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Nitrogen recycling and remobilisation are differentially controlled by leaf senescence and development stage in *Arabidopsis thaliana* under low nitrogen nutrition.

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Key words:

Glutamine synthetase; Senescence; ¹⁵N labelling; Source – sink; Grain filling; Nitrogen metabolism

Abbreviations:

DW: dry weight

FW : fresh weight

QTL: Quantitative trait loci

RIL: recombinant inbred line

SAG: Senescence associated gene

6FL: six first leaves to have emerged

11FL: eleven first leaves to have emerged

YL: young leaves (rosette minus 6FL)

DAS: days after sowing

GABA: γ -aminobutyric acid

%P: partitioning

RSA: Relative Specific Allocation

Abstract

Five recombinant inbred lines (RIL) of *Arabidopsis thaliana*, previously selected by Diaz et al. (2005) from the Bay-0 x Shahdara RIL population on the basis of differential leaf senescence phenotypes (from early-senescing to late-senescing) when cultivated under nitrogen limiting conditions, were analyzed in order to monitor metabolic markers related to nitrogen assimilation and nitrogen remobilisation pathways. In each RIL, a decrease of total nitrogen, free amino acid and soluble protein contents with leaf ageing was observed. In parallel, the expression of markers for nitrogen remobilisation such as cytosolic glutamine synthetase, glutamate dehydrogenase and CND41-like protease was increased. This increase occurred earlier and was more rapidly in early-senescing lines than in late senescing lines. We measured the partitioning of ^{15}N between sink and source leaves during the vegetative stage of development using ^{15}N tracing and showed that nitrogen remobilisation from the source leaves to the sink leaves, was more efficient in the early senescing lines. The N-remobilisation rate was correlated with leaf-senescence severity at vegetative stage. Experiments of ^{15}N tracing at reproductive stage, showed however that the rate of nitrogen remobilisation from the rosettes to the flowering organs and to the seeds, was similar in early and late senescing lines. At reproductive stage, N-remobilisation efficiency did not depend on senescence phenotypes but was related to the ratio between the biomasses of the sink and the source organs.

Introduction

Leaf senescence is a long developmental process leading to the death. Leaf senescence onset starts as leaf expansion stops (Buchanan-Wollaston et al., 2003; Diaz et al., 2005) through the induction of series of metabolic changes allowing nutrient recycling and remobilisation. Since plants are static and most of the time nutrients are limited, Leopold (1961) proposed that leaf senescence was selected through evolution to allow plants to improve nutrient economy and to survive upon starvation. Indeed, nitrogen depletion is known to accelerate leaf senescence and to increase nitrogen recycling and mobilisation (Schulze et al., 1994). Through recycling the endogenous nutrients from the senescing leaves, plants can better support the growth of younger leaves and reproductive organs under nutrient limiting nutrition.

Mechanisms and enzymatic process for nitrogen mobilisation during leaf senescence have been investigated in numerous plant models (Masclaux et al., 2000; Cabello et al., 2006; Martin et al., 2005; Bernhard and Matile, 1994). Rubisco is the main leaf nitrogen storage protein and the degradation of this enzyme during senescence has been widely studied even though Rubisco degradation is still poorly understood. Rubisco proteolysis could be initiated by the deleterious effects of reactive oxygen species (Ishida et al., 1998). Afterwards, the senescence-induced aspartic protease CND41, encoded by the nuclear genome and localised specifically to the chloroplast could degrade Rubisco. *In vitro* analysis confirmed that CND41 showed a Rubisco proteolytic activity at physiological pH values (Nakano et al., 1997; Kato et al., 2004; Figure 1). However, CND41 could not act on Rubisco unless its structure was denatured. Therefore, active Rubisco in the chloroplast would be resistant to CND41 catalysed degradation until leaf senescence is under way. CND41 homologues have been identified in *Arabidopsis* that might also be responsible for the regulation of Rubisco turnover and senescence. Rubisco degradation certainly releases numerous free amino acids available for phloem loading and other interconversions. The way by which amino acids are loaded to the phloem of senescing leaves for translocation is poorly documented (van der Graaff et al., 2006). One possibility is that amino acids would be interconverted to increase the synthesis of amino acids dedicated to transport, like glutamine and asparagine. Indeed, in tobacco, It has been shown that the cytosolic glutamine synthetase (GS1) and the mitochondrial glutamate dehydrogenase (GDH) are induced as the primary nitrogen assimilating enzyme decrease with ageing (Masclaux et al., 2000). Through the catabolism of glutamate, GDH provides ammonium as a substrate for GS1 and cytosolic GS1 is responsible for glutamine neosynthesis (Masclaux-Daubresse et al., 2006). Both GS1 and GDH enzymes are mainly located in the roots. However during senescence, GS1 protein is detected in both leaf mesophyll and companion cells (Brugière et al., 2000). The GS1 and GDH enzymes are octameric and hexameric proteins respectively. Genes encoding their different subunits belong to multigenic families and in *Arabidopsis thaliana*, five genes have been isolated for GS1 (*gln1.1* to *gln1.5*) and three genes (*gdh1* to *gdh3*) for the NADH-dependant GDH. From molecular physiology and gene-expression profiling it appears that *gln1.1*, *gln1.2*, *gln1.4* and *gdh2* belong to the SAG family (Bernhard and Matile, 1994; Guo et al., 2004). However, the precise role of these genes in the nitrogen remobilisation process still remains to be investigated in *A. thaliana*.

In a previous report, the extent and the variability of leaf senescence in *Arabidopsis* was characterized using five genotypes from the Bay-0 x Shahdara RIL population that have

been selected for their differential leaf lifespan, yellowing-symptom earliness and intensity (Diaz et al., 2005). A metabolic profiling of ageing leaves from the five RIL showed that metabolite contents were similarly modified throughout ageing regardless to the genetic background of the RIL (Diaz et al., 2005). Age-related modifications occurred however earlier and were more pronounced in the early senescing lines than in the late senescing lines. From this study, it was shown that the quantities of hexoses, sucrose, starch and individual amino acids in leaves change with ageing and depend on the senescence severity. In particular, γ -aminobutyrate, leucine, isoleucine, tyrosine and arginine accumulate to a greater extent (i) during leaf ageing and (ii) in the leaves of early-senescing lines compare to those of late senescing lines. At the same time glutamate and aspartate decrease more rapidly in early-senescing lines with ageing. This study then showed that trans-aminating activities are certainly differentially expressed during leaf ageing and senescence. Since all the amino acid do not play the same role in plant metabolism and are not mobilized with the same efficiency. All these results lead us to address the question of the leaf nitrogen remobilisation efficiency (NRE) in the five RIL.

In this report, the expression of the N-remobilisation enzymatic and molecular markers have been monitored during leaf ageing in the five genetic background chosen previously by Diaz et al. (2005). The design of the time course experiment as described in Diaz et al. (2005). The aim was to determine if the mechanisms for nitrogen remobilisation and their regulation are similar in *A. thaliana* and in other plant species and if nitrogen-remobilisation depends on leaf ageing and leaf-yellowing severity. To determine if total nitrogen export is correlated with leaf-senescence and/or N-remobilisation markers we determined different carbon and nitrogen pools and measured nitrogen fluxes using ^{15}N tracing.

Results

Comparison of the biomass of five RIL showing differential senescence patterns

Diaz et al. (2005) characterized the Arabidopsis RIL on the basis of leaf senescence phenotypes. RIL310 is the earliest senescing line, RIL083 and RIL232 are early senescing while, RIL045 and RIL272 are late senescing lines. The six first leaves (6FL) of the rosettes that have emerged concomitantly for all the RIL, were used to compare the effect of ageing on nitrogen metabolism between genotypes. Rosettes were dissected to isolate and collect the 6FL, and the new leaves (NL), which are the pool of the leaves that have emerged after the six

first leaves. While the biomass of the 6FL depends only on the biomass of each leaf, the biomass of the NL depended on both the number of leaves that have emerged with time and their growth rate. The 6FL can be considered as a source of N-mobilisation for the NL that will be in turn a further source for the flowering stems.

The dry weight (DW) of the 6FL changed according to a bell shape curve (Figure 1) for all the RIL. The 6FL DW stabilized when leaves attained maturity, then the 6FL DW decreased. This decrease was observed earlier and more rapidly for the early senescing lines than for the late senescing lines. The DW of the NL also changed according to a bell shape curve, but the decreasing phase of this curve was not related to the senesce phenotype of the line but to the emergence of the flowering bud. Interestingly, the total rosette DW (6FL-DW + NL-DW) was higher in the late senescing lines (RIL272, 45 mg and RIL045, 40 mg) than in the early senescing lines (RIL310, 30 mg, and RIL083, 30 mg) thus illustrating the negative correlation between rosette dry matter and leaf yellowing that was found previously by Diaz et al. (2006) when studying the variation of nitrogen use efficiency and senescence traits on the whole RIL population.

Comparison of the chlorophyll and starch content in the 6FL and in the NL of the five RIL

As previously described by Diaz et al. (2005), the total chlorophyll decreased with leaf ageing earlier and more rapidly in the 6FL of early senescing lines than in the 6FL of late senescing lines (Figure 2A). In the 6FL, the extent of chlorophyll decrease paralleled the decrease of starch concentration for all of the lines, except RIL045 (Figure 2B). In the NL, the concentration of chlorophyll was surprisingly higher in the early-senescing lines than in the late-senescing lines (Figure 2C) suggesting that the NL chlorophyll concentration could compensate the higher loss in the 6FL of the same plant. By contrast, the starch concentration in the NL was higher in the late senescing lines (Figure 2D), suggesting that the photosynthetic carbon assimilation was better in the late senescing lines.

Total protein content, total amino acid content, and total nitrogen amount decreased in the 6FL and in the NL of the five RIL

The concentration of total N decreased with rosette ageing in a similar way for all the RIL (Figure 3 A and B). This decrease occurred 24 DAS in the 6FL and 35 DAS in the NL. Surprisingly, the decrease of nitrogen in the 6FL was not delayed in the late senescing lines

compared to early senescing lines. In the NL, the N-decrease was less pronounced in RIL310 than in other lines.

Total nitrogen can be distinguished in structural nitrogen and remobilisable nitrogen. Amino acids and soluble proteins are N-remobilisable pools. With ageing, both free amino acids and soluble proteins concentrations decreased in NL and 6FL (Figures 3C-F). In the 6FL, the decrease in total protein appeared earlier for the hyper-senescing RIL310 line, and was slightly higher for all the early senescing lines compared to the late senescing lines after 50 DAS (Figure 3C). The decrease of amino acid concentration in the 6FL was also similar in the early and late senescing lines, except in RIL310 that remained with higher amino acid concentration (Figure 3E).

In the NL, the soluble protein and the free amino acid concentrations were higher in the early senescing lines than in the late senescing lines, showing an opposite trend as observed for sugars (Figures 3D and 3F).

Changes in nitrogen-enzyme activities in the 6FL of the five RIL during ageing

It has been largely reported that primary nitrogen assimilation enzymes are down regulated with leaf ageing while nitrogen-remobilisation enzyme are induced (Masclaux et al. 2000). Therefore, we measured the activity of GS, GDH and NR on the 6FL of the five RIL. As expected, the NR and GS activities decreased with ageing (Figures 4A and 4B) whereas GDH activity increased (Figures 4C and 4D). However, time courses of NR activity decrease and GDH activity increase were not different between the early and late senescing RIL. By contrast, the total GS activity decreased earlier and more rapidly in the 6FL of early senescing lines than in those of late senescing lines.

Changes in glutamine synthetase, Rubisco and CND41-like protein contents in the 6FL of the five RIL

We measured the abundance of GS1, GS2, Rubisco and CND41 proteins by gel staining and antibodies. The amount of GS1 protein increased with ageing for all the RIL whereas the amount of GS2 protein decreased (Figure 5A). The increase in GS1 protein was sharper in the 6FL of RIL310 at 41 DAS and 45 DAS. Rubisco content decreased with leaf ageing for all the lines, but earlier and more rapidly in the 6FL of RIL 310 and RIL083 (Figure 5B). Antibodies raised against the tobacco CND41 chloroplastic aspartic protease

((Kato et al., 2004)) were used to detect a potential CND41-like protein in Arabidopsis. Very interestingly, the signal detected with the CND41 antibodies was higher in the early senescing lines (Figure 5C). The CND41 signal remained very low in RIL045 and was almost undetectable in the 6FL of RIL272.

Change in the nitrogen-enzyme mRNA contents in the 6FL of the five RIL

The expression of the *cab* gene was used as a marker for senescence genes down-regulation. The decrease in *cab* mRNA expression occurred earlier in the 6FL of RIL310 (27 DAS) and RIL083 (31DAS) than in the 6FL of RIL232, RIL045 and RIL272 (34 DAS). The *cab* mRNA remained detectable in RIL272 and RIL045 late, until 52 DAS and 57 DAS respectively (Figure 6).

By comparison with *cab*, both *nia* and *gln2* mRNA, encoding NR and GS2 proteins respectively, decreased with ageing. The amount of *nia* and *gln2* mRNA decreased earlier and to a greater extent in early senescing lines than in late senescing lines. By contrast, *gln1.1* and *gln1.2* (both encoding GS1 proteins), *gad2* (encoding a glutamate decarboxylase isoform) and *gdh2* (encoding glutamate dehydrogenase) mRNA levels increased with leaf ageing earlier and to a greater extent in the early senescing lines than in the late senescing lines (Figure 6).

Comparison of the nitrogen fluxes and nitrogen remobilisation during the RIL development using ¹⁵N tracing

To determine to what extent nitrogen is mobilized during plant development and to compare this trait between the five RIL, labeling experiments were carried out as described in Material and Methods. Three different labeling protocols were designed in order to determine (i) the level of ¹⁵N remobilisation from leaf to leaf during the vegetative phase of the rosette growth (Exp. 1), (ii) the level of ¹⁵N remobilisation from rosette to flowering organs during the reproductive phase (Exp. 2) and (iii) the level of ¹⁵N remobilised for seed filling (Exp. 3).

In Exp.1 the fluxes of ¹⁵N from the leaves (11FL) labeled during the pulse period to the leaves newly emerged during the chase period (NL) is illustrated by the partition of ¹⁵N at the end of the pulse period (time T0) and at time T1, 10 days later. Between T0 and T1, 6 to 7 new leaves (NL) have emerged. Since these NL have appeared and developed during the

chase period, the ^{15}N they contain can only come from the remobilisation of the ^{15}N absorbed during the pulse period and assimilated in the roots and in the 11FL before the end of the pulse period. The measurement of the total ^{15}N quantity (Q) in each organ at T0 and T1 allowed us to calculate for the whole plant (wp) the quantities of ^{15}N at T0 (Q_{wpT0}) and at T1 (Q_{wpT1}). For all the RIL, Q_{wpT0} is equal to Q_{wpT1} (data not shown), showing that there was no loss of ^{15}N during the chase period.

At T0, the partitioning ($P\% = Q_i/Q_{wp}$) of ^{15}N in roots and in shoots (i.e. 11FL) was quite similar between the five RIL (Figure 7A and 7B). Around 15% of the ^{15}N has been assimilated in the roots during the pulse period while the major part (85 %) was incorporated in the 11FL. The determination of P% in the roots, in the 11FL and in the NL at T1, showed that ^{15}N has been mobilized from the roots, and from the 11FL to the NL. Remobilisation from the 11FL was higher than from the roots for all the RIL (Figure 7A and 7B). Moreover the % of ^{15}N exported from the 11FL to the NL during the chase period was significantly higher for the early senescing lines than for the late senescing lines, thus showing that the extent of ^{15}N remobilisation is linked to leaf-senescence intensity (Figure 7B and 7C). The early senescing RIL310, RIL083 and RIL232 have remobilized 16%, 10% and 12% of N respectively from 11FL to NL between T0 and T1 while the late senescing RIL045 and RIL272 have mobilized 5% and 7% of N respectively, during the same period of time.

In Exp.2 the flux of ^{15}N from the whole rosette and the roots to the flowering stem and reproductive organs was investigated. Since only RIL083 and RIL272 (see Diaz et al. (2005)) flowered in the same time, these lines were chosen for this experiment. Roots and rosettes were labeled with $^{15}\text{NO}_3^-$ until the emergence of the flowering bud (T0), which occurred 59 DAS. At T0, for each line, three labeled plants were separated as root and rosette. During the chase period plants were collected 66 DAS (T1) and 88 DAS (T2). The newly appeared organs between T0, T1 and T2 were then separated as cauline leaves, primary (I) and secondary (II) stems, siliques and flowers. For all the lines, it was verified that $Q_{wpT0} = Q_{wpT1} = Q_{wpT2}$ (data not shown). The partitioning of ^{15}N and the level of ^{15}N mobilized is presented in Table 1. At time T0, the partitioning of ^{15}N in the roots was higher for RIL272 than for RIL083 (25% versus 20%). This difference was maintained at T1 since there was no ^{15}N mobilization from the roots to the rest of the plant for RIL083 and RIL272 between T0 and T1. Between T0 and T1, the ^{15}N exported to the new growing organs was only coming from the rosettes, and the level of the ^{15}N mobilized was similar in the two lines, reaching 12.6% of the whole plant ^{15}N , and 16% of the ^{15}N contained in the rosette at T0. The

ratio of ^{15}N translocated to the stem I, the cauline leaves and the flowers were similar in the two RIL and mainly dedicated to flowers that received almost 60% of the ^{15}N pool that have been remobilised from the rosette, while the stem I and the cauline leaves have received 20% each.

Between T1 and T2, 1.5 % of the whole plant ^{15}N was reallocated from the roots to the flowering stems and reproductive organs, while 24% of the whole plant ^{15}N (or 36% to 40% of ^{15}N contained in the rosettes at T1) was exported from the rosette to the flowering stems and reproductive organs. Remobilisation from the rosettes mainly benefited to the cauline leaves and the flower + silique compartments. The strength of these two sinks appeared slightly more powerful in RIL083 than in RIL272, (Table 1).

Since there was no significant difference in the level of ^{15}N mobilized from the rosettes of the early senescing line RIL083 and of the late senescing line RIL272, while the siliques and flowers sinks seemed to be slightly favored in RIL083, the question of grain filling was addressed. Hydroponic culture mode is not suitable to seed production. Culture on sand was then preferred. Culture on sand did not permit the harvest of roots, however since the remobilisation of ^{15}N from the roots measured in previous experiments have appeared very weak, it was assumed that neglecting the root compartment will not drive large errors. For each RIL, four plants were then cultivated in N limiting condition. The labeled $^{15}\text{NO}_3^-$ was provided to the plants in the watering solution, twice at 40 DAS and 42 DAS, when the rosette growth was exponential. After ^{15}N uptake, sand was carefully rinsed several times with permutated water to remove remaining labeled nitrogen. The transfer of the plants from short day photoperiod to long day photoperiod 56 DAS induced flowering and reduced the difference of flowering time between the RIL. Plant material was collected at the end of the cycle when the whole plants were dry and seeds fully matured (i.e. around 140 DAS). The partition of dry matter (mg DW), total nitrogen (mg) and ^{15}N (μg) between the stover (dry leaves + stems + silique envelopes) and the seeds compartment was investigated (Table 2).

At the end of their life cycle, the total biomass (DW) per plant was similar (around 800 mg per plant) for all the RIL, except RIL083 whose dry matter was slightly lighter. The total N and ^{15}N per plant were not different between the five RIL, thus showing that the nitrogen uptake was similar for all the plants and genotypes. However, the partitioning of dry matter, total N and ^{15}N in the seeds was significantly different between RIL310 and the other RIL. Compared to other genotypes, RIL310 produced large vegetative biomass and very few seeds. For that reason, the quantity of ^{15}N mobilized to the seeds was also very low. Thus,

except in the case of RIL310, the partitioning of ^{15}N in the seed compartment was similar between all the RIL, thus showing that ^{15}N remobilisation to the seeds was as efficient in early and late senescing lines. Around 65% of the ^{15}N that was assimilated in the rosettes during vegetative phase (at 40 and 42 DAS), was remobilised for grain filling. The $\text{P}\% \text{N}_{\text{seed}}$ and the $\text{P}\% ^{15}\text{N}_{\text{seed}}$ were not significantly different for all the RIL. This shows that the plants did not discriminate between ^{15}N and ^{14}N for grain filling.

Exp.3 was carried out twice, and each repeat contained four plant replicates. This allowed us to test the correlation between the partition of dry matter ($\text{P}\% \text{DW}_{\text{seeds}}$) and the partitioning of ^{15}N ($\text{P}\% ^{15}\text{N}_{\text{seeds}}$) in the seeds. The correlation between $\text{P}\% \text{DW}_{\text{seeds}}$ and $\text{P}\% ^{15}\text{N}_{\text{seeds}}$ was high and repetitive: $R^2 = 0.826$ for the first experiment and $R^2 = 0.720$ for the second one. This showed that the ratio between the source and the sink sizes was correlated with the level of the flux of nitrogen to the seeds.

Discussion

To investigate the effect of senescence and leaf ageing on nitrogen remobilisation process in Arabidopsis, the molecular physiology of five recombinant inbred lines from the Bay-0 x Shahdara population was investigated. These lines have been previously studied and analysed for several nitrogen use efficiency and leaf yellowing traits (Loudet et al., 2003; <http://dbsgap.versailles.inra.fr/vnat/Documentation/33/DOC.html>; Diaz et al., 2006) and for metabolic markers allowing senescence severity characterization (Diaz et al., 2005).

The determination of the biomass, the amino acid contents and the sugar contents led us to confirm some correlations and characterizations that have been previously observed on the whole population in response to nitrogen limitation (Loudet et al., 2003; Diaz et al., 2005; Diaz et al., 2006). In the present report, we observed indeed that the early senescing lines showed lower dry matter accumulation in their rosettes than late senescing lines. This was consistent with the negative correlation, found between traits at 35 DAS (vegetative stage) for leaf yellowing and dry matter level, (Diaz et al., 2006).

We observed here that the new leaves (NL) of the early senescing lines were more concentrated in protein and free amino acid than the NL of late senescing lines. By contrast, they contained lower amount of total sugars. The NL composed the major part of the rosette after 35 DAS. This opposite trend between early and late senescing genotypes was consistent with the positive correlation found between leaf-yellowing and total nitrogen concentration

described in Diaz et al. (2006) and with the co-localization of the major QTLs found for nitrogen use efficiency and yellowing. It remains however to be explained why early senescing lines accumulated more nitrogen and chlorophyll and less sugars in their NL, than late-senescing lines. In addition, the reason why the chlorophyll content was higher in the NL of early senescing lines while starch accumulation and dry matter were lower is unclear. One hypothesis is that the whole rosette of early senescing lines has a lower carbon use efficiency.

The total N, amino acids and proteins measured in the NL and the 6FL decreased with ageing in the five RIL. This trend can be attributed to the 'nitrogen dilution' process already described for many plant species (Greenwood et al., 1990; Justes et al., 1994; Plénet and Lemaire, 2000). Soluble proteins concentration decreased earlier and to a greater extent in the 6FL of the early senescing lines, whereas total-N concentration decreased similarly over time between all the RIL. This suggested that N remobilisation and proteolysis have been enhanced earlier and in the same time as senescence symptoms progressed. The measurement of the activities of NR and GDH suggested that there was a decrease in nitrogen assimilation and glutamate catabolism with ageing. The check point between the primary assimilation phase and the remobilisation phase, as described in Masclaux et al. (2000), was around 40 DAS for all the lines. Determination of total GS activity was more informative and facilitated the monitoring of leaf senescence and N-remobilisation process. The change in total GS is the result of both GS2 and GS1 fluctuations. Since GS2 is the most represented GS isoenzyme in the leaves, the decrease of total GS can be attributed to a decrease of GS2, as observed on the western blots and suggested by the down regulation of *gln2*. The decrease of GS activity was detected sooner in early senescing lines (as soon as 34 DAS for RIL310). In parallel with the decrease of N-assimilation transcripts like *cab*, *nia* and *gln2*, the transcripts for the N-remobilisation enzymes like GS1 and GDH accumulated. This accumulation occurred earlier in the early-senescing lines and paralleled senescence symptoms. In accordance with the accumulation of γ -aminobutyric acid observed by Diaz et al. (2005), we observed here the accumulation of the *gad2* transcripts with ageing. In the same time as *gln1.1* and *gln1.2* transcripts accumulated, increase in GS1 protein content was observed. The good correlation between *gln1.2* and *gln1.1* induction, GS1 accumulation and leaf-senescence severity suggested that the nitrogen remobilisation was more efficient and occurred earlier in the early senescing lines. In addition, the similar increase of *gdh2* expression and GDH activity with ageing suggests that GDH participate to amino acid degradation and recycling in Arabidopsis (Masclaux-Daubresse et al., 2006).

Free amino acids present in senescencing leaves are supposed to be released from the intense proteolytic activity induced with ageing (Brouquisse et al., 2000). Indeed, we observed that Rubisco, which is the biggest nitrogen storage protein, decreased with ageing. Antibodies raised against the tobacco CND41 protein allowed us to detect a signal in the 6FL extracts that increased with ageing and paralleled the decrease of Rubisco protein. While Rubisco decrease was stronger in early senescing lines, the CND41-like protein was much more abundant in the leaves of early-senescing lines. This CND41 homologue proved then to be a good marker for leaf senescence severity and its large accumulation in parallel with Rubisco decrease suggests that this protease have an important role in N-remobilisation in *Arabidopsis* (Kato et al., 2005a; Kato et al., 2005b).

All together, results of the molecular physiology of the five RIL showed that N-remobilisation markers were expressed earlier in the early senescing lines. The remaining question was then to determine if the early senescing lines do mobilise nitrogen more efficiently than late senescing lines.

To address this question ^{15}N labelling and tracing experiments were performed. The results showed that NRE was different depending on the source/sink model considered. The level of ^{15}N mobilized from leaf to leaf during the vegetative phase of the rosette growth was higher in early senescing lines than in late senescing lines. N remobilisation during vegetative development of the rosette was then clearly dependent on the senescence-symptoms severity. By contrast, the level of ^{15}N remobilised from the whole rosette to the flowering organs during the reproductive phase did not differ between the lines. The proportion of ^{15}N assimilated during the vegetative phase of leaf development and mobilized to the seeds and flowering stems was not related to the leaf-senescence phenotype. For all the lines except RIL310 approximately 65% of the ^{15}N absorbed during the vegetative growth was remobilised to the seeds. The variation between the lines RIL045, RIL272, RIL083 and RIL232 was weak. In RIL310, the amount of nitrogen remobilised to the seeds was very low and this certainly is a result of the low fitness of this line. Using all the data available on the five lines, a good correlation was found between the partitioning of dry matter in the seeds and the partitioning of ^{15}N in the seeds. This indicated that the efficiency of N-remobilisation to the grain was certainly controlled by the respective sizes of the sink and source compartments. The RIL310, which is a late flowering line, has favored vegetative growth rather than reproduction.

As a result we conclude that leaf senescence influence NRE at vegetative stage only, emphasizes that leaf-senescence and rosette-senescence have to be distinguished as (i)

sequential senescence occurring during the vegetative rosette development and (ii) a monocarpic senescence that occurs at flowering stage. The existence of both sequential and monocarpic senescence in *Arabidopsis* depends on genotype and environment. Monocarpic senescence is more easily observed when plants are grown in long day photoperiod to favour flowering and is related to the whole rosette longevity rather than to individual leaf senescence symptoms (Levey and Wingler, 2005). Sequential leaf senescence observed during vegetative stage varies with genotypes and can be more easily observed when plants are grown in short-day (SD) photoperiod and for example under nitrogen limiting nutrition (Diaz et al., 2006).

Monocarpic and sequential senescence are certainly not controlled by the same genetic basis (Luquez et al., 2006) and do not have the same influence on NRE. Diaz et al. (2006) showed that the most important QTL to explain individual leaf senescence under limiting nitrogen nutrition co-localize with nitrogen use efficiency QTL measured at vegetative stage. In the present report, the absence of genetic variation for the N-remobilisation efficiency to the seeds questions the pertinence of searching for N-remobilisation QTLs in the Bay-0 x Shahdara population. Studying both traits using a population of accessions, or core collections of recombinant inbred lines, would be necessary to assess correlation between both traits.

Material and methods

Plant material and growth conditions for physiological study

The five lines RIL310, RIL272, RIL232, RIL083 and RIL045 selected from the Bay-0 x Shahdara RIL population have been previously described and characterized by Diaz et al. (2005). Plants were grown as described by Diaz et al. (2005), under nitrate limiting conditions and in short days (light/dark cycle 8 h/16 h), under photosynthetic photon flux density of 160 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$. The pots were watered three times per week, by immersion of the base of the pots, in a solution pH 5.5 containing 3 mM nitrate, (Diaz et al., 2005). About 20 days after sowing, the five RIL had formed six leaves plus cotyledons. Diaz et al. (2005) have observed that the time when the six first leaves emerged was similar for all the RIL, whereas the date of emergence of further leaves was different. As physiological studies require a lot of plant material, we decided to use the six first leaves (6FL) of the rosettes. For metabolic and molecular analysis, the six first leaves (6FL) of each rosette were dissected every 3 or 4 days and pooled. At each harvesting time, four different bulks of 6FL were harvested per RIL

between 10:00 and 11:00 AM, and stored at -80°C before further experiments. The remaining part of the rosette, called new leaves (NL), consisting of the young leaves that emerged after the 6FL, was also collected and stored.

Hydropony and ^{15}N tracing

Three different labeling experiments were performed in order to determine ^{15}N remobilisation (i) from leaf to leaf during the vegetative phase of rosette growth (Exp. 1), (ii) from rosette to flowering organs during the reproductive phase (Exp. 2) and (iii) for seed filling at the end of plant cycle (Exp. 3).

Exp. 1 and Exp. 2 were conducted using hydroponic grown plants.

Seeds were vernalized in agar 0.1% for 48 hours at 4°C . The seeds were then sown directly on the top of modified Eppendorf tubes (0.5 ml Eppendorf tubes which bottom was cut and top removed) filled with 0,8% agar (Kalys, Saint Ismier, France) melted in a dilution (1:1 with permutated water) of the ^{15}N nutritive solution. The composition of the ^{15}N nutritive solution was 0.25 mM KH_2PO_4 , 0.321 mM K_2SO_4 , 0.25 mM MgSO_4 , 0.1 mM K^{15}NO_3 (2.5% enrichment), 0.25mM CaCl_2 . The tubes were positioned on floating rafts transferred on diluted (1:1) ^{15}N nutritive solution in a growth chamber under the following environmental conditions: light/dark cycle 8 h/16 h, light intensity $160 \mu\text{mol s}^{-1} \text{m}^{-2}$, temperature $21^{\circ}\text{C}/17^{\circ}\text{C}$, and hygrometry 85%. After 1 week, the diluted (1:1) ^{15}N nutritive solution was replaced by full ^{15}N undiluted nutrient solution. The labelling period of time varied between Exp.1 and Exp.2 and ended with the time zero (T0) of harvest. Exp.1 was conducted on the five RIL while Exp.2 was only conducted on RIL272 and RIL083.

For Exp.1, T0 was 27 DAS, when rosettes have emerged 11 leaves (11FL). At T0, six plants were harvested to form roots (R) and 11FL labelled samples. For the six remaining plants, that have not been harvested and which will pass the chase period, all the leaves emerged during the pulse period, were marked, even if very small, with a small spot using white pen corrector to detect further the 11FL. At 27 DAS (T0), the ^{15}N nutritive solution was replaced by unlabelled ^{14}N nutritive solution with the same nutrient composition, except that K^{15}NO_3 have been replaced by K^{14}NO_3 . Before transferring plants on the unlabelled solution, roots and all material were carefully rinsed using permutated water. A second harvest was done at time T1, 37 DAS, when 6 to 7 new leaves (NL) were emerged during the chase period. Plants were dissected as roots, 11FL and NL. Exp.1 was carried out twice, on four replicates for the former, on six replicates for the second one.

In Exp.2, The ^{15}N and ^{14}N nutritive solutions were the same as in Exp.1. The end of the pulse period at T0 was 59 DAS, when RIL272 and RIL083 emerged concomitantly flowering buds. At T0, three labelled plants were harvested to form roots and whole-rosette T0 labelled samples. For the remaining plants, the ^{15}N nutritive solution was replaced by unlabelled ^{14}N nutritive solution. As for Exp.1, roots and all material were carefully rinsed using permutated water before transferring plants on the unlabelled solution. At time T1, 66 DAS, the roots, rosettes, and the newly appeared organs of three plants were dissected. The new appeared organs were separated as the primary (I) flowering stem, cauline leaves (I) on the primary flowering stem and the flowers (I) carried on the primary flowering stem. At time T2, 88 DAS, three remaining plants were dissected to form the roots, the rosette, the primary flowering stem (I), the secondary flowering stem (II), the cauline leaves and the siliques plus flowers carried on the flowering stem I and II.

Exp. 3 was carried out using plants grown on sand.

Seeds of the five RIL were vernalized in agar 0.1% for 48 hours at 4°C and sown on sand. Plants were grown in growth chamber under the following environmental conditions: light intensity $160 \mu\text{mol s}^{-1} \text{m}^{-2}$, temperature 21°C/17°C, hygrometry 85%, and light/dark cycle 8 h/16 h, for 56 days. After 56 DAS, plants were transferred to long days conditions (light/dark cycle 16 h/8 h) to induce flowering. The pots were watered three times per week, by immersion of the base of the pots, in a solution, pH 5.5, containing 3 mM nitrate, 2.75 mM potassium, 0.5 mM calcium, 0.7 mM chloride, 0.25 mM phosphate, 0.25 mM sulphate, 0.25 mM magnesium and 0.20 mM sodium. At two ^{15}N uptake time points, 40 DAS and 42 DAS, the unlabelled watering solution was replaced by ^{15}N containing solution that had the same nutrient composition except that $^{14}\text{NO}_3$ was replaced by $^{15}\text{NO}_3$ 10% enrichment. Plants were harvested at the end of their cycle when the total seeds were matured and the rosette dry. Samples were separated as (i) dry [rosette + stem + cauline leaves + siliques], named “stover” part and (ii) total seeds. Four replicates (plants) for each RIL were harvested and the experiment was carried out twice.

For all the experiments, unlabelled samples were harvested in order to determine the ^{15}N natural abundance.

Determination of total nitrogen content and ^{15}N abundance and partition

In all the labelling experiment, after drying and weighting of each plant part, material was ground to obtain homogenous fine powder. A sub-sample of 1000 to 2000 μg was

carefully weighted in tin capsules to determine total N content and ^{15}N abundance using an elemental analyzer (roboprep CN, PDZ Europa Scientific Ltd, Crewe, UK) coupled to an isotope ratio mass spectrometer (Twenty-twenty, PDZ Europa Scientific Ltd, Crewe, UK) calibrated measuring natural abundance. The ^{15}N abundance of samples was calculated as atom percent and defined as $A\% = 100 \cdot (^{15}\text{N}) / (^{15}\text{N} + ^{14}\text{N})$ for labeled plant samples and for unlabelled plant controls ($A_{\text{control}}\%$ was ca. 0.3660). The ^{15}N enrichment of the plant material was then defined as $(A_{\text{sample}}\% - A_{\text{control}}\%)$. The absolute quantity of ^{15}N contained in the i sample was defined as $Q_i = \text{DW}_i \cdot (A_i\% - A_{\text{control}}\%) \cdot \%N_i$ with $\%N_i$ the proportion of nitrogen in the i sample. The Q value of the whole plant (wp) can be measured and $Q_{\text{wp}} = \sum Q_i$. The partition $P\%$ of ^{15}N in the organ i was calculated as $\%P_i = [(A_i\% - A_{\text{control}}\%) \cdot \text{DW}_i \cdot \%N_i] / [(A_{\text{wp}}\% - A_{\text{control}}\%) \cdot \text{DW}_{\text{wp}} \cdot \%N_{\text{wp}}] = Q_i / Q_{\text{wp}}$.

Metabolite extraction and analysis

Amino acids and NH_4^+ were determined after extraction in 2% solution of 5-sulfosalicylic acid (50 mg FW. ml^{-1}) by the Rosen colorimetric method using glutamine as a reference (Rosen, 1957). Sugars were determined after ethanolic extraction (Loudet et al., 2003). Sucrose and hexoses were determined as described by Masclaux-Daubresse et al. (2002) in supernatants. Starch content was determined from pellets (Masclaux-Daubresse et al., 2002).

Enzymatic assays

Enzymes were extracted from frozen leaf material stored at -80°C . Nitrate reductase was extracted and the maximal extractable activity measured as described by Ferrario-Méry et al. (1997). Glutamine synthetase (GS) was measured according to the method of O'Neal and Joy (1973). The NADH- and NAD^+ -dependent glutamate dehydrogenases (NADH-GDH and NAD^+ -GDH) were measured as described by Turano et al (1996), except that the extraction buffer was the same as for GS.

Chlorophyll and total protein determinations

Chlorophyll content was determined in crude leaf extracts used for GS activity (Arnon, 1949). Soluble protein content was determined in crude leaf extracts used for GS activity using a commercially available kit (Coomassie Protein assay reagent, BioRad, Hercules, California, USA).

Gel electrophoresis, western blot analysis and gel staining procedure

Proteins were extracted in Hepes (50 mM), MgCl₂ (2 mM), EDTA (0.5 mM), DTT (2mM) and triton X100 0.1% buffer. Proteins from three different extracts were pooled and separated by SDS-PAGE (Laemmli, 1970). The percentage of polyacrylamide in the running gels was 8% for GS and 12% for Rubisco and CND41. An equal amount of protein (10 µg for GS, 20 µg for CND41 and 6 µg for Rubisco) was loaded in each track. Denaturated proteins were electrophoretically transferred to nitro-cellulose membranes or directly stained with Comassie blue for Rubisco detection. Polypeptide detection was performed using polyclonal antiserum raised against tobacco GS2 (Becker et al., 1992) and CND41 (Kato et al., 2004). Relative GS2, GS1, CND41 and Rubisco protein amounts were determined by densitometric scanning of Western blot membranes or stained gels and quantification using the Fujifilm FLA5000 phospho-imager and the MultiGauge Fujifilm image analyser (Fujifilm, Saint Quentin en Yvelines, France).

Extraction of total RNA and northern blot analysis

Total RNA was extracted from plant material stored at –80°C. Northern blot analysis was performed as described previously (Masclaux et al., 2000). 32P-labelled probes were used for mRNA detection. Specific probes raised against *gln2* (*At5g35630*), *gln1.1* (*At5g37600*) and *gln1.2* (*At1g66200*) were obtained through PCR amplification as described by Oliveira and Corruzzi (1999). Specific probe raised against *gdh2* (*At5g07440*) was obtained using the 1,4 kb cDNA *Sall/EcorI* inserts purified from the 188II23T7 EST clone provided by the *Arabidopsis Biological Resource Center* (ABRC, Columbus Ohio, USA). The *gad2* (*At1g65960*) specific probes were kindly provided by Dr Nicolas Bouché (INRA, Versailles, France) and obtained from the cognate ABRC EST vectors after digestion by *KpnI/HindIII* restriction enzymes and purification of cDNA. The *CAB* probe was kindly provided by Dr Dolores Abarca (Abarca et al., 2001). The 18S rRNA probe was the same as used by (Masclaux et al., 2000). High stringency hybridization conditions were used for all the probes.

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Legends of figures

Figure 1:

Comparison of the dry weight (DW, mg per plant) of the six first leaves 6FL (white bars), new emerging leaves NL (black bars), and flowering stems FS (hashed bars) of RIL310, RIL083, RIL232, RIL045 and RIL272. Means and standard errors are presented, n=4 to 6. The arrows indicate the flowering time.

Figure 2:

Total chlorophyll (A and C) and starch (B and D) concentrations in the six first leaves (6FL, A and B) and the new leaves (NL, C and D) of RIL310 (white square), RIL083 (white circle), RIL232 (white triangle), RIL045 (black circle) and RIL272 (black square). Means and standard errors are presented, n=3.

Figure 3:

Total nitrogen concentration (A and B), soluble protein concentration (C and D) and free amino acid concentration (E and F) in the six first leaves (6FL, A, C and E) and the new leaves (NL, B, D and F) of RIL310 (white square), RIL083 (white circle), RIL232 (white triangle), RIL045 (black circle) and RIL272 (black square). Means and standard errors are presented, n=3.

Figure 4:

Determination of nitrate reductase (A), glutamine synthetase (B) and NADH- (C) and NAD- (D) dependent glutamate dehydrogenase in extracts of the six first leaves (6FL, A, C and E) and of the new leaves (NL, B, D and F) of RIL310 (white square), RIL083 (white circle), RIL232 (white triangle), RIL045 (black circle) and RIL272 (black square). Means and standard errors are presented, n=3.

Figure 5:

Analysis of the GS2 and GS1 protein content (A), Rubisco protein content (B) and CND41-like protein content (C) in extracts of the six first leaves of the five RIL. Western blots and gel staining are presented. Quantifications of the signals and relative values are shown for RIL310 (white square), RIL083 (white circle), RIL232 (white triangle), RIL045 (black circle)

and RIL272 (black square). Equal amount of proteins have been loaded in each lane (see Material and Methods).

Figure 6:

Changes in the steady state levels of transcripts for the chlorophyll a/b binding protein (*cab*) senescence marker, NR mRNA (*nia*), GS2 mRNA (*gln2*), GS1 mRNA (*gln1.1* and *gln1.2*), glutamate decarboxylase mRNA (*gad2*) and GDH mRNA (*gdh2*). Each samples contained 10 µg total RNA, and both ethidium-bromide-stained gels and post hybridisation with a 18 S rRNA probe were used as a control for loading standardisation (not shown).

Figure 7:

Change of the partitioning of ^{15}N in the roots (A), 11 first leaves (11FL, B) and newly appeared leaves (NL, C) between T0 (dark bars, end of the labelling period) and T1 (grey bars, T0 + 10 days). Calculation of partitioning is described in Material and Methods. Values are the means of 6 individual plants, standard errors are presented. The % of nitrogen mobilised from roots and 11FL to the NL between T0 and T1 is mentioned on the graph, on the top of the bars. The probability that the the differences between T0 and T1 (between dark and grey bars) was not significant was estimated using ANOVA and is indicated as such * $Pr < 0.05$, ** $Pr < 0.01$, *** $Pr < 0.001$.

Tables

Table 1: Evolution of the partitioning of ^{15}N , $\text{P}\%(^{15}\text{N})$ in the different organs of early senescing line RIL083 and the late senescing line RIL272 between the end of the labelling period (T0, 59 DAS) and two time intervals of chase (T1, 66 DAS and T2, 88 DAS). P% was calculated as described in Material and Methods and permitted the determination of the % of ^{15}N mobilized from the labelled rosettes and roots, to the new organs that have appeared between T0 and T1 and between T1 and T2. Values are the means and standard errors of three plants.

	RIL083			RIL272		
	<i>Partitioning of ^{15}N P%(^{15}N)</i>		<i>% of ^{15}N mobilized from</i>	<i>Partitioning of ^{15}N P%(^{15}N)</i>		<i>% of ^{15}N mobilized from</i>
Time 0 (flowering bud)	Mean	\pmSD		Mean	\pmSD	
Roots	20.2%	\pm 1.0%		25.25%	\pm 7.1%	
Rosette	79.2%	\pm 1.0%		74.75%	\pm 7.0%	
Time 1 (7 days after f.b.)			<i>between T0 & T1</i>			<i>between T0 & T1</i>
roots	20.2%	\pm 1.8%	0%	25.5%	\pm 2.2%	0%
rosette	66.6%	\pm 1.3%	12,6%	62.1%	\pm 0.9%	12,6%
flowering stem I	2.7%	\pm 1.6%		3.3%	\pm 1.2%	
cauline leaves I	2.6%	\pm 0.6%		2.6%	\pm 1.0%	
flowers I	7.73%	\pm 5.3%		7.3%	\pm 3.0%	
Time 2 (1 month after f.b.)			<i>between T1 & T2</i>			<i>between T1 & T2</i>
roots	18.7%	\pm 0.8%	1,5%	24.1%	\pm 1.8%	1,4%
rosette	42.6%	\pm 0.6%	24%	37.2%	\pm 4.9%	24,9%
flowering stem I	6.7%	\pm 0.7%		7.8%	\pm 0.9%	
flowering stem II	5.0%	\pm 2.0%		9.0%	\pm 4.0%	
cauline leaves	13.0%	\pm 0.7%		10.0%	\pm 3.0%	
siliques and flowers	15.0%	\pm 1.0%		10.0%	\pm 1.0%	

Table 2: Comparison of the partitioning of the dry weight (DW, mg per plant), the total nitrogen content (total N, mg per plant) and the total ¹⁵N content (¹⁵N, µg per plant) of all the compartments (seeds, stover and whole plant). The partitioning P% (in % of the whole plant) of the DW, the total N and the ¹⁵N of the seed compartments of the five RIL have been calculated from 4 individual plant repeats. For all the data, means and standard errors, n=4, are presented.

	DW (mg)			total N (mg)			¹⁵ N (µg)			P% in the seeds (as % of the whole plant)		
	Stover	Seeds	WP	Stover	Seeds	WP	Stover	Seeds	WP	P%(DW)	P%(N)	P%(¹⁵ N)
RIL310	756 ± 184	43 ± 19	778 ± 173	11.56 ± 3.0	1.58 ± 0.7	12.35 ± 2.53	53.5 ± 10.0	2.7 ± 3.6	56.2 ± 6.7	3.2 ± 4.5	14.4 ± 10.6	5.4 ± 7.5
RIL083	382 ± 70	231 ± 51	670 ± 51	3.3 ± 0.3	7.8 ± 1.9	11.13 ± 2.00	15.6 ± 2.9	32.0 ± 4.6	47.7 ± 4.0	37.5 ± 2.4	69.1 ± 6.5	67.1 ± 6.0
RIL232	577 ± 56	235 ± 15	812 ± 57	4.45 ± 0.6	8.0 ± 0.7	12.46 ± 0.94	16.78 ± 1.8	29.2 ± 2.9	46.0 ± 2.0	29.0 ± 2.5	64.3 ± 3.6	63.4 ± 4.5
RIL045	618 ± 123	200 ± 59	819 ± 66	7.4 ± 3.8	7.0 ± 1.8	14.4 ± 2.46	17.7 ± 7.7	30.9 ± 9.8	48.5 ± 9.6	25.0 ± 8.7	50.6 ± 16.5	63.64 ± 14.6
RIL272	512 ± 44	155 ± 19	767 ± 62	4.2 ± 0.3	9.3 ± 0.8	13.55 ± 0.95	15.3 ± 2.0	34.6 ± 7.9	49.9 ± 10.7	33.2 ± 1.0	68.5 ± 1.6	69.1 ± 2.2











