Chapter 3

# Plant Senescence and Nitrogen Mobilization and Signaling

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Additional information is available at the end of the chapter

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## 1. Introduction

### 1.1. Senescence

Very early during their reproductive phase, annual plants initiate the process of senescence. Monocarpic senescence describes the last steps in these plants' development; senescence on organ level starts shortly after entering reproductive phase while after anthesis the whole plant undergoes senescence and dies.

In the following we will focus on leaf senescence. Two different processes can be distinguished in annual plants relying on different genetic programs. Before anthesis, sequential leaf senescence recycles nutrients from old to developing leaves which is mainly under the control of the growing apex and is arrested when no more new leaves develop and when the plant starts to flower and sets fruit. Monocarpic leaf senescence recovers valuable nutrients from the leaves during flower induction and anthesis to provide these to the developing reproductive organs [1, 2]. The latter is crucial for fruit and seed development and has a major impact on yield quantity and quality. In wheat salvaged nitrogen (N) from the leaves accounts for up to 90% of the total grain N content [3]. A complex regulation of many different metabolic pathways and expression of numerous genes underlies this process. How coordination and interplay of many controlling factors, like hormones, genetic reprogramming, biotic and abiotic stresses are achieved is far from being understood, but it is already clear that this regulatory network is highly complex and dynamic.

Thousands of genes are differentially regulated during senescence induction and progression. To date forward and reverse genetic approaches as well as large-scale transcript profiling have identified almost 6.500 genes being differentially expressed during the course of leaf senescence including up-regulated as well as down-regulated genes [4]. The high num-

ber of differentially regulated senescence-associated transcription factors (TF) demonstrates the dimensions of genetic reprogramming taking place. These TFs include 20 distinct families of which NAC-, WRKY-, C2H2-type zinc finger, EREBP- and MYB-families are most abundant [5]. Recently, Breeze et al. (2011) [4] published extremely important results of a high-resolution temporal transcript profiling of senescing Arabidopsis leaves giving insight into the temporal order of genetic events. One of the first steps at the onset of senescence is a shift from anabolic to catabolic processes. Amino acid metabolism and protein synthesis are down-regulated while expression of autophagy- and reactive oxygen species-related, and water-response genes is enhanced. In contrast to the following elevation of abscisic acid (ABA) and jasmonic acid (JA) signaling-related gene expression, cytokinin-mediated signaling is lowered just as chlorophyll and carotenoid biosynthesis. The next phases include down-regulation of carbon utilization and enhanced expression of cystein-aspartat proteases, carotene metabolism-associated genes and pectinesterases which is then followed by the reduction of photosynthetic activity and degradation of the photosynthetic apparatus coinciding with the increased activity of lipid catabolism, ethylene signaling and higher abundance of cytoskeletal elements [4].

Hormonal control of senescence is conveyed especially by ethylene, jasmonic and salicylic acid, cytokinin and auxin. Many mutants with a delayed senescence phenotype could be traced back to impaired or up-regulated ethylene or cytokinin signaling, respectively [6, 7]. In adition, ABA acts as a positive regulator of leaf senescence. Recently a membrane-bound, leucine rich repeat containing receptor kinase (RPK1) has been identified to play an important role in ABA-mediated senescence induction in an age-dependent manner. Strikingly, *rpk1* mutant lines did not show significant alterations in developmental processes, which have been reported for numerous other ABA signaling defective mutant lines [5], except slightly shorter growth [8]. This kinase has been indentified to integrate ABA signals during seed germination, plant growth, stomatal closure and stress responses. Overexpression lines showed enhanced expression of several stress and $H_2O_2$-responsive genes [9, 10]. Mutant lines showed a delayed senescence phenotype with slower progression of chlorophyll degradation and cell death.

Induction and progression of leaf senescence demands a tight regulation of numerous processes. Integration of nutritional cues, biotic and abiotic influences, plant development and age has to take place for the correct timing of onset and temporal advance of this complex developmental process. Despite the enormous efforts and achievements in this field, many of the regulatory mechanisms remain elusive.

### 1.2. Nitrogen and agriculture

The nowadays growth of population and thus increasing demand for food and oil crops forces agricultural industry to increase quantitative as well as qualitative yields. Until 2050 world's population is predicted to be as high as 9-10 billion people [11] and grain requirement is projected to be doubled, mostly resulting from a higher demand for wheat fed meat [12]. As most of the cropping systems are naturally deficient in nitrogen, there is a fundamental dependency on inorganic nitrogen fertilizers. 85-90 million tons of these fertilizers

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are applied annually worldwide [13-15]. However, 50-70% of these nitrogenous fertilizer are lost to the environment [16], mostly due to volatilization of $N_2O$, NO, $N_2$ and $NH_3$ and leaching of soluble $NO_3^-$ into the water. Thus nitrogen is not only one of the most expensive nutrients to provide, but it also has a strong detrimental impact on the environment. Since surrounding ecosystems and potable water supply are endangered by oversaturation with nitrogenous compounds, it is necessary to improve application techniques and plant's nitrogen use efficiencies.

Several different definitions of nitrogen and nutrient use efficiencies are on hand. The most common is the *nitrogen use efficiency* (NUE), which is defined as shoot dry weight divided by total nitrogen content of the shoots. The *usage index* (UI) takes absolute biomass into account as it is denoted by the shoot fresh weight times the NUE. Likewise, the *N uptake efficiency* (NUpE) takes into account the whole N content of the plant and the N supplied by fertilization per plant. The fraction of the N taken up, which is then distributed to the grain, can be obtained by calculating the *N utilization efficiency* (NUtE) (Grain weight per total N content). Other efficiencies, which seem to be more suitable for the use in applied sciences, are the *agronomic efficiency* (AE), *apparent nitrogen recovery* (AR) and the *physiological efficiency* (PE). AE, AR and PE do require an unfertilized control to be calculated. While AE measures the efficiency to redirect applied nitrogen to the grains, AR defines the efficiency to capture N from the soil. PE puts the N uptake into relationship with the outcome of grain (reviewed in [15]). *Nitrogen remobilization efficiency* (NRE) describes the plant's capacity to translocate already assimilated nitrogen to developing organs. Finally, the *harvest index* (HI) and the *nitrogen harvest index* (NHI) are often used terms. HI is the total yield weight per plant mass, while NHI states the grain N content per whole plant N content.

Emission of nitrogen to the environment could be strongly reduced by application of 'best management techniques' in agricultural practice like e.g. rectifying the rate of appliance by accounting for all other possible sources of nitrogen influx (carryover from previous crops, atmospheric deposits etc.), ameliorating the timing and also changing the method of appliance to reduce atmospheric losses [13]. Food production has doubled in the last 40 years. Most of this increase could be achieved by selection of new strains, breeding and application of greater amounts of fertilizer and pesticides and other techniques [12]. Amending of nutrient use efficiencies of the crop plants was mostly accomplished via breeding programs by now. QTL selection for higher yields, increased oil or protein content has been pursued for decades. In wheat for example, increasing yield and grain protein content has been extensively studied, but improving both is restrained by the negative genetic relationship between these traits [17, 18].

## 2. Nitrogen uptake, assimilation and distribution

Nitrogen sources vary extremely encompassing organic and inorganic forms, small peptides and single amino acids, thus uptake systems need to be adjusted and well regulated in spatial and temporal activity. Although the predominant form in which N is taken up mainly

depends on the plants adaption to the given environment and influences like fertilization, soil pH, temperature, precipitation and others [14, 19], most plants cover their N demand primarily through soil nitrate being provided by fertilization, bacterial nitrification and other processes [15]. However, a wide range of different uptake system has evolved in plants. For example, oligopeptides can be taken up via OPT-proteins (oligopeptide transporters), ammonium via ammonium transporters (AMTs) and amino acids via amino acid transporters and amino acid permeases. Besides the *AtCLC* (ChLoride Channel) gene family, comprising 7 members of which two (*AtCLCa* and *AtCLCb*) have been shown to encode tonoplast located $NO_3^-/H^+$ antiporters [20, 21], two families of nitrate transporters have been identified in higher plants (NRT1 and NRT2), representing low- and high affinity transporter systems, respectively. Moreover the *NRT1*-family has been shown to comprise also di-/tripeptide transporters (PTR) [22].

### 2.1. Nitrogen transporter systems

Four constituents of nitrate uptake are known, constitutive (c) and inducible (i) high- (HAT) and low-affinity (LAT) transporters, respectively. The high-affinity system's $K_M$ ranges from ~ 5-100 μM, varying with plant species, and a maximal influx via this system of 1 μMol * $g^{-1}$ * $h^{-1}$ has been determined [23, 24]. At nitrate concentrations of 10 mM the influx rate via the LATs can reach up to ~24 μMol * $g^{-1}$ * $h^{-1}$ [24].

The *NRT1*-family comprises 53 genes in Arabidopsis which are classified as LATs. *AtNRT1-1* (*CHL1*) was the first member to be identified in 1993 and has been shown to encode a proton-coupled nitrate transporter [25]. Studies with *Xenopus* oocytes have shown that this transporter protein possesses two different states, one serving low-affinity and the other one high-affinity nitrate uptake [26, 27], thus the distinction between high and low-affinity nitrate transporters is overridden in this case. Switching between these two modes of action is conferred by phosphorylation of threonin at position 101 [28]. *AtNRT1-1* is expressed in the cortex and endodermis of mature roots and in the epidermis of root tips. Additionally, a nitrate sensing function regulating the plants primary nitrate response has been strongly indicated for the AtNRT1-1 protein by several lines of evidence. The *chl1-5 (atnrt1-1-5)* mutant, a deletion mutant with no detectable *CHL1* transcript, is deficient in nitrate uptake and initiation of the primary nitrate response. The *chl1-9* mutant is defective in nitrate uptake but not in the primary nitrate response. The *chl1*-9 mutant has a point mutation between two transmembrane domains. When threonine 101 was mutated to mimic or repress phosphorylation and transformed into the *chl1-5* background, primary nitrate response could be repressed or enhanced [29]. Constitutive expression identifies AtNRT1-2 as part of the Arabidopsis cLATs. Its transcript is only found in root epidermal cells [22]. Expression of *AtNRT1-5* is nitrate inducible; however, the response to nitrate is much slower than for *AtNRT1-1*. AtNRT1-5 has been shown to be a pH-dependent, bidirectional nitrate transporter, with subcellular localization in the plasma membrane of root pericycle cells near the xylem implicating an involvement in long-distance nitrate transport [30]. Experimental evidence suggests nitrate storage in leaf petioles to be associated with the function of AtNRT1-4. Here, nitrate content is relatively high, while nitrate reductase (NR) activity is

low. Additionally, *AtNRT1-4* is predominantly expressed in the leaf petiole and the *atnrt1.4* mutant shows a nitrate content decreased by half in the petiole [22, 31]. *AtNRT1-6* is expressed in the silique's and funiculus' vascular tissue and thought to play a nitrate providing role in early embryonic development [32]. AtNRT1-8 functions in nitrate unloading from the xylem sap and is mainly located in xylem parenchyma cells within the vasculature [33], whereas AtNRT1-9 facilitates nitrate loading into the root phloem from root phloem companion cells [34].

High affinity nitrate uptake is conducted by members of the *NRT2*-family, comprising 7 genes in Arabidopsis. AtNRT2-1 has been shown to be one of the main components of the HATs. Mutant *atnrt2-1* plants displayed a loss of nitrate uptake capacity up to 75% at HAT-specific $NO_3^-$ concentrations [35]. Furthermore, lateral root growth is repressed under low nitrate combined with high sucrose supply, where NRT2-1 acts either as sensor or transducer [36]. Experiments with *Xenopus* oocytes revealed the requirement of a AtNAR2 protein for AtNRT2-1 function [37]. Mutants of either of these two components showed impaired nitrate uptake at HAT-specific concentrations and hampered growth with display of N-starvation symptoms, in which, remarkably, the *atnar2* mutant phenotype appeared to be more pronounced [38]. The phenotype of the *atnrt2-7* mutant is similar to the phenotype of the *atclca* mutant. The *AtCLCa* gene has been shown to encode a $NO_3^-/H^+$ antiporter enabling accumulation of nitrate in the vacuole. Mutation of either of these resulted in lower nitrate content. Ectopic overexpression of *AtNRT2-7* led to higher nitrate contents in dried seeds, where the gene is highly expressed under wild type conditions, and an increase in the nitrate HATs uptake capacity by 2-fold. However, normal development was not impaired in the mutants as well as overexpressor plants [14, 20, 39]. Despite its high homology to *AtNRT2-1, AtNRT2-4* is not dependent on the function of *AtNAR2*. *AtNRT2-4* expression is highly induced upon nitrogen starvation in the outermost layer of young lateral roots [40].

Members of the *AMT1*- and *AMT2*-subfamilies are thought to be the main high affinity ammonium transporters in plants. Due to ammonium's toxic nature and convertibility from $NH_4^+$ to $NH_3$ and the thus varying membrane permeability, its uptake and transport needs to be tightly regulated [41, 42]. AMTs are regulated transcriptionally by N-supply, sugar and daytime and provide an additive contribution to ammonium transport [41]. AtAMT1-1 contributes 30-35 % as does AtAMT1-3, while AtAMT1-2 provides only 18-25% [43, 44]. AtAMT1-1 transports ammonium as well as its analog methyl-ammonium. Additionally, its activity is regulated posttranscriptionally via the availability of nitrate [42].

### 2.2. Nitrogen assimilation

Assimilation of $NO_3^-$ and $NH_4^+$ almost always includes incorporation into amino acids (AA). The most abundant transport forms are glutamine, glutamate, asparagine and aspartate [45] although direct transport of $NO_3^-$ and $NH_4^+$ also takes place but to a much lesser extend [46]. Nitrate assimilation thus requires reduction to ammonium. Nitrate reductase (NR) realizes the first step by reducing $NO_3^-$ to $NO_2^-$. This reaction takes place in the cytoplasm, while the reduction of nitrite to ammonium is carried out in the plastids. Here, nitrite reductase (NiR) converts $NO_2^-$ to $NH_4^+$ making it readily available for the in-

corporation into AAs in a NADH-dependent manner. Assimilation of ammonium into AAs involves chloroplastic glutamine synthetase 2 (GS2) and glutamate synthase (Fd-GOGAT), which generates glutamine and glutamate (for detailed review see [14]). Glutamine as well as glutamate serve as ammonium donor for the synthesis of all other amino acids including aspartate and asparagine, which in turn function as active $NH_4^+$ donor or as long-range nitrogen transport and storage form, respectively [47]. Alternatively carbamoylphosphate synthase can be involved in ammonium assimilation by producing carbamoylphosphate and successively citrulline and arginine. Assimilation in non-green tissues is achieved in plastids in a similar manner, although here GOGAT depends on NADH instead of ferredoxin. Carbon skeletons are essential for the acquisition of inorganic nitrogen in AAs. Especially the demand for keto-acids has to be met (see [14] and references within). These are predominantly obtained from the TCA-Cycle in the form of 2-oxoglutarate (2OG) [47, 48]. 2OG is used for incorporation of photorespiratory ammonium, resulting in the production of glutamate, which in turn can be utilized by GS1 and GS2 to produce glutamine. This displays the intricate interconnection between carbon and nitrogen metabolism, in which N uptake and assimilation is also influenced via photosynthetic rates [47]. Besides direct assimilation into AAs, nitrate can also be stored in the vacuole and in the chloroplast. Vacuolar nitrate concentrations can vary enormously, as vacuolar nitrate also contributes to turgor maintenance and might have a nitrate storage function to maintain the cytosolic nitrate concentrations which are more constant [49].

## 3. Senescence induction and nitrogen mobilization

As mentioned above, induction of senescence is a highly complex regulated and dynamic process. Besides developmental cues, there are numerous other possible impacts. Nutritional starvation, photosynthetic activity, pathogen infections, carbon accumulation, carbon to nitrogen ratio, photoperiod and various other cues can lead to senescence induction on either organ or whole plant level. Both natural and stress induced senescence are accompanied with the remobilization of valuable nutrients from various organs of the plant. In the following we will again focus on the situation in leaves.

### 3.1. Senescence induction

Correct timing of leaf senescence is crucial for proper plant development. Too early senescence induction would decrease the ability to assimilate $CO_2$, while too late induction would reduce the plant's capacity to remobilize nutrients from old leaves to developing organs [50]. Nevertheless, timing of senescence can also be regarded as an active adaption to the given nutritional and environmental conditions. For example under limited nutrition, continued growth of vegetative tissues would result in a reduced ability to develop reproductive organs.

Nutritional limitation, especially in concerns of nitrogen, has been shown to be able to enhance leaf senescence. Sunflower (*Helianthus annuus*) plants grown under low nitrogen sup-

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ply showed a stronger decline of photosynthetic activity and more pronounced senescence symptoms than plants sufficiently supplied with nitrogen [51]. Furthermore these plants showed a more pronounced and earlier drop in (Glu+Asp)/(Gln+Asn) ratio during the progression of senescence indicating an additional adaption to low nitrogen conditions through enhanced nitrogen remobilization. In this experiment, also a significant increase in the ratio of hexose to sucrose was observed at the beginning of senescence which was higher in N-starved plants. This indicates that sugar-related senescence induction is dependent on the availability of nitrogen [51]. However, high sugar contents repress photosynthesis and can induce early SAG expression while late SAG expression is repressed. Diaz et al. (2005) [52] showed sugar accumulation to be lower in some recombinant inbred lines which display early leaf yellowing, thus pointing out a mayor function for sugar accumulation alone but the regulating function of the C/N balance during induction of monocarpic senescence is widely discussed. Recently, trehalose-6-phosphate (T6P) was identified as a main signaling component in this pathway. T6P inhibits the activity of Snf1-related protein kinase (SnRK1). Zhang et al. (2009) [53] showed the T6P/SnRK1 interaction in Arabidopsis seedling extracts and other young tissues treated with T6P. Additionally, Delatte et al. (2011) [54] confirmed the inhibition of SnRK1 by T6P with plants overexpressing the SnRK1 catalytic subunit gene *KIN10.* These plants were insensitive to trehalose treatments. Further verification of T6P as signaling molecule was provided by several studies. In wheat the interaction of T6P and SnRK1 has been suggested to play a role during grain filling [55]. Wingler et al. (2012) [56] conducted a study with *otsA* and *otsB* expressing Arabidopsis plants *otsA* encodes the bacterial T6P synthase gene, *otsB* the T6P phosphatase gene; therefore, overexpression leads to increasing or decreasing T6P contents, respectively. A significantly higher accumulation of glucose, fructose and sucrose was observed in *otsB* expressing plants and these plants displayed a delayed senescence phenotype. But most interestingly, these plants were rendered less susceptible to the induction of senescence-associated genes by sugar feeding in combination with low nitrogen supply, whereas *otsA* plants induced senescence and anthocyanin synthesis upon external supply of 2% glucose.

Another signaling component involved in senescence induction is light quantity and quality. Senescence can be induced by the darkening of individual leaves. However, darkening of the whole plant resulted in delay rather than in induction of leaf senescence in Arabidopsis and sunflower plants [57, 58]. Brouwer et al. (2012) [59] recently revealed the involvement of photoreceptors in dark and shading induced leaf responses. They applied different shading conditions to single leaves of Arabidopsis plants. Depending on the amount of light perceived, different biological programs were induced, leading to either acclimation to the new light conditions or leaf senescence. Furthermore, *phytochrome A* mutant lines displayed accelerated chlorophyll degradation under all shading conditions except darkness, displaying its involvement in the perception of and adaption to changing light conditions [59].

A tight linkage between stress response and leaf senescence is demonstrated by the function of several members of the NAC- and WRKY-family [60]. For example, *At NTL9 (NAC TRANSCRIPTION FACTOR LIKE 9)* mediates osmotic stress signaling during leaf senes-

cence [61] and *At VNI2 (ANAC083)* has been shown to integrate abscisic acid (ABA)-mediated abiotic stress signals into leaf senescence [62].

Besides various other cues like the stage of plant development, pathogens, extreme temperatures, source-sink transitions and drought, the action of reactive oxygen species (ROS) has been shown to have a severe impact on the induction of leaf senescence. Cellular $H_2O_2$ levels increase at the onset of senescence due to a complex regulation of hydrogen peroxide scavenging enzymes [63]. The increase in intracellular $H_2O_2$ levels is initiated via the down-regulation of the expression of the hydrogen peroxide scavenging enzyme CATALASE2 by the transcription factor GBF1 (G-Box binding factor 1). In *gbf1* knock-out plants, the senescence specific elevation in $H_2O_2$ levels is absent leading to a significant delay of leaf senescence [64]. We have demonstrated recently using a specific *in vivo* hydrogen peroxide monitoring and scavenging system that the pivotal role of $H_2O_2$ during the induction of developmental leaf senescence in Arabidopsis is depending on the subcellular localization and concentration. Furthermore, a similar senescence-specific up-regulation of $H_2O_2$ levels and down-regulation of the respective scavenging enzymes was also observed in *Brassica napus* [65]. Knock-out and overexpression plants of *AtOSR1* (*ANAC059* or *ATNAC3*) or *AtJUB1* (*ANAC042*), which are both highly inducible by $H_2O_2$, were delayed or accelerated, respectively, concerning the onset of leaf senescence in which JUB1 also modulates cellular $H_2O_2$ levels [66, 67]. Besides their important role in disease resistance [68], several WRKY transcription factors have been suggested to have a striking role in the regulation of leaf senescence. For example AtWRKY53, a $H_2O_2$-responsive transcription factor, has been indicated to have a function as important control element during the onset of leaf senescence [69].

Conclusively, leaf senescence is governed not only by developmental age but a wide range of various different external and internal factors, biotic and abiotic influences, molecules and cues, which have to be integrated. Despite its enormous agricultural importance, our knowledge of these integrative mechanisms is still limited and needs much more efforts to get complete insight into the regulatory network controlling the onset and progression of leaf senescence.

### *3.1.1. N-uptake during senescence*

Nitrogen uptake and partitioning after beginning anthesis varies greatly between different species and even between ecotypes. An analysis of different Arabidopsis accessions revealed that the fate of nitrogen absorbed during flowering can be different, depending on general N availability and accession. At low nitrogen concentrations most of the N assimilated post-flowering was allocated to the seeds, while under high N regimes the main part of it was distributed to the rosette leaves and successively lost in the dry remains, except for four tested accessions. N13, Sakata, Bl-1 and Oy-0 allocated the nitrogen taken up post-flowering also to the seeds under high N supply [70]. In wheat, a minor portion of grain N is derived from N uptake post-flowering, whereas up to 90-95% is remobilized from other plant tissues [3, 71]. In oilseed rape (*Brassica napus*) the induction of the reproductive phase is accompanied with a drastic down-regulation of nitrogen uptake systems. HATs and HATs + LATs activities are decreased thus almost resulting in an arrest of nitrogen uptake during seed fill-

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ing and flowering [14, 72-74]. Grown under non-limiting nitrogen conditions, Arabidopsis displays a lowered nitrate influx during the reproductive stage in comparison to the influx during the vegetative stage [14]. Although many plants seem to continue N uptake during seed filling, this nitrogen is not always allocated to the seeds, thus rendering nitrogen remobilization from senescing organs a central component for the proper development of reproductive organs.

## 3.2. Nitrogen mobilization

### *3.2.1. Senescence associated proteases*

Protein degradation is most likely the most important degradation process that occurs during senescence [75]. With a combined $^{15}N$ tracing/proteomics approach, Desclos et al. (2009) [76] have shown that HSP70, chaperonin10 and disulfide isomerase are synthesized during the whole progression of senescence in *B. napus* illustrating the necessity to prevent the aggregation of denatured proteins. In addition, almost all protease families have been associated with some aspects of plant senescence [75].

The aminopeptidase LAP2 has been characterized as exopeptidase liberating N-terminal leucine, methionine and phenylalanine. Arabidopsis *lap2* mutants displayed a significant change in amino acid contents. In particular, nitrogen rich AAs like glutamate and glutamine were dramatically reduced while leucine levels were the same as in wild type plants. Furthermore, a premature leaf senescence phenotype was observed in these plants. Different recombinant inbred lines, which are also modified in Glu, Gln, Asp and Asn contents, also show a senescence phenotype tempting the authors to speculate that the senescence phenotype of *lab2* might be related to a decreased turnover of defective proteins and the marked decrease of nitrogen rich AAs [77].

Chloroplast targeted proteases comprise proteases of the Lon, PreP, Clp, FtsH and DegP type [78-80]. Their substrates include, besides others, chlorophyll apoproteins like LHCII, the D1 protein of the photosystem II reaction center and Rubisco. The Clp protease complex is the most abundant stromal protease, where PreP is also located [78, 79]. Several catalytic subunits of the Clp proteases display up-regulated expression during dark-induced senescence in Arabidopsis, like e.g. ClpD/ERD1 and ClpC1. They possess sequence similarity to the chaperon HSP100 indicating that they might function as recognition subunit in the Clp protease complex to recruit denatured proteins [80]. FtsH proteases are thylakoid bound facing the stroma while Deg proteases are also thylakoid bound but facing stroma as well as thylakoid lumen [81, 82]. DegP2 is responsible for an initial cleavage of the D1 protein, where after FtsH proteases complete the full degradation [80, 83]. These two proteases belong to the family of serine proteases. In wheat, serine proteases are the most important family of proteases participating in N remobilization [84]. Subtilases have been reported to be highly expressed in barley during natural and senescence induced via artificial carbohydrate accumulation. Additionally induced proteases were SAG12, CND41-like, papain-like, serine carboxypeptidase III precursor, aspartic endopeptidases and others [85]. Roberts et al. (2012) [75] suggest a classification of senescence-associated proteases according to their expression

profile and probable function during natural senescence. Class I includes all proteases being expressed in non-senescent and in senescent tissue. Although no senescence specific expression change can be observed, their continued expression in a catabolic environment displays their significance for a normal progression of senescence. Class II contains proteases being expressed at a low level in non-senescent tissue and induced upon senescence onset. Class III comprises proteases which are induced exclusively during senescence. This suggests a role in the late stages of senescence and a probable function in cell death execution. Class IV proteases constitute proteases transiently expressed during onset of senescence which could be involved in early breakdown processes like e.g. chloroplast dismantling. Finally, class V proteases are down-regulated during senescence. These enzymes are likely to fulfill housekeeping protein turnover and other proteolytic functions, which are no longer needed during the progression of leaf senescence [75].

*3.2.2. Chloroplast dismantling*

Chloroplasts are the first organelles to show visible symptoms of degradation processes during senescence. Containing up to 75% of total leaf nitrogen, chloroplasts are the main source for its remobilization [86]. Four different pathways have been proposed for chloroplast and chloroplastic protein degradation: I) endogenous proteases degrade proteins intra-plastidial, II) degradation of stroma fragments in an extraplastidic, non-autophagic pathway, as well as III) extraplastidic degradation by autophagy-associated pathways, and IV) autophagic degradation of entire plastids [87]. Chloroplast breakdown is not a chaotic decay, but rather an organized and selective process. As chloroplasts are one of the plants main ROS-producing organelles, and due to the potential phototoxicity of many chloroplastic components and their degradation intermediates, a coordinated dismantling process is necessary to prevent severe cell damage [88, 89].

Within these organelles Rubisco represents the most abundant protein. Its abundance exceeds the requirements for photosynthesis by far, thus a nitrogen storage function has been suggested for it [90]. In chloroplasts isolated from senescing leaves a 44 kDa fragment of Rubisco's large subunit accumulates, but seems not to be degraded further [91]. The chloroplastic aspartate protease CND41 has been shown to degrade denatured Rubisco, but not active Rubisco *in vitro* [92]. This protease might be involved directly in Rubisco degradation, as accumulation of CND41 correlates with loss of Rubisco [93]. However, tobacco CND41 antisense lines also show a dwarf phenotype, reduced gibberellin levels and reduced leaf expansion, thus this correlation could be an indirect effect through gibberellin homeostasis [94]. Rubisco containing bodies (RCB) have been found to be shuttling from the chloroplast to the central vacuole via an autophagy-dependent pathway [95]. The autophagy-dependency of these bodies was shown using *atg4a4b-1*mutants which are impaired in autophagy. Chloroplast fate was investigated in individually darkened leaves (IDLs) of wild type plants and *atg4a4b-1* mutants since individual darkening of leaves has been shown to rapidly induce senescence [57]. Wild type plants showed a decrease in chloroplast number and size as well as formation of RCBs. A*tg4a4b-1* mutant lines also displayed a decrease in chloroplast size but RCB formation was abolished and also the count of chloroplasts stayed constant.

However, Rubisco, nitrogen, soluble protein and chlorophyll contents decreased at almost the same rate in wild type plants as in *atg4a4b-1* mutant plants. This suggests alternative, autophagy-independent protein degradation pathways [96]. Lastly, despite the earlier mentioned 44 kDa Rubisco fragment observed in isolated chloroplasts, oxidative stress conditions might also initiate degradation of Rubisco's large subunit, as under these conditions the large subunit is split into a 37 and a 16 kDa fragment [97].

Besides the RCBs, senescence-associated vacuoles (SAVs) have been described. These vacuoles, enclosed by a single membrane layer, are enriched in Rubisco and display a high proteolytic activity at a pH more acidic than the central vacuole´s. SAVs are structurally not related to RCBs which posses a double layer membrane [95, 98]. The double layer membrane enclosing the RCBs appears to be derived from the chloroplast envelope [95]. Furthermore, SAV development seems to be autophagy-independent, as Arabidopsis *atapg7-1* mutant lines show normal SAV formation [98]. SAVs are only formed in leaf mesophyll cells. They are approximately 700 nm in diameter and can be labeled with antibodies against the (H+)-pyrophosphatase, an Arabidopsis vacuolar marker indicating these organelles indeed to be vacuoles [98]. Accumulation of stromal proteins in the SAVs was proven via plastid localized GFP which localized in SAVs in senescing tobacco leaves. In addition, high levels of chloroplastic glutamine synthase could be detected within these vacuoles [89]. Although the chlorophyll degradation pathway has been elucidated to a large extend and the first steps are regarded to occur within the plastid [88, 99], chlorophyll *a* has been found in SAVs under certain conditions, thus an alternative degradation pathway can be proposed [89]. Despite SAG12 has been shown to localize in SAVs, *sag12* mutant lines did neither show impairment in SAV formation nor in the proteolytic activity within the SAVs [98].

Even though chlorophyll represents about 2% of the total cellular nitrogen content [86], N fixed in chlorophyll is not exported from the leaf but rather remains in the vacuole [100]. However, around 20% N are fixed in proteins associated with or directly binding chlorophyll [88] and removal of chlorophyll seems to be a prerequisite for degradation of the corresponding apoproteins [88]. Pheophorbide a oxygenase (PAO) is an iron-dependent monooxygenase localized to the inner envelope of maturing gerontoplasts and catalyzes the conversion of pheophorbide a to red chlorophyll catabolites, one of the first steps during chlorophyll degradation. It represents a key control point in regulation of chlorophyll degradation [88, 101, 102]. In *pao* mutants and other stay green mutants affected in PAO activity and thus impaired chlorophyll degradation, this retention is accompanied with the accumulation of chlorophyll apoproteins like LHCII (see [88] and references within).

### *3.2.3. Autophagy*

Autophagy plays a crucial role for nitrogen remobilization. The most striking phenotype of all *atg* mutants is hypersensitivity to nitrogen starvation ([103] and references within). Furthermore, an age dependent early senescence phenotype can be observed. As autophagy is involved in molecule degradation one would expect delayed senescence if this pathway is blocked. One hypothesis explaining this contradiction is that the autophagy pathway is normally activated at an early stage of senescence starting to degrade plastid proteins while

leaving the photosynthetic apparatus intact. However, when autophagy is blocked, it is speculated that autophagy-independent pathways for chloroplast protein degradation might be activated untimely leading to premature chloroplast and chlorophyll degradation and thus to an early senescence phenotype [104]. Recently, Guiboileau et al. (2012) [103] conducted a study on the impact of *atg* mutants *(atg5, atg9* and RNAi18) on nitrogen remobilization. These plants were grown under ample and low nitrate conditions. In comparison to wild type plants the dry weight as well as the seed weight was lowered. However, when calculating the harvest index, *atg* mutants did not display a significant difference, except for the *atg5* mutant line at low N conditions. When the nitrogen use and remobilization efficiency was investigated via $^{15}N$ tracing experiments, all *atg* lines showed a decrease in this feature. It was demonstrated that remobilization was significantly impaired, as N contents in the plants dry remains were enriched and $^{15}N$ previously partitioned to the leaves was not mobilized to the seeds. To verify that this impairment rests on an autophagy defect and not on premature senescence and cell death symptoms, *atg5* mutants were combined with two SA signaling mutants, *sid2* and *nahG*, overriding the early senescence phenotype. These mutants reached nearly wild type biomass levels, but did not compensate the decrease in NRE. These results and the finding that autophagy regulates SA levels in a negative feedback loop [105] suggest, that the premature senescence phenotype in *atg* mutants is at least in part mediated by increased SA levels [104]. Conclusively, blocked autophagy pathways might result in an early senescence phenotype because of the accumulation of damaged and thus potentially toxic molecules in combination with a missing negative feedback on SA levels leading to cell death and activation of alternative pathways for bulk protein degradation.

### *3.2.4. Re-assimilation and translocation of salvaged nitrogen*

As mentioned above, chloroplastic glutamine synthetase (GS2), GOGAT, NiR and Rubisco are targeted for rapid degradation already during early phases of senescence, disrupting primary nitrogen assimilation. Proteolysis in the vacuole feeds into the cellular pool of free AAs during the progression of senescence. The steady-state concentrations of free AAs depend on the rate of their release due to proteolysis and their efflux into growing structures [106]. Soudry et al. (2005) [106] have utilized a bioluminescence assay combined with auxotrophic bacteria for the detection of free tryptophan levels. They assumed that tryptophan reflects the overall pool of free AAs, as it is not modified before its export into sink organs. An accumulation of free AAs was observed in detached oat and Arabidopsis leaves. While attached oat leaves showed a gradual decrease in tryptophan levels during further progression of senescence, the attached Arabidopsis leaves did not or only due to membrane leakage resulting from the experimental procedure. The authors concluded that not only source strength but also sink strength is important for successful nutrient remobilization and suggested that the small reproductive organs of Arabidopsis exerted too weak sink strength. However, these findings might be related to the experimental design, as Arabidopsis does indeed remobilize N for seed filling [70] and Diaz et al. (2005) [52] reported decreasing levels for several AAs during the progression of leaf senescence in Arabidopsis. Protein breakdown increases free AAs in the cell. While some seem to be exported without prior modification, many are probably modified, hydrolyzed or interconverted. Expressional

profiling revealed that, besides others, the cytosolic GS1, glutamate dehydrogenase (GDH) and asparagine synthetase (AS) are specifically induced during senescence [14]. A series of transamination reactions would result in an accumulation of glutamate, which could serve as substrate for GDH. Deamination of glutamate via GDH provides then 2OG and ammonia. $NH_3$ could then in turn be used as substrate for cytosolic GS1, giving rise to glutamine, which is one of the major nitrogen transport forms during nutrient remobilization. In fact many studies strengthen a positive correlation between GS activity and yield as well as grain and stem N content. Martin et al. (2006) [107] identified two cytosolic glutamine synthetase isoforms in maize which have a major impact on kernel size and yield. In wheat, GS activity was also positively linked with grain and stem N content [71]. Recently, two rice varieties with different levels of GS2 activity were analyzed and plants with higher activity displayed less $NH_3$ emission due to photorespiration and a better ability to recycle and reassimilate ammonia within the plant [108]. In barley amino acid permeases (AAP) seem to play a significant role during N retranslocation and grain filling. Recent RNA-Seq data revealed an overrepresentation of this gene family in both source and sink tissues. Furthermore, the grain-specific HvAAP3, which was also identified in this study, has high sequence similarity to Arabidopsis AAP1 and AAP8, which have been already shown to be involved in seed N supply (see [109] and references within).

Asparagine amounts also increase significantly in whole rosettes darkened for several days as well as in senescent leaves (see e.g. [1, 80]). Besides the senescence specific up-regulation of AS, pyruvate orthophosphate dikinase (PPDK) expression is also significantly increased during dark-induced senescence [80]. PPDK might have a role in carbon salvage after lipid degradation, thus Lin and Wu (2004) [80] also investigated other pathways possibly involved in this process. Remarkably, they found only a few components of these pathways to be up-regulated and many others even down-regulated. Based on their expressional profiling data, they postulated a alternative pathway for asparagine synthesis, where PPDK delivers metabolic precursors [80]. Additionally, seed protein contents were elevated and viability of seedlings was increased on nitrogen-limiting media in Arabidopsis *ASN1* overexpressor lines (*35S*::*ASN1*). Furthermore, they observed more Asn to be allocated to flowers and developing siliques and also higher Asn contents in phloem exudates [80, 110].

Nitrogen is not only remobilized from older leaves via amino acids. Nitrate and ammonia are also translocated to developing sink tissues. Fan et al. (2009) [111] identified a nitrate transporter (NRT1-7) which is involved in remobilization processes. Arabidopsis *nrt1-7* mutants displayed retarded growth under nitrogen starvation conditions. Also the spatial expression of this transporter in phloem tissue of older leaves and the expressional induction upon nitrogen starvation points out its function in nitrogen remobilization. Finally, the inability of *nrt1-7* mutants to remobilize $^{15}N$ from old to young leaves and the high accumulation of nitrate in old leaves in this mutants further underlines this assumption [111]. Another nitrate transporter involved in remobilization is NRT2-4. This transporter acts in the high-affinity range and its expression is also induced upon nitrogen starvation. Additionally, *nrt2-4* mutant lines had lower phloem sap nitrate contents. However, *nrt2-4* mutants were not altered in growth or development, indicating that the decreased $NO_3$-levels

are not limiting for the adaption to N starvation and most likely functionally redundant transporter systems exist [40].

## 4. Reactive oxygen and nitrogen species in signaling

Reactive oxygen and nitrogen species (ROS, RNS) play a central role in many aspects of plant development and response to environmental influences. These include among others responses to wounding, pathogen infection, drought and water stress, high salinity, cold and heat. In the case of ROS, research has focused especially on $H_2O_2$. As this reactive oxygen molecule is relatively long lived (~1 ms half life), small and uncharged, and thus is able to pass membranes, a central position in various signaling pathways has been attributed to it. Nitric oxide (NO) has been shown to be involved in many of the $H_2O_2$-mediated pathways in either a synergistic or antagonistic mode of action. In the following we will briefly introduce the production and scavenging mechanisms for this two reactive oxygen and nitrogen compounds and their interplay in regulation of developmental processes, stress responses and senescence will be outlined.

### 4.1. ROS and RNS: Molecule types, production and scavenging

Many of the reactive oxygen species in the cell are formed as toxic byproducts of metabolic processes. Photorespiration and β-oxidation of fatty acids produce $H_2O_2$ in peroxisomes and glyoxisomes, which is normally scavenged by an extensive protection system mainly consisting of catalases (CAT) and ascorbate peroxidases (APX). Xanthine oxidase generates superoxide anions in the peroxisomes, which is converted by superoxide dismutases (SOD) into $O_2$ and $H_2O_2$. Chloroplasts are the main site for ROS production in plants. Due to the photooxidative nature of many of their components they can give rise to superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen. ROS produced in the chloroplasts are mainly scavenged by the ascorbate-glutathione cycle [112]. SODs scavenge superoxide anions and dismutate them to $O_2$ and $H_2O_2$, which is then in turn reduced to water by the action of ascorbate peroxidase and ascorbate. The resulting monodehydro-ascorbate (MDHA) is regenerated either via the MDHA reductase (MDHAR) under the use of NADPH or it spontaneously converts into dehydroascorbate (DHA) which is then reduced to ascorbate again via the DHA reductase (DHAR). DHAR uses glutathione (GSH) as second substrate. The reduced state of GSH is reconstituted by glutathione reductase (GR). Excess oxidized GSSG seems to be exported from the cytosol to the central vacuole and the chloroplasts to maintain a reduced environment and redox homeostasis in the cytosol and possibly the nucleus [113]. Finally, superoxide radicals can be produced as a byproduct during respiration in mitochondria. Here, also SOD and the ascorbate-glutathione cycle removes the ROS. Further ROS scavenging in this organelle is mediated by peroxiredoxins and thioredoxins, as it is also observed in chloroplasts. Additionally, non-enzymatic components like tocopherols, flavonoids, ascorbic acid and others are employed in the extensive and elaborate ROS detoxification system (reviewed in [114-119]). Under optimal growth conditions,

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ROS production is relatively low; however, during stress, the production of ROS is rapidly enhanced [120].

Active production of ROS or the so called "oxidative burst" is initiated upon several stresses and developmental stimuli. The main enzymes generating these ROS are the respiratory burst oxidase homologs (RBOH) [121]. In a NADPH-dependent reaction they form $O_2^-$ in the apoplast. This is then converted by SODs to $H_2O_2$. The function of the 10 different RBOH proteins identified in Arabidopsis [122] is important in various developmental and regulatory processes. Root elongation is reduced in *atrbohD/F* mutants [122]. ROS produced upon pathogen attack are generated by RBOHs (see for example [123]). Also the response to heavy metals seems to be at least in part mediated by RBOH proteins. Cadmium treated sunflower leaf discs showed an altered expression and activity of the NADPH oxidase [124]. The function of these proteins is often associated with the action of $Ca^{2+}$. Arabidopsis *rbohC/rhd2* mutants displayed lowered ROS contents in growing root hairs and a distortion in $Ca^{2+}$ uptake due to a missing activation of $Ca^{2+}$ channels [125], although for the rice RBOHB homolog calcium was needed to activate the oxidase itself [126].

Reactive nitrogen species (RNS) comprise NO and NO-derived molecules as di-nitrogen trioxide, nitrogen dioxide, peroxynitrite, S-nitrosothiols and others [127]. NO production in plant cells is under continuous debate. Especially the existence of a plant nitric oxide synthase (NOS) is a controversial topic. Until today, there is no clear proof for the existence of NOS in plants although there is indirect evidence through the application of NOS inhibitors, which have been established for mammalian cells (e.g. L-NAME a L-arginine analogue) in combination with NR inhibitors, or the measurement of NOS-like activity, like the conversion of L-arginine to citrulline, where NO is assumed to be produced at the same time [128-130]. AtNOS1 was identified in 2003 by Guo et al. (2003) [131], but is under controversial discussion ever since. Indeed, *atnos1* mutant plants do exhibit significantly lower NO contents, a chlorotic phenotype in seedlings which can be rescued by NO application and an early-senescence phenotype, but expression of the corresponding genes from Arabidopsis, maize and rice revealed no NOS activity *in-vitro*, and even the mammalian orthologous displayed no NOS function [132]. Thus *AtNOS1* was renamed to *AtNOA1* (NO associated 1). Nevertheless, there are other enzymatic ways known to produce NO. NR was found to be able to generate NO. It was shown to be involved in NO generation during the transition to flowering Arabidopsis *nr1* and *nr2* mutants display a low endogenous NO content [133, 134]. Additionally, a NR- and NiR-independent pathway of NO production has been proposed via electron carriers of the mitochondrial respiratory chain [135] and an oxidation-associated pathway for NO synthesis has been suggested as hydroxylamines can be oxidized by superoxide and $H_2O_2$ generating NO [136].

Despite all the controversy on the topic of NO generation, it seems clear, that there are many ways to generate NO in plant cells and the pivotal role in many regulatory pathways cannot be denied. Involvement in fruit ripening, leaf senescence, flowering and stomatal closure and many other processes has been shown ([129] and references within).

### 4.2. ROS and RNS: Signaling

The role of $H_2O_2$ and NO during the onset of leaf senescence has been investigated in many studies. Recently, an upstream regulator of the ROS network during ABA-mediated drought-induced leaf senescence has been identified. The drought-responsive NAC transcription factor AtNTL4 (ANAC053) has been shown to promote ROS production by directly binding to promoters of genes encoding ROS biosynthetic enzymes [137]. In guard cells, an ABA-$H_2O_2$-NO signaling cascade has been proposed for stomatal closure. $H_2O_2$-induced generation of NO in guard cells has been reported for mung bean [138], Arabidopsis [139] and other plant species (see for example [140]). Removal of $H_2O_2$ as well as the blocking of calcium channels was able to suppress NO generation [138, 141]. A further interaction of NO and $H_2O_2$ was studied in tomato (*Lycopersicon esculentum* Mill. cv. "Perkoz") where the effect of application of exogenous NO scavengers and generators was analyzed in combination with *Botrytis cinerea* inoculation. NO generators specifically reduced $H_2O_2$ generation and thus allowed the infection to spread significantly under control conditions and in comparison to NO scavenger pre-treated leaves [142]. Moreover, cytoplasmic $H_2O_2$ can also directly activate a specific Arabidopsis MAP triple kinase, AtANP1, which initiates a phosphorylation cascade involving two stress AtMAPKs, AtMPK3 and AtMPK6 [143]. A direct interaction between AtMPK6 and AtNR2 during lateral root development has been shown *in-vitro* and *in-vivo*. During this interaction MPK6 phosphorylates and thus activates NR2 resulting in enhanced NO production [144]. Finally, another point of crosstalk between the NO and $H_2O_2$ signaling pathways has been referred to by positional cloning of the rice *NOE1*. This gene codes for a rice catalase, a knock-out leads to increased $H_2O_2$ contents which in turn enhance the activity of NR, resulting in elevated NO concentrations. The removal of excess NO ameliorated the cell death symptoms of the *noe1* mutants pointing out a cooperative function of $H_2O_2$ and NO during induction of PCD. Here, specifically S-nitrosylated proteins were identified, and overexpression of a rice S-nitrosoglutathione reductase could also alleviate the cell death symptoms [145].

Senescence-inhibiting features of NO have long been recognized, while $H_2O_2$ has often been attributed with senescence-promoting features. Exogenous NO application extends postharvest life of fruits and vegetables and, during leaf maturation in pea, NO contents gradually decrease [146, 147]. Furthermore, NO-deficient mutants display an early-senescence phenotype and the heterologues expression of an NO-degrading enzyme in Arabidopsis also leads to early leaf senescence and SAG up-regulation, which could be inhibited by external supply of NO [148]. Remarkably, the senescence delaying features of NO might be achieved due its ability to scavenge various kinds of ROS. In barley aleuron cells, NO has been shown to act as an antioxidant and thus alleviating GA-mediated PCD induction [149].

### 4.3. Specificity in ROS and RNS signaling

Some amino acids are more susceptible for modification by ROS and RNS than others. For example cysteins are often found to be preferentially oxidized. These residues are sensitive for ROS-derived protein carbonylation and RNS-mediated nitrosylation (-SNO) and glutahionylation. Additionally, sulfenic acid and disulfide formation also can be mediat-

ed via ROS and RNS on these residues. Tryptophane residues have been shown to be specifically di-oxygenized in plant mitochondria, thus forming N-formylkynurenine. The proteins found to be specifically oxygenized did, with one exception, all posses redox-activity or were involved in redox-active proteins [150]. Another good example for this specificity is Rubisco. Preferential oxidation of certain cysteine residues mediates the binding of Rubisco to the chloroplast envelope, thus causing catalytic inactivation and marking it for degradation [151, 152]. Recently, it has been shown, that chloroplast peroxidases are present in an inactivated form and become activated in part by proteolytic cleavage upon a $H_2O_2$ signal; in combination with newly synthesized peroxidases, they regulate plastidial ROS content in neem (*Azadirachta indica* A. juss) chloroplasts [153]. This displays a specificity of ROS induced processes, rather than undirected, detrimental impacts. However, how the cell responds differentially to the variety of $H_2O_2$ signals in different signaling pathways is still unclear. With regard to leaf senescence induction, a dependency of $H_2O_2$-mediated effects on the subcellular location was discovered. By using an *in-vivo* $H_2O_2$-scavenging system, we manipulated $H_2O_2$ contents in the cytosol and peroxisomes in Arabidopsis. While both lines showed lowered $H_2O_2$ contents and a delayed leaf senescence phenotype, the delay of the cytoplasmic line was more pronounced, despite the higher expression of the peroxisomal transgene [65]. Furthermore, lowering mitochondrial $H_2O_2$ production by blocking cytochrome *c* dependent respiration with the fungal toxin antimycin A had no effect on induction of leaf senescence [154]. Since senescence is predominantly regulated on transcriptional level, the cytoplasmic compartment might have a direct influence on redox regulation of transcription factors. Expression of the MAP triple kinase1 (*MEKK1*) of Arabidopsis can also be induced by $H_2O_2$ and shows its expression maximum during onset of leaf senescence [155]. Whether $H_2O_2$ induced expression of SAGs is transduced by MAPK signaling or directly by redox-sensitive transcription factors has yet to be elucidated.

Moreover, the already mentioned evidence of numerous selective oxidation reactions on specific amino acid residues depending on the type of ROS/RNS might lead to the degradation of the damaged proteins, thus generating distinct peptide patterns. These peptides would contain information being ROS- and source-specific (see [150] and references within). Spatial control might also be a source of specificity, as for example RBOH proteins are membrane bound and, therefore, localization of the ROS signal could be highly specific. Additionally, through the extensive detoxification system, ROS signals also might be spatially confined. In contrast, ROS signal auto-propagation over long distances via RBOHD induced by various stimuli has been shown in Arabidopsis [156]. Interestingly, temporal oscillation of ROS bursts has been observed to modulate root tip growth of Arabidopsis root hairs [157]. Finally, integration of metabolic reactions also seems to be a convenient way of specific signaling. Local blockage or enhancement of certain pathways would lead to the accumulation of intermediates, which in turn could serve for signaling functions (reviewed in [158]).

## 5. Concluding remarks

The intriguing connection between efficient nutrient remobilization and progression of leaf senescence is obvious. The correct timing of onset and progression of senescence has great influence on seed and fruit development and viability. Therefore, manipulating leaf senescence seems to be a promising trait to increase yield in various crop species. Functional stay green traits can prolong carbon assimilation and thus increase yield. However, a too strong delay in leaf senescence might hamper nutrient and especially nitrogen remobilization from the leaves. For various wheat mutants, Derkx et al. (2012) [159] speculated that the stay green phenotype might be associated with a decrease in grain N sink strength. Gpc-B1, a QTL locus in wheat, which is among others associated with increased grain protein content, has been shown to encode a NAC transcription factor (*NAM-B1*). It accelerates senescence and enhances nutrient remobilization from leaves. RNA interference mediated silencing of multiple homologues resulted in a delay of leaf senescence by approximately 3 weeks and decreased grain protein, iron and zinc content by more than 30% [160]. This indicates that the relation between senescence and nitrogen mobilization is very complicated and cannot be modified as easy as expected.

Besides QTL selection, transgenic approaches to increase nitrogen use efficiency in crop plants have been extensively studied. For example expression of alanine aminotransferase and asparagine synthase often resulted in enhanced seed protein content and higher seed yield. Increased cytokinin biosynthesis almost always resulted in delayed senescence and was sometimes associated with higher seed yield, seed protein content and increased biomass. Expression of amino acid permease from V*icia faba* under the *LeB4* promoter increased the seed size by 20-30%, as well as the abundance of nitrogen rich AAs and the content of seed storage proteins in the seeds (reviewed in [161]).

Nevertheless, although transgenic approaches have proven to enhance nitrogen use efficiencies and yield quantity as well as quality, these techniques have to cope with general skepticism on the consumer's side. Although approval for the agricultural use of genetically modified organisms has been extensively performed like e.g. in the Swiss National Research program NRP 59 (Benefits and risks of the deliberated release of genetically modified plants) clearly indicating a low risk and a enormously high potential of transgenic crop plants, problems with the acceptance of this technology, especially in Europe, still have to be faced.

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