Wu P, Xia M, Wu Z, Chen Q, Liu F. 2002. Identification of genes enriched in rice roots of the local nitrate treatment and their expression patterns in split-root treatment. *Gene* **297,** 93-102.

Waters BM, Uauy C, Dubcovsky J, Grusak MA. 2009. Wheat (*Triticum aestivum*) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain. *Journal of Experimental Botany* **60**, 4263-4274.

Weaver LM, Gan S, Quirino B, Amasino RM. 1998. A comparison of the expression patterns of several senescenceassociated genes in response to stress and hormone treatment. *Plant Molecular Biology* **37**, 455-469.

Williams L, Miller A. 2001. Transporters responsible for the uptake and partitioning of nitrogenous solutes. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 659-688.

Wingler A, Delatte TL, O'Hara LE, Primavesi LF, et al. 2012. Trehalose 6-Phosphate Is Required for the Onset of Leaf Senescence Associated with High Carbon Availability. *Plant Physiology* **158**, 1241–1251.

Wingler A, Purdy S, MacLean JA, Pourtau N. 2006. The role of sugars in integrating environmental signals during the regulation of leaf senescence. *Journal of Experimental Botany* **57**, 391-399.

Winter H, Lohaus G, Heldt HW. 1992. Phloem Transport of Amino Acids in Relation to their Cytosolic Levels in Barley Leaves. *Plant Physiology* **99,** 996-1004.

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. *Journal of Cell Science* **126**, 4823-4833.

Ying-Hua S, Yu-Bo L, Xian-Sheng Z. 2011. Auxin–Cytokinin Interaction Regulates Meristem Development. *Mol Plant* **4,** 616-625.

Zalewski W, Galuszka P, Gasparis S, et al. 2010. Silencing of the HvCKX1 gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to higher plant productivity. *Journal of Experimental Botany* **61,** 1839-1851.

Zdunek E, Lips SH. 2001. Transport and accumulation rates of abscisic acid and aldehyde oxidase activity in Pisum sativum L. in response to suboptimal growth conditions. *Journal of Experimental Botany* **52** (359), 1269-1276.

Zeevaart JAD, Creelman RA. 1988. Metabolism and physiology of abscisic acid. Ann. *Rev. Plant Physiol. Plant Mol. Biol.* **39,** 11439-11473.

Zhang H, Forde BG. 1998. An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. *Science* **279**, 407-409.

Zhang H, Forde BG. 2000. Regulation of *Arabidopsis* root development by nitrate availability. *Journal Experimental Botany* **51,** 51-59.

Zhang K, Gan S. 2012. An abscisic acid-AtNAP transcription factor-SAG113 protein phosphatase 2C regulatory chain for controlling dehydration in senescing Arabidopsis leaves. *Plant Physiology* **158**, 961-969.

Zhang X, Jiang L, Wang G, Yu L, et al. 2013. Structural insights into the abscisic acid stereospecificity by the ABA receptors PYR/PYL/RCAR. *PLoS ONE* **8,** e67477.

Zhang Y, Primavesi LF, et al. 2009. Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant Physiology* **149**, 1860-1871.

9. Abbreviations

2-oxo: 2-oxoglutarate

3PGA: 3-Phosphoglycerat

AAO or AO: Aldehyde oxidase

AAP: Amino acids permease

AAs: Amino acids

ABA: Abscisic acid

AB15: ABA response gene

ABA-GE: ABA-glucose ester

ABI3, ABI4, and ABI5: ABA insensitive transcription factors 3; 4; 5

AGPase: ADP-Glc pyrophosphorylase

AGPs: Arabinogalactans-proteins

AHK: Arabidopsis Histidine Kinase

AMP, ADP, or ATP: adenosine (momo, di, or tri) 5'-phosphates

AMTs: Ammonium transporters

AP2/EREBP: APETALA2 /Ethylene-responsive element binding protein

AQC: 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate

ARF: Auxin response factor

ARR: A response regulator

AtIPT3: At-Isopentenyl transferase 3

AtGLR1.1: Arabidopsis glutamate receptor, 1.1

AUX/IAA: auxin/indole-3-acetic acid

pmol: Pico mole

bop1-1: blade on petiole 1-1

C: Carbon

CAT: Cationic amino acid transporter

Chl-Content: Chlorophyll content

Chip-seq: Chromatin immunoprecepitation sequencing

Cit: Citarte

Cis-Acc: cis-aconitate

CKs: Cytokinins

CKX: CK oxidase

C/N ratio: Carbon/Nitrogen ratio

Cont.: Control

cRNA: Complementary RNA

Ct: Control

CYP735A1 and CYP735A2: Cytochrome P450 (735A1; 735A2)

cZ: cis-zeatin

D2;3: Drought stage 2;3

DAG: Day after germination

DAS: Days after sowing

DH: Dehydrogenase

DNA: Deoxyribonucleic acid

DPA: Diphaseic acid

DW: Dry weight

DZ: dihydrozeatin

EA: Elemental analyzer

EDTA: Ethylenediaminetetraacetic acid

EIN2: Ethylene-insensitive 2

FC: Fold change

Fum: Furmarte

FW: Fresh weight

Gb: Giga base pairs

GDH: Glu dehydrogenases

g (FW): Gram (Fresh weight)

GOGAT: Glutamine oxoglutarate aminotransferase (Glu synthase)

GR-RBPs: Glycine-rich RNA-binding proteins

GS1;2: Glutamine synthetase 1;2

Hv: Hordeum vulgare

HXK1: HEXOKINASE-1

IC: Ion chromatography

IC-MSMS: Ion chromatography-mass spectrometry

INV: Invertase

IP: N^6-(Δ^2-isopentenyl)-adenine

IPR: N^6-(Δ^2-isopentenyl)-adenine riboside

IPT: Isopentenyl transferase

Isocit: Isocitrate

JUB1: JUNGBRUNNEN1

KIN10: SNF1 kinase homolog 10

L.: Leaf

LN: Low N

Mal: Malate

µg: Mikrogramm

Min: Minute

Ml: Milliliter

µl: Mikroliter

mM: Millimolar

µM: Mikromolar

MAX: MORE AXILLARY GEOWTH

miR164: microRNA164

miR319: MicroRNA 319

miRNAs: micro RNAs

MYB: Myeloblastosis

N: Nitrogen

NAC: NAC is an acronym derived from the names of the three genes first described as containing the domain, namely NAM (no apical meristem), ATAF1,2 and CUC2 (cup-shaped cotyledon)

NCED: 9-cis-epoxycarotenoid dioxygenase

NiR: Nitrite reductase

NLPs: NIN-like protein

NR: Nitrate reductase

NRT1:2: Nitrate transporter superfamily 1;2

NUE: nitrogen use efficiency

NTL4: Arabidopsis NAC transcription factor

OAA: Oxaloacetate

OPP: Oxidative pentose phosphate

ORE1: ORESARA1

OtsB: T6P phosphatase gene

PA: Phaseic acid

PDH: Pyruvate dehydrogenase

PEP: Phosphoenolpyruvate

PK: Pyruvate kinase

PPDK: Pyruvate orthophosphate dikinase

PGI: Phosphoglucoisomerase

PSII: Photosystem II

PYR1: PYRABACTIN RESISTANT 1

qPCR: Quantitative PCR

RNA: Ribonucleic acid

RN: Resupply N

RPK1: ABA-inducible receptor kinase

S3: Senescence stage 3

SAG12: Senescence associated gene12

SAGs: senescence associated genes

SD: Standard deviation

SDGs: senescence downregulated genes

SIRK: senescence –induced receptor like kinase gene

siRNAs: small –interfering RNAs

SNF1: Sucrose non-Fermenting 1

SnRK1: Sucrose-nonfermentation1-related protein kinase1

SPAD: Soil Plant Analysis Development

SPP: Sucrose-phosphate phosphatase

SPS: Sucrose-phosphate synthase

Succ: Succinate

Susy: Sucrose synthases

SUTs: Sucrose transporters

T6P: Trehalose-6-phosphate

TAE: Tris-acetate-EDTA

TCA cycle: The tricarboxylic acid cycle

TCP4: TEOSINTE BRANCHED1/CYCLOIDEA/PRO-LIFERATING CELL FACTOR 4

TPP: T6P phosphatase

TPS: Trehalose-6-P synthase

tZ: trans-zeatin

tZR: trans-zeatin riboside

UPLC: Ultra-performance liquid chromatography

XDH: Xanthine dehydrogenase

v/v: volume per volume

w/v: weight per volume

ZR: Zeatin riboside

10. Appendix

Table 10. 1. Primers sequence

Gene	Forward primer	Reverse primer
HvGR-RBP1	CCGACGGATACTGAAGGAACTG	TGCGATCAAAAACACAAACGAG
HvGR-RBP2	TTGCCGTATGTTGCATTGTG	GCGTTTTTGTAACTTCAGGCA
HvARR6-1	GTGATCATGTCATCGGAGAA	CGTACTGGTTTCAGGAAGAA
Hv-S40	GTCTGCCCCGGTCCTCGTG	GTTCCTCTTCGCGTCGTTGG
Hv-PP2A	CACCATTTCTCAGCTTGTATTG	CACCCCTTTGTTATTGTTTGTTG
Hv-GS2	AGCGCGATCTCACAGGTCG	ATCGTCGTCTCTACGTACTTGC
Hv-actin	GGAAATGGCTGACGGTGAGGAC	GGCGACCAACTATGCTAGGGAAAAC
Hv-NAC013	ATG CCG CCG CAC ATG ATG TAC	ACA GGT CGC CGG AAT TAG CG
HvPK1	CTTTTCCTGCATCTCCCCTGA	TGGTCACAGGCAAGCACATA
HvPK2	CGTGTAATTAGTGGTTTTCGTCGTC	GGCCAACAAACAACAGCTAATGATA
HvPDH1	CATCGGGAACTCCATCAAGAAGA	AGTGTCCAGCTCATCCCAGAAG
HvPDH2	CGACAAAACACAAAGGCGATAAAC	GAGGAGGAGATGGATGGAGAGGAC
HvPDH3	CAAAGCAGGACGCCATCATC	GGATCCACCCTTGCCCTTAG
HvPDH4	CATCGTCCTCCTCGGCTAATCC	GGTTCCCTTGCTGTTGTTGTTG
HvPPdK1	CACTTTCCCCACCAGCCCTTAG	ATTCCCTCTGCCCCATTCTTCC
HvPPdK2	CACGCAGATGACATTTGGTTACA	CAAAGGGGTCATGCTGGAGGATA
HvPPdK3	TTGCACTCACTAATGGCTCC	GTCTTTCATGGCCTTGTTGC
HvPPdK4	AGAACGACACCGACCTGACTG	CTCCTTTGGCTTCGACATAGAC

Table 10. 2. List of the all regulated and highly regulated genes by N status. You can find the table in the attached CD to this thesis. The relative expression was determined by microarray. The values are presented $\log_2$ (fold changes (FC)). Values of Low N are relative to corresponding time point of control. Whereas Values of N resupply are relative to Low N (20 DAG).

Table 10. 3. Multiple reaction monitoring (MRM) of potentials of MS/MS transitions used in negative mode. Nitrogen was used as carrier gas, nebulizer gas, heater gas and collision gas. Ion spray voltage and the capillary temperature were -3500 V and 350°C, respectively. Pre ion=precursor ion, Pro ion=product ion, Dwell=dwell time, Frag=fragmentor, CE= collision energy, CAV= Cell accelerator voltage.

Compound Name	Prec Ion	Pro Ion	Dwell	Frag	CE	CAV	Polarity
Acetyl-CoA	808.1	460.9	20	380	33	5	Negative
Acetyl-CoA	808.1	403.9	20	380	20	5	Negative
NADPH	744	159	20	380	53	5	Negative
NADPH	744	79	20	380	80	5	Negative
NADH	664	408	20	380	33	5	Negative
NADH	664	346	20	380	29	5	Negative
ADPGlc	588	346	20	380	29	5	Negative
ADPGlc	588	241	20	380	29	5	Negative
ADPGlc	565	322.9	20	380	25	5	Negative
ADPGlc	565	158.9	20	380	53	5	Negative
ATP	505.9	408	20	380	21	5	Negative
ATP	505.9	159	20	380	33	5	Negative
UTP	483	402.9	20	380	21	5	Negative
UTP	483	384.2	20	380	17	5	Negative
ADP	426	328	20	380	17	5	Negative
ADP	426	159	20	380	21	5	Negative
Tre6P	421	240.9	20	380	25	5	Negative
Tre6P	421	139	20	380	29	5	Negative
Suc6P	421	96.8	20	380	33	5	Negative
Suc6P	421	79.1	20	380	53	5	Negative
UDP	403	272.8	20	380	21	5	Negative
UDP	403	110.9	20	380	17	5	Negative
AMP	346	134.2	20	380	37	5	Negative
AMP	346	106.9	20	380	61	5	Negative
Fru 1.6BP	339	97	20	380	21	5	Negative
Fru1.6BP	339	79	20	380	57	5	Negative
Glc1P	259	96.8	20	380	9	5	Negative
Glc1P	259	78.9.8	20	380	44	5	Negative
Fru6P	259	96.8	20	380	9	5	Negative
Fru6P	259	78.9	20	380	44	5	Negative
Glc6P	259	78.9	20	380	44	5	Negative
Glc6P	259	96.8	20	380	44	5	Negative
Rib5P	229	97	20	380	5	5	Negative
Rib5P	229	79	20	380	44	5	Negative
Gluca	193	113	20	380	5	5	Negative
Gluca	193	85	20	380	13	5	Negative
Isocit	191	110.8	20	380	9	5	Negative
Isocit	191	87	20	380	13	5	Negative

Table 10.3. continued

Compound Name	Prec Ion	Pro Ion	Dwell	Frag	CE	CAV	Polarity
3PGA	185.1	97	20	380	13	5	Negative
3PGA	185.1	79	20	380	41	5	Negative
Cacnt	173.1	128.9	20	380	1	5	Negative
Cacnt	173.1	85	20	380	9	5	Negative
Tacnt	173.1	111	20	380	5	5	Negative
Tacnt	173.1	128.9	20	380	1	5	Negative
PEP	167	78.9	20	380	13	5	Negative
PEP	167	63.2	20	380	77	5	Negative
2-oxo	145.1	101	20	380	5	5	Negative
Mal	133	115	20	380	5	5	Negative
Mal	133	71	20	380	9	5	Negative
Succ	116.9	73	20	380	5	5	Negative
Fum	115	70.9	20	380	1	5	Negative
13C-Pyr	89.9	89.9	20	380	1	5	Negative
13C-Pyr	89.9	45.1	20	380	5	5	Negative
Pyr	87.1	87	20	380	5	5	Negative

Table 10. 4. Primary metabolites concentrations in primary leaves under different N regimes. Values indicate mean in nmol. gFW^{-1} or $\mu mol.gFW^{-1}$± SD (n= 5-9). Letters (a-g) represent the significant; Means with a common letter are not significantly different ($p > 0.05$). DAG, day after germination.

	Cont. 14 DAG	Cont. 17 DAG	Cont. 20 DAG	Cont. 23 DAG	Cont. 26 DAG	Low N 14 DAG	Low N 17 DAG	Low N 20 DAG	Low N 23 DAG	**Resupply N 20 DAG**	Resupply N 23 DAG
2-Oxo nmol.gFW^{-1}	65.96 ± 15.2 bc	78.21 ± 9.48 b	59.82 ± 6.74 c	57.62 ± 11.27 cd	43.82 ± 10.00 de	91.46 ± 16.81 a	74.07 ± 10.05 b	46.01 ± 4.63 d	25.01 ± 6.05 f	31.51± 4.93 ef	32.01 ± 4.17 ef
3PGA nmol.gFW^{-1}	4.05± 0.82 e	6.34± 1.24 e	2.98± 0.45 e	2.71± 0.75 e	4.01± 1.28 e	138.71 ± 23.56 a	85.9 ± 18.41 b	57.5 ± 7.86 c	24.13 ± 4.38 d	69.25± 6.46 c	62.78 ± 9.26 c
Suc-6P nmol.gFW^{-1}	2.52± 0.54 cd	4.19± 0.61 c	3.41± 0.82 cd	2.13± 0.54 d	2.71± 0.24 cd	8.05± 1.27 b	12.75 ± 2.9 a	11.94 ± 1.1 a	8.79± 3.38 b	9.56± 0.95 b	8.15± 0.71 b
UDP-Glc nmol.gFW^{-1}	41.49 ± 18.74 cd	77.23 ± 4.12 a	68.93 ± 3.78 a	46.31 ± 7.04 bc	52.23 ± 6.83 b	43.27 ± 3.32 bcd	35.61 ± 7.01 de	31.11 ± 4.17 e	29.59 ± 6.05 e	44.18± 4.91 bcd	39.16 ± 7.54 cde
ADP-Glc nmol.gFW^{-1}	2.34± 0.79 b	4.29± 0.78 a	2.75± 0.44 b	1.23± 0.28 c	1.18± 0.26 c	2.21± 0.41 b	1.11± 0.29 c	0.58± 0.18 cd	0.25± 0.05 d	0.96± 0.18 cd	0.84± 0.21 cd
Glc-1P nmol.gFW^{-1}	7.13± 3.2 e	10.5± 1.4 d	10.7± 2.1 d	8.4± 2.99 de	8.0± 1.2 de	17.48± 1.33 c	19.5± 3.99 ab	20.1± 2.79 ab	22.6± 3.42 a	19.4± 3.49 bc	15.6± 2.02 c
ADP nmol.gFW^{-1}	1.54± 0.59 de	2.75± 0.47 ab	1.17± 0.16 e	2.84± 1.49 ab	1.93± 0.63 bcde	2.22± 0.43 bcd	1.78± 0.62 cde	2.44± 0.59 bc	1.27± 0.17 de	3.56± 0.59 a	1.73± 0.43 cde
NADPH nmol.gFW^{-1}	0.79± 0.73 ef	2.4± 0.68 a	0.25± 0.05 f	0.2± 0.07 f	0.28± 0.13 f	1.71± 0.42 abcd	1.13± 0.4 de	1.32± 0.15 cde	1.62± 0.7 bcd	1.91± 0.99 abc	2.15± 0.21 ab
PEP nmol.gFW^{-1}	0.21± 0.06 f	0.54± 0.12 f	0.25± 0.05 f	0.15± 0.05 f	0.36± 0.22 f	32.2± 4.54 a	24.2± 4.35 b	19.6± 3.59 c	8.57± 2.34 e	14.08± 1.72 d	13.22± 1.91 d
AMP nmol.gFW^{-1}	17.8± 2.66 b	26.0± 2.55 a	25.4± 3.39 a	18.0± 3.34 b	18± 1.08 b	11.37± 1.13 c	6.09± 1.19 d	4.22± 0.41 de	2.74± 0.54 e	5.49± 0.35 de	4.9± 0.69 de
Furmarate nmol.gFW^{-1}	26.41 ± 3.65 c	55.85 ± 8.34 a	52.61 ± 5.66 ab	47.68 ± 9.99 b	47.64 ± 2.41 b	25.71 ± 2.92 c	10.47 ± 1.94 d	11.67 ± 1.9 d	8.64 ± 1.54 d	25.27± 2.41 c	24.93 ± 4.18 c
Ribolose 5P nmol/gFW	2.58± 0.66 cd	4.08± 0.78 a	2.95± 0.22 bc	2.36± 0.3 cde	1.97± 0.35 de	3.53± 0.48 ab	2.62± 0.39 cde	2.24± 0.41 cde	2.87± 0.6 bc	1.9± 0.36 e	2± 0.21 de

Table. 10.4. Continued

	Cont. 14 DAG	Cont. 17 DAG	Cont. 20 DAG	Cont. 23 DAG	Cont. 26 DAG	Low N 14 DAG	Low N 17 DAG	Low N 20 DAG	Low N 23 DAG	Resupply N 20 DAG	Resupply N 23 DAG
Hex-6P nmol.gFW^{-1}	14.19 ± 3.04 de	17.89 ± 2.19 cd	17.65 ± 2.11 cd	12.61 ± 2.59 e	12.69 ± 1.81 e	24.09 ± 5.2 ab	24.56 ± 3.6 a	18.47 ± 1.8 c	20.31 ± 4.2 bc	22.72 ± 4.48 ab	21.26 ± 1.98 abc
T6P nmol.gFW^{-1}	0.53± 0.17 cd	0.2± 0.08 d	0.28± 0.16 cd	0.25± 0.16 d	0.34± 0.15 cd	1.47± 0.42 c	2.23± 0.69 ab	2.43± 0.45 ab	2.59± 0.46 a	1.78± 0.78 b	0.26± 0.04 d
Cis Acc- nmol.gFW^{-1}	3.44± 0.7 bc	5.37± 0.84 a	2.53± 0.55 cd	4.69± 0.54 ab	3.98± 0.48 bc	1.76± 0.16 d	1.07± 0.16 d	1.11± 0.2 d	1.39± 0.27 d	1.66± 0.1 d	1.86± 0.31 d
Pyruvate nmol.gFW^{-1}	262.1 ± 46.95 ab	155.6 ± 11.2 cd	106.5 ± 15.8 de	229.7 ± 12.3 bc	272.2 ± 66.4 a	192.4 ± 20.9 c	102.5 ± 21.6 de	51.5± 8.5 ef	31.3± 6.7 f	64.6± 8.3 def	177.6± 27.3 c
Malate nmol.gFW^{-1}	2213 ± 662.4 b	2977 ± 217.6 a	2920 ± 98.2 a	3251 ± 256.3 a	3191 ± 363.5 a	1013 ± 61.8 c	631.9 ± 119.9 cde	544.6 ± 72.5 de	272.6 ± 38.5 e	926.9 ± 45.7 cd	1027 ± 157.8 c
Citrate nmol.gFW^{-1}	1943 ± 570.9 c	2590 ± 162.9 ab	2706 ± 108.7 b	2911 ± 143.9 a	2754 ± 400 ab	681 ± 84.1 de	415 ± 81.8 de	380 ± 47.9 e	357 ± 60.4 e	613.8 ± 27 de	813.7 ± 108 d
Iso- Citrate nmol.gFW^{-1}	64.3 ± 46.4 c	152 ± 20.6 a	107.2 ± 14.4 b	108.9 ± 20.3 b	87.7 ± 16.9 bc	25 ± 0.5 d	9.4 ± 8.2 d	ND	ND	ND	ND
Glc µmol/ gFW	0.93± 0.24 bcd	1.28± 0.69 abc	0.89± 0.25 cd	0.81± 0.13 d	0.61± 0.1 d	1.35± 0.3 ab	1.47± 0.4 a	1.44± 0.54 a	1.27± 0.68 abc	0.96± 0.37 bcd	0.73± 0.43 d
Frc µmol/ gFW	0.46± 0.13 de	0.66± 0.23 cd	0.68± 0.08 cd	0.56± 0.19 cd	0.47± 0.16 de	0.73± 0.23 bc	1.18± 0.33 a	0.97± 0.17 ab	1.18± 0.44 a	0.52± 0.13 cde	0.25± 0.09 e
Suc µmol/ gFW	10.19 ± 1.6 def	12.62 ± 2.27 c	10.64 ± 1.45 de	9.98 ± 1.78 def	8.87 ± 0.97 fg	11.37 ± 0.91 cd	16.84 ± 2.38 a	14.63 ± 2.61 b	9.55 ± 2.05 ef	7.81 ± 0.85 g	8.67 ± 0.9 fg
Starch µmol.gFW^{-1}	13.17 ± 3.16 a	9.1 ± 0.69 b	5.23 ± 2.72 c	2.85 ± 1.03 de	2.29 ± 0.69 de	14.01 ± 5.13 a	8.09 ± 1.79 b	4.1 ± 2.04 cd	1.28 ± 0.9 e	2.54 ± 1.2 de	3.35 ± 1.39 cde

Table 10. 5. Number of upregulated and downregulated genes that were classified to the different functional groups.

	Low N (17 DAG)			Low N (20 DAG)			N resupply (20 DAG)		
Group	**Regulated genes**	**Up-regulated**	**Down-regulated**	**Regulated genes**	**Up-regulated**	**Down-regulated**	**Regulated genes**	**Up-regulated**	**Down-regulated**
Protein	206	92	114	222	100	122	627	399	228
RNA	143	53	90	157	81	76	328	209	119
Misc	90	52	38	139	53	86	214	94	120
Transport	87	73	14	118	61	57	203	54	149
Signalling	82	30	52	83	43	40	239	47	192
Stress	57	24	33	83	36	47	107	44	63
Cell	47	19	28	54	33	21	88	37	51
Development	44	28	16	41	15	26	77	42	35
DNA	39	9	30	20	12	8	67	47	20
Hormone metabolism	37	27	10	53	25	28	64	25	39
Lipid metabolism	27	15	12	37	23	14	71	25	46
Amino acid metabolism	26	12	14	34	12	22	68	40	28
Secondary metabolism	26	21	5	44	14	30	78	30	48
Redox regulation	24	4	20	22	2	20	41	31	10
PS	22	0	22	44	1	43	84	79	5
Nucleotide metabolism	20	4	16	26	13	13	51	27	24
Cell wall	14	6	8	23	8	15	24	15	9
Minor CHO metabolism	11	1	10	11	2	9	21	15	6
Major CHO metabolism	7	2	5	9	3	6	19	11	8
Mito. electron transp. ATP	6	3	3	5	3	2	14	5	9
Glycolysis	5	2	3	5	1	4	11	8	3
Tetrapyrrole synthesis	5	0	5	12	1	11	25	22	3
TCA	5	0	5	8	0	8	12	10	2
Co-factor/Vitamines	3	0	3	6	1	5	15	13	2
Gluconeogenese /Glyoxalate cycle	2	2	0	3	3	0	6	1	5
OPP	2	0	2	4	2	2	3	2	1
Metal handing	1	1	0	4	3	1	7	6	1
N metabolism	1	1	0	3	2	1	7	3	4
Polyamine metabolism	1	0	1	1	1	0	0	0	0
Biodegradation xenobiotics	1	0	1	2	1	1	3	1	2
C1 metabolism	1	1	0	2	0	2	5	4	1
Fermentation	0	0	0	2	1	1	3	1	2
S assimilation	0	0	0	0	0	0	1	1	0
miRNA/antisense	0	0	0	0	0	0	0	0	0
Not assigned	595	281	314	685	345	340	1546	882	664

11. Curriculum Vitae

Nazeer Fataftah

Personal Details

Date of Birth: November 26th, 1985
Nationality: Palestinian

Contact Details

Address: Richard-Paulick Str.13, Wnr: 1022
06124 Halle (Saale), Germany
Mobile: +4917687846221
Email: nazeer.fataftah@pflanzenphys.uni-halle.de

Education

- **PhD in Plant molecular Physiology.** Nov, 2012- 2016.
  Leibniz-Graduate School for Yield Formation in Cereals, in a close cooperation of researchers from the Leibniz Institute for plant genetics and crop plant research (IPK) and the Martin-Luther University Halle-Wittenberg (MLU).

- **M.Sc. in Horticultural Genetics and Biotechnology.** Oct, 2009 - Jan, 2012.
  Mediterranean Agronomic Institute of Chania (MAICh), Greece.
  Evaluation: A, with cum maxima laude distinction.

- **B.Sc. in Biology.** Sept, 2004 – June, 2008.
  Biochemistry and Biology Dept., Birzeit University, Palestine.
  Evaluation: Very Good.

Work Experience

- **Oct, 2008- Oct, 2009**. Biology teacher. Palestine
- **Nov, 2012- up to date.** PhD researcher in IPK and MLU, Germany

Conferences and scientific meetings

- **Nov. 10-14, 2014.** 7th European workshop on plant senescence. Aarthus University, Denmark.
  Nazeer Fataftah, Mohammad Hajirezaei, Nicolaus von Wirén, Klaus Humbeck. A wider perspective on the barley leaf senescence connecting whole plant development and nitrogen availability.
  Presented as a poster (winning poster prize)

- **Sept. 10-12, 2014.** International conference of the German society of plant nutrition, MLU, Germany.
  Nazeer Fataftah, Mohammad Hajirezaei, Nicolaus von Wirén, Klaus Humbeck. A wider perspective on the barley leaf senescence connecting whole plant development and nitrogen availability.
  Presented as a poster

- **Feb. 20-21, 2015.** Middle Germany plant physiology meeting, Leipzig University, Germany.

- **Feb. 14-15, 2014.** Middle Germany plant physiology meeting, Dreseden University, Germany.
  Nazeer Fataftah, Mohammad Hajirezaei, Nicolaus von Wirén, Klaus Humbeck. Regulation of the barley leaf senescence connecting whole plant development and nitrogen availability.
  Presented as an oral talk

- **Feb. 15-16, 2013.** Middle Germany plant physiology meeting, MLU, Germany.

- **Jun. 2-5, 2014.** The 10th plant science student conference. IPK, Germany.
  Nazeer Fataftah, Mohammad Hajirezaei, Nicolaus von Wirén, Klaus Humbeck. Regulation of the barley leaf senescence connecting whole plant development and nitrogen availability.
  Presented as an oral talk

Training Courses

- **Mar. 26-27, 2015.** Saccharomyces cerevisiae - a versatile tool to study transport proteins. IPk, Germany.
- **Sept. 16-25, 2013.** Plant-based bioeconomy. MLU, Germany.
  Covered: Part1- Scientific presentation and interdisciplinary thinking and part2 – from idea to realization.
- **Sept. 9-12, 2013.** Barlomics summer school. IPK, Germany**.**

Covered the following modules: the barley gene bank collection, Phenomics, Proteome, Transcriptome and genome analysis, and genetic engineering of barley plant.

- **Jun. 10-14, 2013.** Confocal microscopy, IPK, Germany.
- **Oct-Nov, 2008.** Educational qualification program by Palestinian ministry of education.
- **Oct-Dec, 2008.** Using scientific items in Science Laboratories by Palestinian ministry of education and The United Nations Children's Fund – UNICEF.

Stays at other research center

- **Oct. 1- Nov.1, 2013.** IPK institute, Germany; Phytohormones, sugars, and amino acids level measurements.
- **Feb. 20- Mar. 27, 2014.** IPK institute, Germany; Phytohormones, sugars, amino acids, anions and metabolites level measurements.
- **Nov. 17-27, 2014.** IPK institute, Germany; Microarray analyses.

Publications

Nazeer Fataftah, Mohammad Hajirezaei, Nicolaus von Wirén, Klaus Humbeck. A wider perspective on the barley leaf senescence connecting whole plant development and nitrogen availability. (In preparation).

Activities

- **June, 2012.** Helping in organizing an Environmental Day at Birzeit University, Palestine.

Scholarship

- PhD position Nov, 2012- up to date. Martin-Luther University Halle-Wittenberg (MLU).
- Master study full Scholarship, Mediterranean Agronomic Institute of Chania - MAICh, a constituent institute of International Centre for Advanced Mediterranean Agronomic Studies (CIHEAM), Greece, 2009-2011.

12. Affirmation

I hereby declare that the submitted work has been completed by me, the undersigned, and that I have not used any other than permitted reference sources or materials or engaged any plagiarism. All the references and the other sources used in the presented work have been appropriately acknowledged in the work. I further declare that the work has not been previously submitted for the purpose of academic examination, either in its original or similar form, anywhere else.

Hiermit erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die den benutzten Hilfsmitteln wörtlich oder inhaltlich entnommenen Stellen habe ich unter Quellenangaben kenntlich gemacht. Die vorliegende Arbeit wurde in gleicher order ähnlicher Form noch keiner anderen Institution order Prüfungsbehörde vorgelegt.

Nazeer Fataftah

Halle (Saale), Germany.