Transcriptome analysis of *Medicago truncatula* leaf senescence: similarities and differences in metabolic and transcriptional regulations as compared with *Arabidopsis*, nodule senescence and nitric oxide signalling

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Summary

- Here, for the first time, a comprehensive transcriptomics study is presented of leaf senescence in the legume model *Medicago truncatula*, providing a broad overview of differentially expressed transcripts involved in this process.
- The cDNA-amplification fragment length polymorphism (AFLP) technique was used to identify > 500 genes, which were cloned and sorted into functional categories according to their gene ontology annotation.
- Comparison between the datasets of *Arabidopsis* and *M. truncatula* leaf senescence reveals common physiological events but differences in the nitrogen metabolism and in transcriptional regulation. In addition, it was observed that a minority of the genes regulated during leaf senescence were equally involved in other processes leading to programmed cell death, such as nodule senescence and nitric oxide signalling.
- This study provides a wide transcriptional profile for the comprehension of key events of leaf senescence in *M. truncatula* and highlights a possible regulative role for MADS box transcription factors in the terminal phases of the process.

Introduction

Senescence is the last stage of development of an organ. In leaves, this process is accompanied by a gradual loss of chlorophyll with consequent yellowing and degradation of proteins, lipids and nucleic acids (reviewed by Buchanan-Wollaston *et al.*, 2003). Leaf senescence is a slow process, which has been divided into three steps: initiation, execution (regulated degenerative process) and terminal phases (Yoshida, 2003). During degeneration, cell components are degraded and the resulting nutrients are redirected to other plant organs. Senescing tissues eventually die by programmed cell death (PCD). Despite some unique features of senescence among the various types of plant PCDs, such as the slowness of the process and the recycling of nutrients, the terminal phase of senescence shares some similarities with other PCD events. Both senescence and hypersensitive response, for example, are

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characterized by an increase in reactive oxygen species (ROS) and nitric oxide (NO) production, which are signal molecules involved in cell death (Carimi *et al.*, 2003, 2005; Zimmermann & Zentgraf, 2005; Zaninotto *et al.*, 2006). Being a genetically controlled process, leaf senescence is transcriptionally active; despite a general decrease of the RNA concentrations, the expression of a set of genes, generally referred to as senescence-associated genes (SAGs), is enhanced during the various stages of senescence (Gan & Amasino, 1997). An estimated 10% of the total genes are expected to be SAGs (He *et al.*, 2001) and many of them encode catabolic enzymes, transcription factors or components of signalling pathways. As senescence is a degenerative process involving several aspects of cell metabolism, large-scale expression studies are particularly indicated to identify *SAG*s and unravel important regulatory networks. In the past few years, several genome-wide transcriptional analyses of leaf senescence were conducted in *Arabidopsis* (Swidzinski *et al.*, 2002; Buchanan-Wollaston *et al.*, 2003; Gepstein *et al.*, 2003; Guo *et al.*, 2004; Lin & Wu, 2004), aspen (Bhalerao *et al.*, 2003; Andersson *et al.*, 2004), wheat (Gregersen & Holm, 2007) and barley (Jukanti *et al.*, 2008).

Understanding the senescence mechanism might lead to agronomical improvements, for example, increment of biomass or a delay in post-harvest deterioration of crops. Legumes represent major components in human and animal nutrition because of the high protein content of their seeds and leaves. Senescence studies are therefore potentially important in these plants, where key events of the process are a massive degradation of proteins and a consequent reallocation of nitrogen to grains. *Medicago truncatula* is a model organism for legumes; its genome is undergoing sequencing, and large collections of ESTs are available. Because of the macro- and microsynteny among legumes (Young *et al.*, 2005), information obtained in this species is likely to be easily transferred to agronomically important crops. *M. truncatula* is closely related to alfalfa (*M. sativa*), the most widely cultivated forage legume, and, as such, is a good candidate for the study of senescence in the Fabaceae family. In a previous article, we identified morphological and biochemical features associated with senescence in *M. truncatula* cell cultures (Zottini *et al.*, 2006). Recently, a transcriptional analysis of nodule senescence was performed in this species (Van de Velde *et al.*, 2006). To date, however, a study of the transcriptome of legume senescing leaves is lacking.

We present, for the first time, a large-scale gene identification study of leaf senescence in *M. truncatula*, providing a broad overview of differentially expressed transcripts involved in this process. We used a modified cDNA-amplified fragment length polymorphism (AFLP) analysis (Breyne *et al.*, 2003). This technique does not require pre-existing information at the molecular level, thus allowing a comprehensive study of phenomena in species such as *M. truncatula* for which complete genome platforms are not yet available. This technique, being PCR-based, is also able to detect low abundant transcripts, such as the ones encoding transcription factors. Since the cDNA-AFLP technique was already used successfully in *M. truncatula* for studying nodule senescence (Van de Velde *et al.*, 2006) and the nitric oxide signalling pathway (Ferrarini *et al.*, 2008), we used these datasets to make a direct comparison of the genes differentially expressed in various processes leading to PCD: leaf, nodule senescence and nitric oxide pathway. The genes identified in this study were annotated and sorted into gene ontology categories. Comparison with *Arabidopsis thaliana* leaf senescence revealed differences between the two species in the transcription factor families involved and the mechanism of nitrogen mobilization, which we investigated in detail.

Materials and Methods

Plant material and growth conditions

After scarification, seeds of *Medicago truncatula* L. cv. Jemalong (genotype 2HA) were placed at 4°C for 4 d in about three volumes of distilled water. For germination, seeds were transferred to Petri dishes containing wet filter paper and placed in the dark at room temperature for 3–4 d. Young seedlings were then transferred to 18-cm-diameter pots in a potting mixture prepared with commercially available garden soil and sand (1 : 1, v/v). Plants were grown in a Conviron PGR-15 growth chamber (Controlled Environments Limited, Winnipeg, MB, Canada) at 23 : 20°C day : night temperatures, at 60–70% relative humidity, with a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1} and a 16 : 8 h photoperiod. The plants were irrigated when necessary. Leaves of different developmental stages were harvested from 10 nodulated plants and immediately frozen in liquid nitrogen.

Chlorophyll quantification

Fifteen milligrams of leaves were ground in liquid nitrogen and chlorophylls were extracted with 1.9 ml of absolute ethanol in the dark by agitation for 30 min. After a brief centrifugation, supernatants were collected (modified from Weaver & Amasino, 2001). Concentrations of chlorophylls were determined by measuring their absorbance, as in Wintermans & DeMots (1965). Reactions were conducted in triplicate and averaged.

RNA extraction and cDNA-AFLP analysis

RNA from leaves at different stages was extracted using the TriZol reagent (Invitrogen, Carlsbad, CA, USA) and quantified spectrophotometrically.

The cDNA-AFLP analysis was performed as in Breyne *et al.* (2003). Briefly, total RNA from mature green (MG) leaves and a pool of leaves of three senescent (S) stages (each

represented by an equal amount of fresh weight) was reverse-transcribed using the Superscript II reverse transcription kit (Invitrogen) according to the manufacturer's instructions. Second-strand synthesis was performed by strand displacement with *Escherichia coli* ligase, DNA polymerase I and RNase H (Invitrogen). cDNA was purified with the aid of a QIAquick PCR purification kit (Qiagen, Germantown, MD, USA) and digested with the restriction enzymes BstYI and MseI; fragments were then ligated to adaptors and amplified by PCR. Adapter and primer sequences were as in Breyne *et al.* (2003). Primers spanned the adapter region and the cutting site of the restriction enzymes. The BstYI primers were characterized by a selective pyrimidine nucleotide at its 3′ end, thus reducing the original cDNA complexity by twofold. A second PCR amplification was then performed, using the same primers as before plus a selective nucleotide at the 3′ end (+1, +1). BstYI primers were tagged with [^{33}P]ATP for autoradiographic detection of bands. A total of 32 different combinations were therefore employed, which allowed a clear band separation on a 6% polyacrylamide gel in a 33 × 61 cm vertical electrophoresis apparatus (Genomyx, Foster City, CA, USA) by running for 3 h at 2700 V, 150 W at 50°C. The gel image was acquired by a gel scanner apparatus (Genomyx, Foster City, CA, USA). Band intensities were quantified by ImageJ software (NIH, Bethesda, MD, USA). The raw data were corrected for differences in total lane intensities. To that end, the total intensity value of the S lane was divided by the value of the MG lane, for each primer combination, to yield the correction factors. Subsequently, all raw data were divided by these correction factors. Next, the corrected band intensities of the S lane were divided by the corresponding intensities of the MG lane, to yield an S : MG ratio. Differentially expressed bands with an S : MG ratio greater than 1.1 or lower than 0.91 (cut-off = 10%) were excised from the gel, using a virtual grid (Genomyx) super-imposed on the gel image for the precise location of the band in the gel. The rather small 10% cut-off was chosen in order to recover those transcripts expressed only in a short temporal window late in senescence. This was necessary as the pooling strategy under-represented the final stages, when the RNA content was especially low.

The cDNAs were eluted from the bands in 100 μl sterile distilled water, and an aliquot of 5 μl was used as template for reamplification with nonlabelled primers identical to those employed for selective AFLP amplification. PCR products were cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA) and three different *Escherichia coli* clones were collected for each tag. All of the clones were then sequenced by the dideoxy termination method (Sanger *et al.*, 1977) at BMR Genomics (Padova, Italy).

Bioinformatic analysis

cDNA sequences were manually checked for overall quality. Vector and primer contamination were trimmed using a custom-made tool based on Blast (Altschul *et al.*, 1997) vs the UniVec Database (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html). After this first screening, sequences were clustered based on Megablast (Zhang *et al.*, 2000) and then assembled using CAP3 (Huang & Madan, 1999) to produce longer and more complete consensus sequences. Sequences coming from the same gel band, but belonging to different assemblies, were discarded in a semi-automated way and manually checked. The remaining sequences were compared against the Release 8 of *M. truncatula* tentative consensus sequences (*Medicago truncatula* Gene Index, MTGI, http://compbio.dfci.harvard.edu/tgi/) to get longer sequences. Before proceeding with the *in silico* extension of the genes, the coding sequences extracted from MTGI were further screened to remove vector contamination and clustered with CD-HIT (Li & Godzik, 2006) at 95% of identity cut-off to reduce redundancy. Gene ontology (Ashburner *et al.*, 2000) annotation was performed using an in-house automatic procedure (http://genomics.research.iasma.it/argot/index.html, Velasco *et al.*, 2007) based on thorough analysis of Blast results vs Uniprot database (release 9.3) (Schneider *et al.*, 2005) and semantic similarities among GO terms associated with the hits found. The annotation was validated for every single sequence of the starting subset and the functional classifications were carried out on the basis of the GO plant slim (http://www.geneontology.org/GO.slims.shtml release of January 2007) and manually refined. Highly similar *Arabidopsis* hits with a Blast E-value < $1e^{-5}$ are reported in the Supporting Information, Table S1, which shows all genes considered in this work with their annotation and expression level. Sequences from nodule senescence (Van de Velde *et al.*, 2006) and nitric oxide treatment (Ferrarini *et al.*, 2008) were associated to our dataset using the clustering/assembly approach (see earlier and Tables S1, S5, and S6).

Relative-quantitative RT-PCR

Total RNAs extracted from leaves at different stages were treated with DNAseI (Ambion, Foster City, CA, USA). First-strand cDNA was synthesized using the PowerScript Reverse Transcriptase (Clontech, Mountain View, CA, USA) and Random Decamers (Ambion) and diluted 1 : 5.

Relative-quantitative reverse transcription (RT)-PCR was carried out with 5 μl of first-strand cDNA using the 18S rRNA as an internal standard (QuantumRNA 18S Internal Standards Kit; Ambion). The 18S primers : competimers ratios, the number of PCR cycles and the specific primers for each gene are listed in Table S2. The cycling parameters were as follows: 20 s at 94°C, 20 s at 66°C and 30 s at 72°C. Taq DNA polymerase by Eppendorf (Hamburg, Germany) was used. Densitometric analysis of ethidium bromide-stained agarose gels (0.5 μg ml^{-1}) was performed using ImageJ software (NIH, USA). The relative abundance of the transcript within the samples was calculated as the ratio of the intensities of the gene amplicon relative to the 18S rRNA amplicon. The

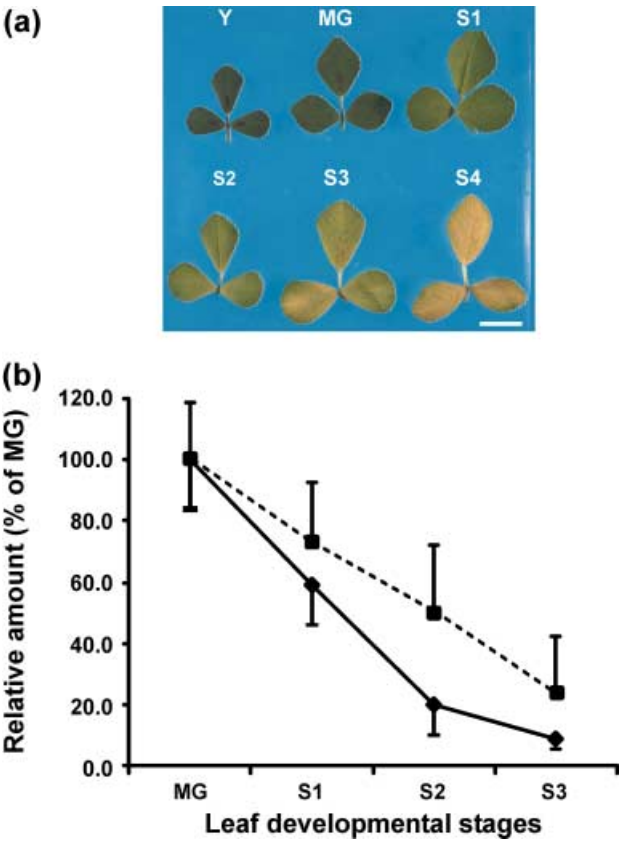

Fig. 1 Progression of leaf senescence in *Medicago truncatula*. (a) Stages of leaf senescence: Y, young leaf; MG, mature green leaf; S1, *c.* 40% loss of chlorophyll; S2, *c.* 80% loss of chlorophyll; S3, > 90% loss of chlorophyll; S4, precedes abscission. (b) Changes in the relative amount of chlorophyll (Chl; diamonds, solid line) and RNA (squares, dashed line) during leaf senescence. All values are relative to those of MG leaves, set as 100%. Data presented are mean values ± SD of three independent analyses of pool of leaves collected from 10 plants. Bar, 1 cm.

resulting values of the S samples were then divided by the value of the MG sample. Reactions were performed in triplicate and averaged.

Results and Discussion

Phenology of *M. truncatula* leaf senescence

Following a previous paper on *Arabidopsis* by Lohman *et al.* (1994), we divided *M. truncatula* leaf development into stages based on morphological appearance, chlorophyll and RNA contents (Fig. 1a,b). However, leaf senescence in *M. truncatula* appears to be more uniform than in *Arabidopsis*, at least under our growing conditions; therefore, a subdivision of leaf senescence in more than four phenological stages did not seem feasible.

Young, small and hairy leaves (Y, 20 d after sowing (das)) develop into a mature green stage (MG, 30 das) characterized by the highest amount of chlorophyll. As senescence starts, leaf colour becomes uniformly paler (S1, 40 das), and eventually yellow zones appear, especially in the outer areas of the leaves (S2, 60 das, and S3, 80 das). The last stage of senescence (S4, 100 das) is characterized by a complete yellowing of the leaf blade, and it precedes abscission and death of the tissue (Fig. 1a). During the entire senescence process, chlorophyll and RNA contents gradually drop (Fig. 1b), as observed in *Arabidopsis* (Lohman *et al.*, 1994). Because of the dramatic loss in RNA content in S4, here we present S3 as the last analysable stage of leaf development.

cDNA-AFLP transcriptome analysis and gene ontology

In order to isolate differentially expressed genes, the transcriptome of mature, green leaves (sample MG) was compared with the one derived from a pool (sample S) of three senescent stages (S1, S2 and S3) using a modified cDNA-AFLP technique (see the Materials and Methods section).

Among *c.* 6000 gel bands revealed by the analysis, 846 showed differential expression and were excised and cloned. After selection with stringent criteria (see the Materials and Methods section), 545 sequence clusters were retained. We will refer to these clusters as genes for simplicity.

To verify the data obtained with cDNA-AFLP analysis, the expression of a subset of 10 genes, randomly chosen among the differentially expressed genes, was monitored by relative-quantitative RT-PCR. In all cases, the expression profiles were consistent with the results obtained with cDNA-AFLP analysis (Fig. 2).

The 545 genes were then analysed for functional annotation. Genes were clustered with tentative consensus sequences of the *M. truncatula* Gene Index database (see the Materials and Methods section) to obtain longer sequences and increase the probability of including coding sequences. Blastx searches were performed against the UniProt databank with default E-value cut-offs. The results were parsed and automatically analysed to obtain functional information (see the Materials and Methods section) and manually checked for every hit. The extracted biological process terms of the gene ontology were retrieved for *c.* 62% of the whole subset. The similarity with *Arabidopsis* exceeds 56%, showing that it is possible to find a high conservation among the proteins of these two plants despite the phylogenetic distance between them and the stringent criteria used. The 545 genes, split into 346 senescence-enhanced and 199 repressed genes, were grouped into 23 functional categories (Fig. 3) according to a gene ontology classification (Table S1). For 21.1% (115) of the genes (13.4% up-regulated and 7.7% down-regulated), no significant sequence similarity was found; these genes fall in the category 'no hit' (Fig. 3). The sequences with a certain identity with genes of unknown function (*unknown* in Fig. 3) comprise 19.4% (106), of which 11.7% are up-regulated and 7.7% are down-regulated.

Id	Category	cDNA-AFLP	RT-PCR	cDNA-AFLP S	cDNA-AFLP MG	RT-PCR S	RT-PCR MG
16-2	Biological regulation	1.67	1.24 ± 0.11				
78-2	Catabolic process	0.44	0.42 ± 0.06				
113-1	Unknown	2.16	1.83 ± 0.26				
146-3	Photosynthesis	0.62	0.18 ± 0.08				
221-2	Protein modification process	0.47	0.20 ± 0.03				
273-2	Metabolic process	1.82	1.94 ± 0.07				
363-2	Unknown	1.63	1.12 ±0.01				
482-2	Cellular component organization and biogenesis	0.31	0.2 ± 0.04				
637-2	Metabolic process	0.54	0.21 ± 0.01				
639-2	Carbohydrate metabolic process	1.69	2.17 ± 0.1				

Fig. 2 Expression profiles of 10 randomly selected genes. The cDNA-AFLP and the RT-PCR expression values are expressed as the ratio of the corrected values S : MG, as explained in the Materials and Methods section. RT-PCR values are means ± SD of three independent RT-PCRs. The RT-PCR gel blots shown are representative; in each blot, the upper band is 18S rRNA, used as internal control, and the lower band is the specific amplicon.

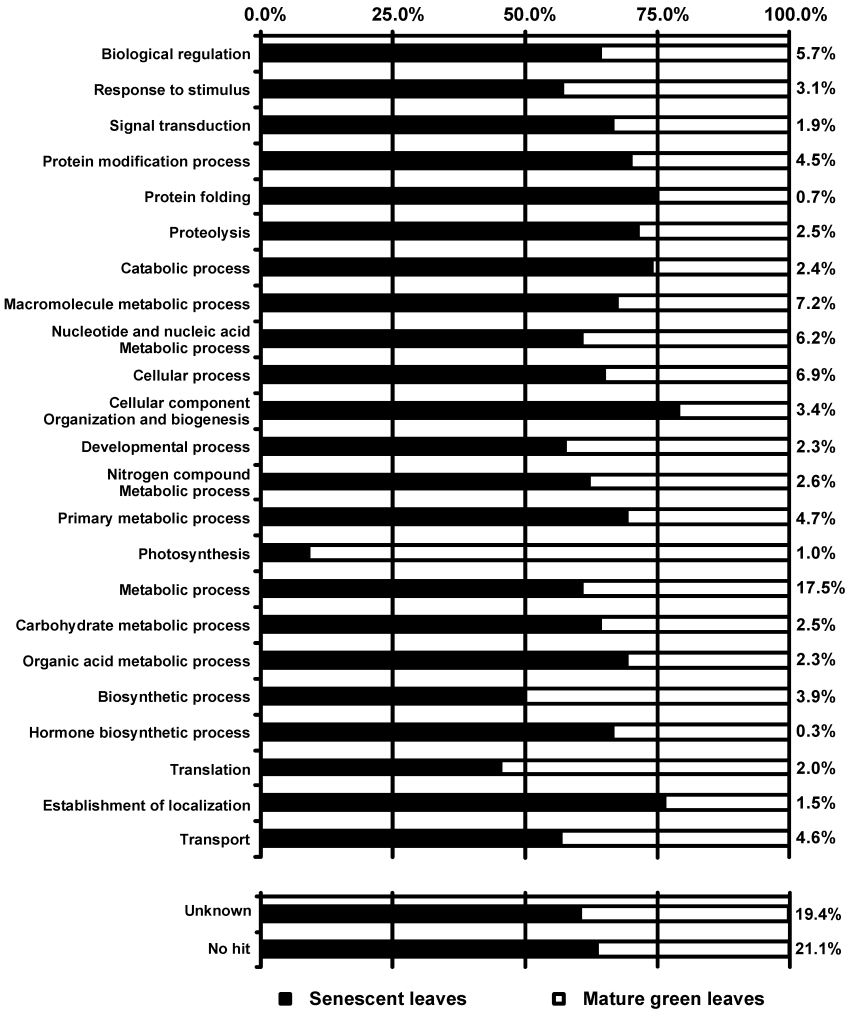

Fig. 3 Distribution of genes in the Gene Ontology (GO) biological process categories. The percentages shown on the right indicate the abundance of each category within the whole dataset; the percentages in the upper part of the diagram and the lengths of the bars represent the relative abundance of genes induced (black bars) or repressed (white bars) during leaf senescence for each category. Genes with unknown function (unknown) and those with no homology to any sequence (no hit) are also shown, although they do not fit into any GO category.

Interestingly, 131 genes of the dataset (24%) have no similarity with any known *M. truncatula* transcript. Among them, only 36 have an associated gene ontology term, whereas 95 are completely unknown.

The large number of novel genes isolated in this work supports once again the validity of the cDNA-AFLP approach to study the transcriptome of organisms, such as *M. truncatula*, whose genome is not yet fully sequenced. This result also

indicates that the microarray platforms available for this species, based on ESTs sets and incomplete genomic sequence, are not the ultimate tool for global expression surveys.

Medicago truncatula leaf senescence is similar to *Arabidopsis* but shows peculiarities in the regulation and execution of the terminal phase

The molecular basis of leaf senescence has been extensively studied in *Arabidopsis*. Recently, a comprehensive large-scale transcriptional analysis identified 827 genes up-regulated more than threefold in senescent leaves (Buchanan-Wollaston *et al*., 2005). The comparison with our *M. truncatula* dataset revealed 150 (27.5%) matches (Fig. 4a, Table S3), which were grouped on the basis of their annotation and presented in Table 1. Additionally, *c*. 30% (43) of these 150 common genes showed discrepant expression, meaning that they are induced in *Arabidopsis* but repressed in *M. truncatula*. Among the remaining 395 clusters showing no similarity with genes in the *Arabidopsis* dataset (Fig. 4a), 89 (23%) are annotated as 'unknown' and 115 (29%), classified as 'no hit', correspond to expressed sequences with no similarity to proteins in the Uniprot database (Table S3). Senescence in legumes might therefore differ in some metabolic pathways with respect to other plant families, and more extensive studies are recommended in order to characterize these newly isolated legume-specific players.

Nevertheless, it seems that, as a general process, leaf senescence is similar in *Arabidopsis* and *M. truncatula*, as the same functional categories are affected (Fig. 3, Table 1): signal transduction and protein modification (41 genes are common, equal to 57.7% of the members in the categories); response to stimulus (19, 54.3%); biological regulation (27, 42.2%); catabolic process and proteolysis (26, 47.3%). Among them, typical senescence players are represented. These include the genes encoding no apical meristem and cup-shaped cotyledon (NAC)-like transcription factors (Guo *et al*., 2004), cysteine proteases, lipases, ribonucleases, glutathione-S transferases (GST), translation initiation factor 5A-1 (Thompson *et al*., 2004), phosphatases 2C, 26S proteasome components and autophagy-related proteins. To better define the temporal involvement of some of these markers, we evaluated their expression pattern by RT-PCR along the developmental stages (Fig. S1). Two cysteine proteases (TC100972 and TC106627), two NAC-like transcription factors (TC96387 and TC95634), the ERF-like transcription factor TC109441, and the translation initiation factor 5A (TC94697) all showed an increase of the transcript level, particularly at the last stage of leaf development. Therefore, these genes can truly be considered SAGs in *M. truncatula* too. The category 'photosynthesis', on the other hand, is under-represented (Fig. 3); this result was expected because of the dramatic decrease of the transcripts related to this process and chloroplast degradation during senescence (Buchanan-Wollaston *et al*., 2003).

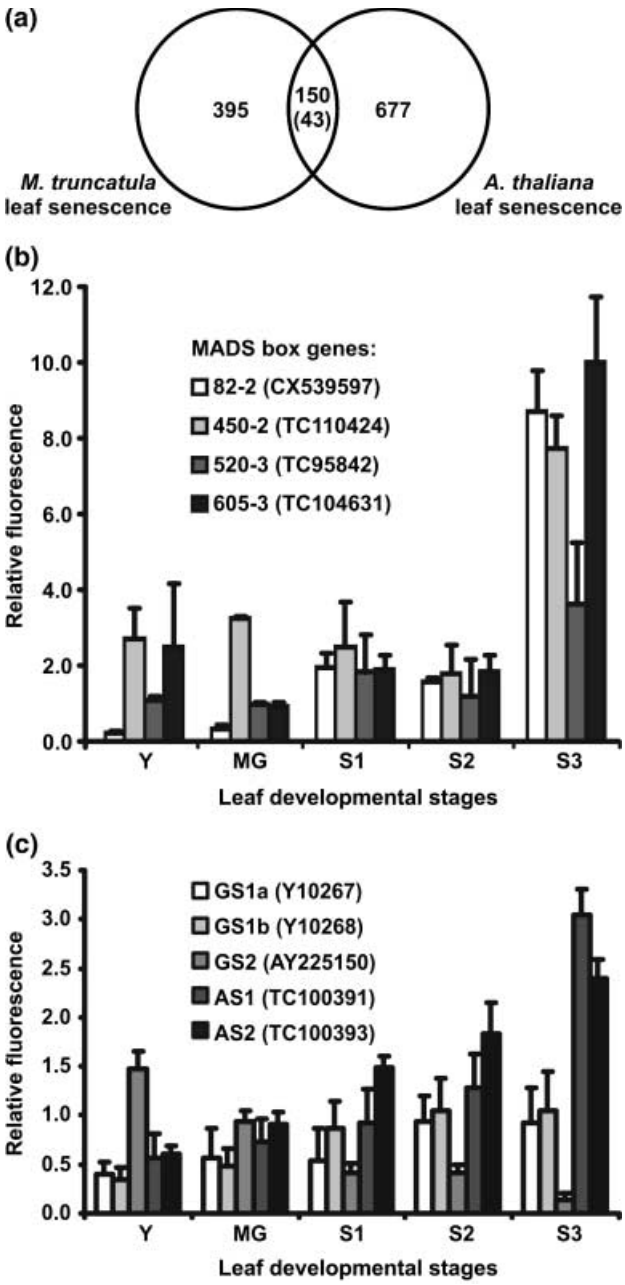

Fig. 4 Comparison with *Arabidopsis* leaf senescence. (a) Venn diagram of distribution of genes among leaf senescence in *Medicago truncatula* (induced and repressed genes, listed in Table S1) and *Arabidopsis thaliana* (threefold induction in Buchanan-Wollaston *et al*., 2005). Numbers in brackets are *M. truncatula* leaf genes with discordant expression patterns compared with the *Arabidopsis* dataset. (b) Expression patterns of the four MADS box transcription factors genes (CX539597, TC110424, TC95842, TC104631) during different leaf developmental stages. (c) Expression patterns of the three glutamine-synthetase (*GS1a*, *GS1b*, *GS2*) and the two asparagine-synthetase (*AS1*, *AS2*) genes during different leaf developmental stages. Bars represent the amount of transcripts measured by densitometric analysis of relative-quantitative RT-PCR products. Values are means ± SD of three independent RT-PCRs. Y, young leaf; MG, mature green leaf; S1, *c*. 40% loss of chlorophyll; S2, *c*. 80% loss of chlorophyll; S3, > 90% loss of chlorophyll.

Table 1 Comparison between *Medicago truncatula* and *Arabidopsis thaliana* leaf senescence datasets

Category	% common members[a] (no. of genes)	% concordant[b] (no. of genes)	% discordant[c] (no. of genes)	Examples[d]
Biological regulation	48.4 (31)	32.8 (21)	15.6 (10)	Transcription factors (MYB, ZINC finger KNOX, NAC, ethylene-responsive transcriptional coactivator, BTB/POZ, HD-ZIP); glutaredoxin II; *transcription factors (bZIP)*
Catabolic process – proteolysis	49.1 (27)	41.8 (23)	7.3 (4)	Cystein proteinase; endochitinase (pathogenesis-related protein PR3); defense-related lipase (patatin-like); RING finger
Response to stimulus	57.1 (20)	3.42 (12)	22.9 (8)	GST; RNaseS; monogalactosyldiacylglycerol (MGDG) synthase; glutathione S-conjugate ABC transporter; DNAJ heat shock protein; *peroxidase; HSP90-LIKE; ABC transporter*
Signal transduction – protein modification	57.8 (41)	45.1 (32)	12.7 (9)	Ubiquitin conjugating enzyme RING finger; phosphatase 2C; LRR kinases; CPK; CBL-interacting protein kinase (CIPK); autophagy related protein; *kinases*
Unknown	15.6 (18)	11.3 (13)	4.3 (5)	

[a]Percentage of sequences, relative to each category (100%), showing significant similarity (Blastx E-value < $1e^{-5}$) with *Arabidopsis* genes induced > threefold in leaf senescence (Buchanan-Wollaston *et al*., 2005).
[b]Percentage of transcripts, relative to each category, up-regulated in senescent *M. truncatula* and *Arabidopsis* leaves.
[c]Percentage of transcripts, relative to each category, down-regulated in *M. truncatula* and up-regulated in *Arabidopsis* senescent leaves.
[d]Italics show proteins coded by *M. truncatula* genes with discordant expression patterns with respect to *A. thaliana*.

MADS box transcription factors are induced in the last phase of senescence In our survey, we isolated 34 transcription factors (Table S4) and 67% of them showed enhanced expression in senescence. While some, belonging to the classes MYB (MYB-HD), CONSTANS-like (CCT), ethylene responsive factors (ERF), and NAC, are also involved in *Arabidopsis* senescence (Guo *et al*., 2004), half of the identified putative transcription factors (16) found no match in the *Arabidopsis* dataset. Among them are four members of the MADS box family (CX539597, TC110424, TC95842, TC104631). The up-regulation of this class of genes in leaf senescence is unexpected since these transcription factors are known to delay senescence, as demonstrated in *Arabidopsis* flowers (Fang & Fernandez, 2002) and poplar leaves (Hoenicka *et al*., 2008). Only in *Iris* and carnation has the induction of an AGAMOUS-like MADS box (van Doorn *et al*., 2003; Hoeberichts *et al*., 2007) been reported in flower senescence. Although no MADS box transcription factors have been found up-regulated in leaf senescence so far, we have observed that the *TC110424* gene is similar to the *Arabidopsis At2g22540* gene that is induced not only during floral development (Schmid *et al*., 2005) but also in leaf senescence (Arabidopsis eFP browser; Winter *et al*., 2007). This gene is not in the *Arabidopsis* dataset used in this analysis because of the high cut-off applied by Buchanan-Wollaston *et al*. (2005).

In order to characterize the expression profiles of the four MADS-box genes identified in *M. truncatula* leaf senescence, we analysed their induction in detail by relative-quantitative RT-PCR at the different leaf developmental stages (Y, MG, S1, S2, S3, Fig. 4b). The transcript level of all these genes was particularly high at the last stage of senescence (S3). This expression profile, and the fact that overexpression of this class of genes often delays senescence, suggests that in legumes the last, degenerative phase of leaf senescence is naturally prolonged by MADS box transcription factors, and could account for a more effective reallocation of nitrogen in these protein-rich plants.

Asparagine-synthetases are involved in nitrogen mobilization during the nutrient reallocation step of leaf senescence As a consequence of their ability to fix atmospheric nitrogen, legumes usually have high protein content in their seeds and leaves. During leaf senescence, proteins are extensively degraded, and their amine moieties are recycled to other parts of the plant, such as developing seeds. The molecular shuttles for nitrogen are glutamine and asparagine, formed by the enzymes glutamine-synthetase (GS) and asparagine-synthetase (AS), respectively. The use either of glutamine or asparagine to transport reduced nitrogen depends on the plant species, the organ and the developmental stage. The xylem of nodulated temperate legumes, such as *M. truncatula*, is rich in asparagine, and this seems to be the major nitrogen transport molecule involved under those physiological conditions (Shi *et al*., 1997). However, Carvalho *et al*. (2000) demonstrated the induction

of the cytosolic GS genes during leaf senescence in *M. truncatula*. Among the genes up-regulated during leaf senescence, we found a GS (identical to *GS1a*, Y10267) and two AS coding sequences (*AS1*, TC100391, and *AS2*, TC100393). This latter class of enzymes is known to be induced in detached asparagus and darkened leaves of alfalfa, pea and *Arabidopsis*, and also in sugar-starved *Arabidopsis* cell cultures (Tsai & Coruzzi, 1990; Lam *et al.*, 1994; King *et al.*, 1995; Shi *et al.*, 1997; Buchanan-Wollaston *et al.*, 2005); all of these treatments are known to induce degenerative processes similar to natural senescence (Buchanan-Wollaston *et al.*, 2003). ASs are expressed in senescent leaves of asparagus and sunflower but not in *Arabidopsis* (King *et al.*, 1995; Buchanan-Wollaston *et al.*, 2005; Herrera-Rodrìguez *et al.*, 2006). Considering that no AS genes had been studied in *M. truncatula*, and the importance and peculiarity of nitrogen metabolism in legumes, we decided to investigate in detail the expression pattern of the various *M. truncatula* isoforms of AS and GS genes during leaf senescence. To that end, we performed relative-quantitative RT-PCRs on the different leaf developmental stages by using primers designed from the sequences of the two AS isoforms isolated in this study and the three GS genes already available in GenBank database: cytosolic GS1a and Gs1b (Y10267 and Y10268, Carvalho *et al.*, 1997) and plastidial GS2 (AY225150, Melo *et al.*, 2003). As expected from the cDNA-AFLP analysis, *AS1* and *AS2* are strongly induced during senescence (Fig. 4c). The genes encoding the cytosolic isoforms of GS, *GS1a* and *GS1b*, are also induced during senescence, although *GS1a* shows only a slight increase. The plastidial isoform GS2, on the other hand, presented a sharp decrease in the abundance of its transcript, which was likely the result of the breakdown of chloroplasts during senescence. If enzyme activities also mirror the transcriptional changes, and being ASs glutamine-dependent enzymes, we hypothesize that the basal activity of GSs along leaf development provides glutamine for ASs, which are greatly induced late in senescence and synthesize asparagine for nitrogen exportation. Accordingly, *M. truncatula* xylem is rich in asparagine (Shi *et al.*, 1997).

Common regulative pathways are shared by different programmed cell death processes: comparison among leaf and nodule senescence and nitric oxide signalling

A distinctive characteristic of legumes is their ability to form nodules in the roots. Recently, a cDNA-AFLP analysis was performed on *M. truncatula* nodule development (Van de Velde *et al.*, 2006). The authors compared the processes of nodule senescence and *Arabidopsis* leaf senescence, and stated that a high degree of overlap exists between the two processes at the level of gene families involved. To further investigate the parallel between leaf and nodule senescence, we compared our collection of genes with the 508 sequences of the nodule senescence dataset and found 34 common clusters, corresponding to 7% of the leaf dataset (Fig. 5, Table S5). It is

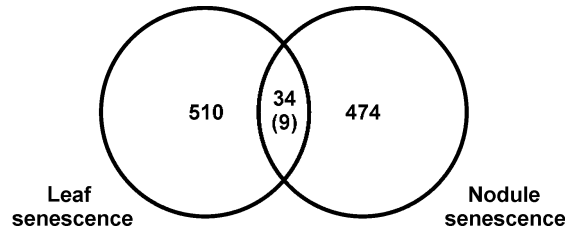

Fig. 5 Venn diagram of distribution of genes among leaf senescence in *Medicago truncatula* (induced and repressed genes, listed in Supporting Information, Table S1) and nodule senescence (induced and repressed genes listed in Van de Velde *et al*., 2006). The number in brackets refers to *M. truncatula* leaf genes with discordant expression patterns compared with nodule dataset.

evident, therefore, that at the level of single genes involved, nodule and leaf senescence differ to a degree higher than was previously supposed. However, since the two cDNA-AFLP analyses were conducted on two different lines (H2A and J5) of the same cultivar of *M. truncatula*, we cannot exclude that part of the transcriptional discrepancy is the result of the slightly different genotypes.

Given that the cDNA-AFLP technique permits the isolation of both up- and down-regulated transcripts, and that several nodule zones were considered in Van de Velde *et al.* (2006), only expression data coming from the senescent nodule zone (addressed as PSN2) were used in this analysis. Nine genes (1.7% of the total and 26% of the common clusters) showed discrepant expression between leaf and nodule senescence, meaning that they are induced in one process and repressed in the other. Despite the low number of common genes detected in this analysis, most (22, 63%) of those modulated in both leaf and nodule senescence processes encode proteins involved in the regulation of leaf senescence (i.e. MAP kinases, Cbl interacting protein kinase, ubiquitin cycle components, phosphatase 2C, GST, transcription factors) or in the degeneration phase (proteolysis, 20%, i.e. cysteine proteases and components of the 26S proteasome) (Table 2). Most of these proteins were already reported in *Arabidopsis* leaf senescence, suggesting a common basal mechanism for both leaf and nodule senescence (Table S5).

Nitric oxide (NO) is a gaseous reactive molecule with a role in the regulation of plant PCD events that is becoming increasingly apparent. It has a fundamental role in triggering the hypersensitive response after interaction with incompatible pathogens (Zaninotto *et al.*, 2006) and participates in the PCD during the development of tracheary elements (Gabaldón *et al.*, 2005). NO might therefore be a key player in the signalling of different types of PCD, modulating the expression of a common core of genes. As senescence eventually terminates with PCD of the tissue, NO might as well be an important molecule in the regulation of this degenerative process. Accordingly, we previously observed that NO is involved in senescence of *Arabidopsis* cell cultures (Carimi *et al.*, 2005).

Table 2 Comparison between *Medicago truncatula* leaf and nodule senescence datasets

Category	% common members[a] (no. of genes)	% concordant[b] (no. of genes)	% discordant[c] (no. of genes)	Examples[d]
Biological regulation	17.2 (6)	8.6 (3)	8.6 (3)	Transcription factors (BTB/POZ, homeobox-like); DNA helicase-like; *transcription factors (TGA type bZIP, bZIP)*
Catabolic process – proteolysis	20.0 (7)	17.1 (6)	2.9 (1)	Cystein proteinase; vacuolar-processing enzyme; 26S proteasome AAA-ATPase subunit; endochitinase (pathogenesis-related protein PR3); defense-related lipase (patatin-like); *pathogenesis-related protein 3*
Response to stimulus	14.3 (5)	11.4 (4)	2.9 (1)	DNA helicase-like; late embryogenesis abundant-like protein; MGDG synthase; ripening-responsive protein; *glutathione S-transferase*
Signal transduction – protein modification	28.6 (10)	17.2 (6)	11.4 (4)	Ubiquitin carrier protein; phosphatase 2C; CBL-interacting protein kinase; *lectin-like protein kinase; SOS2-like protein kinase; MAP3K delta-1 protein kinase*
Unknown	8.6 (3)	8.6 (3)	–	

[a]Percentage of sequences, relative to each category (100%), showing significant similarity (identity > 95%) with genes coming from the senescent nodule analysis (Van de Velde *et al*., 2006).
[b]Percentage of transcripts, relative to each category, with concordant expression pattern between datasets.
[c]Percentage of transcripts, relative to each category, with discordant expression pattern between datasets.
[d]Italics show proteins coded by genes with discordant expression patterns respect to nodule senescence.

In order to shed light on the role of NO in leaf senescence, we compared our transcriptomic dataset with two collections of genes obtained by infiltration of *M. truncatula* leaves with two different NO donors, sodium nitroprusside (SNP) and nitrosoglutathione (GSNO) (Ferrarini *et al*., 2008). The results, presented in Fig. 6, show that a high proportion (*c*. 68%) of genes are shared by the two donors, which is a good indication that NO is the real modulator of the transcriptional changes in infiltrated leaves. About 50 genes (9% of the senescent dataset) are modulated both in senescence and by either NO donor (Fig. 6a, Table S6). Noticeably, 37% of these transcripts are annotated as components of signalling and regulative pathways and code for transcription factors, protein kinases, DNA repair proteins and lipases; another 27% have unknown functions (Table 3). Fourteen genes, mostly of unknown function or related to regulatory pathways, are also expressed during *Arabidopsis* senescence. We further analysed by RT-PCR the expression profile of two NO-regulated genes that showed high expression in senescence and that were similar to previously identified SAGs, of *Arabidopsis* (TC102268) and *Pisum sativum* (TC107754). Their overexpression along the senescence stages was confirmed (Fig. 6b). Overall, the high proportion of regulative genes expressed in both NO-treated and senescent leaves suggests a conserved mechanism in the onset and execution of PCD events. Accordingly, Quirino *et al*. (1999) found a significant overlap in gene expression between the hypersensitive response (NO signalling) and senescence.

Conclusions

Senescence is a complex process, regulated by several endogenous and environmental factors. It starts with signalling events leading to synthesis of the catabolic enzymes responsible for the disassembling of the cells. Being a conservative process, during the execution phase, nutrients are reallocated from dying cells to living organs (i.e. young leaves, seeds).

The scheme presented in Fig. 7 shows representative genes, isolated in this work, that are similar to *Arabidopsis* genes involved in leaf senescence, and their putative allocation among the different phases of senescence. It is noteworthy that, despite the low cut-off (10%) applied to our data, the differentially expressed genes that we identified cover the entire process, from the sensing of the triggering signals to the nutrient reallocation. These results confirm that the use of a low cut-off together with the RT-PCR checking has been a good strategy to discard false positives and to isolate early and late players of senescence, other than new SAGs. In fact, as it can be easily checked by using a filter in Table S1, with a higher cut-off (i.e. twofold cut-off commonly used in large-scale data analysis) most of the genes that we demonstrated as being involved in senescence by RT-PCR (MADS box, asparagine-synthetase,

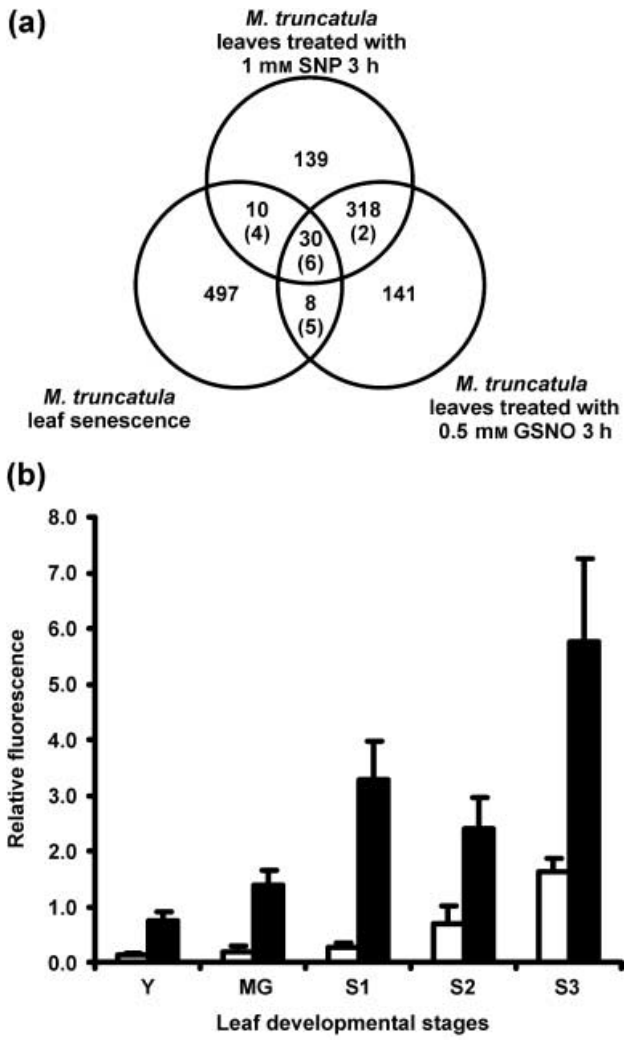

Fig. 6 Comparison with nitric oxide-treated leaves. (a) Venn diagram of distribution of genes among *Medicago truncatula* leaf senescence and nitric oxide treatment (Ferrarini *et al*., 2008). Numbers in brackets refer to genes with discordant expression pattern. (b) Expression patterns of the two genes (TC107754, open bars; TC102268, closed bars) highly induced by nitric oxide. Bars represent the amount of transcripts measured by densitometric analysis of relative-quantitative RT-PCR products. Values are means ± SD of three independent RT-PCRs. Y, young leaf; MG, mature green leaf; S1, *c*. 40% loss of chlorophyll; S2, *c*. 80% loss of chlorophyll; S3, > 90% loss of chlorophyll.

Table 3 Comparison between *Medicago truncatula* senescent and nitric oxide infiltrated leaves datasets

Category	% common members[a] (no. of genes)	% concordant[b] (no. of genes)	% discordant[c] (no. of genes)	Examples[d]
Biological regulation	33.3 (10)	23.3 (7)	10.0 (3)	Transcription factors (MYB, HD); *beta-carotene hydroxylase; translationally controlled tumour protein*
Catabolic process – proteolysis	20.0 (6)	13.3 (4)	6.7 (2)	L-lactate dehydrogenase; homogentisate 1,2-dioxygenase; 26S proteasome AAA-ATPase subunit; *glyceraldehyde-3-phosphate-dehydrogenase; epoxide hydrolase-like protein*
Response to stimulus	16.6 (5)	13.3 (4)	3.3 (1)	DNA repair protein RAD51; defense-related patatin-like lipase; *ripening-related protein-like*
Signal transduction – protein modification	20.0 (6)	16.7 (5)	3.3 (1)	Kinases; ubiquitin-cycle; armadillo-like
Unknown	43.3 (13)	33.3 (10)	10.0 (3)	

[a]Percentage of sequences, relative to each category (100%), showing significant similarity (identity > 95%) with genes coming from nitric oxide-infiltrated leaf analysis (Ferrarini *et al*., 2008).
[b]Percentage of transcripts, relative to each category, with concordant expression pattern between datasets.
[c]Percentage of transcripts, relative to each category, with discordant expression pattern between datasets.
[d]Italics show proteins coded by genes with discordant expression patterns with respect to nitric oxide treatment.

glutamine-synthetase), or commonly known to be involved in the process (eIF-5A, cysteine proteases), are lost.

In summary, our data indicate that the senescence process in *M. truncatula* leaves involves many functional gene categories and is roughly similar to what is observed in *Arabidopsis*. However, important differences exist in some of the transcription factors involved and in the mechanisms of amine mobilization, which might reflect the peculiarity of nitrogen biology in legumes. In addition, we have observed that a minority of

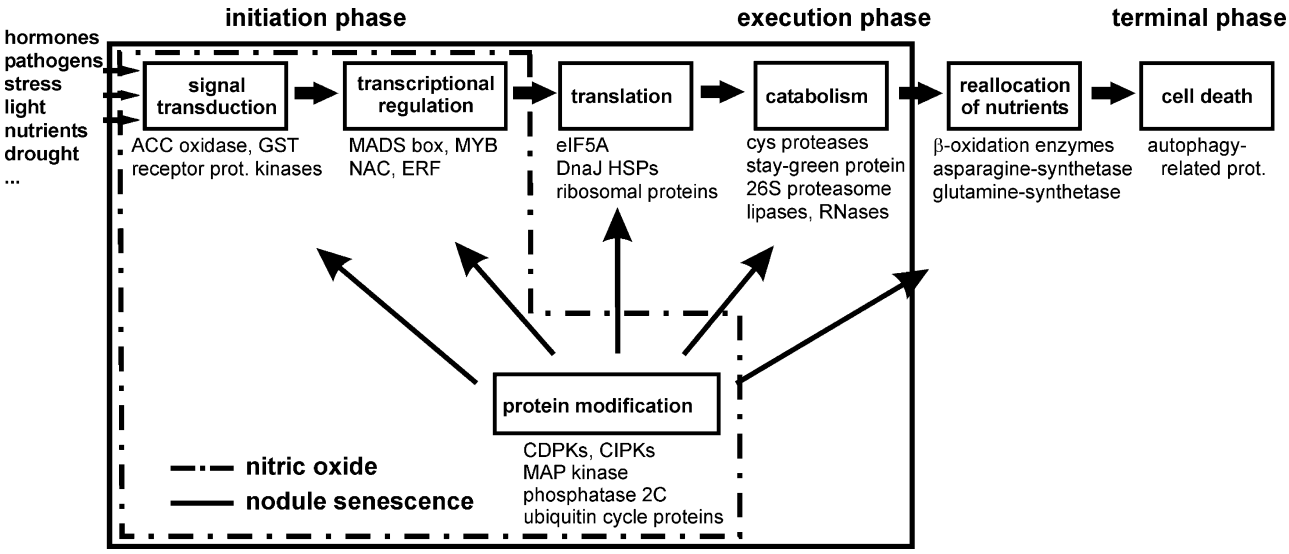

Fig. 7 Schematic illustration of the three phases of the senescence process. For each, the main steps are shown. Examples of genes isolated in this work and known as players in senescence are provided and listed for each step. Boxes comprise phases and steps that we found were also involved in nodule senescence and nitric oxide (NO) signalling.

the genes regulated during leaf senescence are equally involved in nodule senescence or NO treatment; nevertheless, a large proportion of these genes have regulative functions, suggesting a conserved mechanism in orchestrating the different processes.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Expression profiles of genes associated with senescence (cysteine proteases, eIF5A, ERF, NAC-like).

Table S1 Complete list of genes with accession numbers, annotations and comparisons with *Arabidopsis* leaf senescence, *Medicago truncatula* nodule senescence and nitric oxide treatment

Table S2 Primer sequences and conditions used in semi-quantitative RT-PCR analysis shown in Figs 2, 4, 6 and S1

Table S3 Comparison between *M. truncatula* and *A. thaliana* leaf senescence datasets

Table S4 List of transcription factors coding genes isolated in this work

Table S5 Comparison between leaf senescence and nodule senescence datasets

Table S6 Comparison between leaf senescence and nitric oxide treatment datasets

Table S7 List of identification and accession numbers of genes excluded from the analysis because of the expression level under the cut-off applied

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