RESEARCH ARTICLE

Transcriptome analysis of leaf senescence in red clover (*Trifolium pratense* L.)

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Abstract Red clover (*Trifolium pratense* L.) is an important cool-season legume plant, which is used as forage. Leaf senescence is a critical developmental process that negatively affects plant quality and yield. The regulatory mechanism of leaf senescence has been studied, and genes involved in leaf senescence have been cloned and characterized in many plants. However, those works mainly focused on model plants. Information about regulatory pathways and the genes involved in leaf senescence in red clover is very sparse. In this study, to better understand leaf senescence in red clover, transcriptome analysis of mature and senescent leaves was investigated using RNA-Seq. A total of about 35,067 genes were identified, and 481 genes were differentially expressed in mature and senescent leaves. Some identified differentially expressed genes showed similar expression patterns as those involved in leaf senescence in other species, such as *Arabidopsis*, *Medicago truncatula* and rice. Differentially expressed genes were confirmed by quantitative real-time PCR (qRT-PCR). Genes involved in signal transduction, transportation and metabolism of plant hormones, transcription factors and plant senescence were upregulated, while the

downregulated genes were primarily involved in nutrient cycling, lipid/carbohydrate metabolism, hormone response and other processes. There were 64 differentially expressed transcription factor genes identified by RNA-Seq, including ERF, WRKY, bHLH, MYB and NAC. A total of 90 genes involved in biosynthesis, metabolism and transduction of plant hormones, including abscisic acid, jasmonic acid, cytokinin, brassinosteroid, salicylic acid and ethylene, were identified. Furthermore, 207 genes with direct roles in leaf senescence were demonstrated, such as senescence-associated genes. These genes were associated with senescence in other plants. Transcriptome analysis of mature and senescent leaves in red clover provides a large number of differentially expressed genes. Further analysis and identification of senescence-associated genes can provide new insight into the regulatory mechanisms of leaf development and senescence in legume plant and red clover.

Keywords Red clover · Early stage of leaf senescence · RNA-Seq · Senescence associated gene

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Introduction

Leaf senescence is the ultimate phase of plant development, and it negatively affects plant quality and yield. With a gradual loss of chlorophyll, leaves turn yellowish or yellow, and leaf senescence occurs via degradation of proteins, carbohydrates, lipids, nucleic acids and the mobilization of micronutrients. However, leaf senescence is also a crucial life process for plants after suffering stress (Hortensteiner and Feller 2002), and it is a protective mechanism that decreases yield and quality in crop plants.

Recently, many advances have been made in exploring the mechanisms of leaf senescence at the molecular level, and a large number of genes involved in this process have been cloned and identified in many plant species. In a senescing leaf, senescence-associated genes (SAGs) are upregulated, and SAGs are considered to be markers of cellular senescence (Wistrom and Villeponteau 1992). In *Arabidopsis thaliana*, *SAG12*, which encodes a cysteine protease, is expressed only in senescent tissues, and this gene is used as a molecular marker for the study of developmental senescence (Noh and Amasino 1999). Overexpression of the acyl hydrolase activity gene, *SAG101* from *Arabidopsis* caused precocious senescence in the leaves of transgenic plants (He and Gan 2002). *SAG113* is induced in senescing leaves and confirmed as a key factor that promotes leaf senescence by controlling water loss in *Arabidopsis thaliana* (Zhang et al. 2012). The aluminum-induced senescence-associated gene *AhSAG* was isolated from peanuts, and it could induce or promote the occurrence of programmed cell death (PCD) in transgenic plants (Zhan et al. 2013).

Transcription factors are proteins that positively or negatively regulate gene expression by binding to *cis* elements of a target gene; many transcription factors are involved in plant senescence (Guo and Gan 2006; Jaradat et al. 2013; Kong et al. 2006; Kou et al. 2012; Rinerson et al. 2015; Robatzek and Somssich 2001; Xu et al. 2010; Yang et al. 2011; Zhang et al. 2011). Overexpression of a NAC family transcription factor gene, *AtNAP*, causes precocious senescence of transgenic plants, showing that *AtNAP* plays an important role in leaf senescence (Guo and Gan 2006). Expression profiles of WRKY genes in switchgrass were analyzed by RNA-Seq, and 23 genes showed elevated transcription levels during the onset of flag leaf senescence (Rinerson et al. 2015). It was reported that a MYB transcription factor gene, *AtMYBL*, demonstrated elevated expression in old leaves and upon addition of ABA. Transgenic plants with this gene displayed a markedly promoted leaf senescence phenotype, decreased Chl content and enhanced expression of senescence-related genes (Zhang et al. 2011). A CCH-type zinc finger protein transcription factor gene, *OsDOS*, delays leaf senescence by negatively regulating the JA pathway (Kong et al. 2006).

In addition, genes involved in biosynthesis, metabolism and signal transduction of plant hormones are hot spots for plant senescence. It is well known that abscisic acid (ABA) can promote plant senescence. Recently, it was reported that an *Arabidopsis* ABA receptor gene *PYL9* could promote drought resistance and leaf senescence (Zhao et al. 2016). In 2014, it was reported that *RhHBI* isolated from rose petals mediates the antagonistic effect of gibberellic acid (GA) on ABA and ethylene during rose petal

senescence. *RhHBI* blocked expression of a GA biosynthesis gene, *RhGA20ox1*. Silencing of *RhHBI* delayed ABA- or ethylene-mediated senescence, and treatment with an inhibitor of GA biosynthesis repressed these delays (Lu et al. 2014). Cytokinin plays a role in delaying plant senescence (Gan and Amasino 1995; Mayak and Halevy 1970; Zwack and Rashotte 2013). The gene encoding cytokinin biosynthetic enzyme isopentenyltransferase (*IPT*) was transformed into canola, and overexpression of the *IPT* gene delayed leaf senescence in transgenic plants (Kant et al. 2015).

Red clover (*Trifolium pratense* L.) is an important legume plant that plays a key role in sustainable intensification of livestock farming systems (Sullivan and Quesenberry 2015). Red clover is native to Europe, Western Asia and northwest Africa, but it is planted in many other regions because it is adapted to a wide range of soils (Sullivan and Quesenberry 2006; Yeung and Gubili 2008). As a legume plant, it has a higher protein content because of nitrogen fixation by nodulation via symbiosis with the soil microbe *Rhizobium leguminarum*, and its reduced need for nitrogen fertilizer input can reduce the environmental footprint of grassland-based agriculture (Taylor and Quesenberry 1996). Red clover is a diploid ($2n = 14$) species that is naturally cross-pollinated and the draft genome was reported recently (Istvanek et al. 2014). Research on red clover was focused mainly on isoflavone synthase and tolerance to biotic or abiotic stresses (Bipfubusa et al. 2016; Flythe and Kagan 2010; Hu et al. 2015; Kaurinovic et al. 2012). The isoflavone synthase (*IFS*) gene of red clover was cloned, and sequence analysis indicated that the *IFS* gene had high homology with similar genes from other plants (Hu et al. 2015). Previous studies on leaf senescence in red clover are very limited, and how leaf senescence in red clover is affected by plant hormones, SAGs and transcription factors remains unknown. The mechanisms and global transcriptional regulation of leaf senescence in red clover remain poorly understood. Next-generation high-throughput sequencing technology, known as transcriptome analysis or RNA-Seq, has emerged as a revolutionary tool to better understand differential gene expression and regulatory mechanisms. In this approach, there is no strict requirement for a reference genome sequence, so it is suitable for model or non-model species. With this tool, transcriptome analysis of leaf senescence was accomplished in many plant species, such as *Arabidopsis*, wheat, cotton, and sorghum (Gregersen and Holm 2007; Lin et al. 2015; Woo et al. 2016; Wu et al. 2016). The new technology was also applied in red clover. In 2014, de novo assembly of a red clover transcriptome from leaves of drought and non-drought plants provided a rich source for gene identification and the genetic basis of drought tolerance (Yates et al. 2014). Recently, a de novo

transcriptome assembly of red clover was constructed to study transcriptional networks in different tissue types (Chakrabarti et al. 2016). De novo assembly of red clover transcriptome was used because less information about genomic resources for red clover was available at that time. The draft genome of red clover was published in 2015, and this is the second genome assembly of a forage legume (De Vega et al. 2015). In this study, RNA-Seq was performed on senescent or non-senescent leaves, and the global changes in the developing leaf transcriptome were analyzed. We identified SAGs, TFs and hormone-related genes, which gave us novel insight into biological processes, metabolic pathways and the key regulatory components of the senescence process in red clover. This work will serve as the foundation for further analysis of the regulatory mechanisms of leaf senescence in red clover and will provide a rich resource for further identification of genes involved in leaf senescence.

Materials and methods

Plant materials and growth conditions

Red clover seeds (cv. Common) received as gifts from Top Green Group (Beijing, China) were surface-sterilized with 75% ethanol for 10 min and transferred to sow in nutrition medium containing peat, vermiculite and perlite (1:1:1) until germination and red clover plants were grown at 25/23 °C (day/night) with a 16-h photoperiod in growth chambers. Three individual lines were selected, and the clones of each line were used for further experiments. In the squaring period, the stems were cut into 8–10 cm segments and soaked in 20 mg/L IBA (indole-3-butyric acid) for 20 min before cutting propagation. Clones for individual lines were separated and transplanted in nutrition medium (mentioned above), and grown at 25/23 °C (day/night) with a 16-h photoperiod in growth chambers. Mature and old leaves of red clover plants were harvested from the same clones. All samples were frozen in liquid nitrogen and stored at – 80 °C for further experiments.

Chlorophyll, carotenoids, malondialdehyde and soluble sugar measurements

Chlorophyll (Chl) and carotenoids (Car) was extracted from each sample, as described previously (Xing et al. 2010). Briefly, mature and old leaves (0.05–0.08 g) were harvested from red clover plants, weighed, and ground in 95% ethanol solution (8 ml). All extraction solutions were combined into one tube (10 ml) and debris was removed by centrifugation at 12,000 g for 10 min. A volume of 1 ml of the supernatant was transferred into a cuvette with a

pipette. Absorbance of the final solution at 470, 665 and 649 nm was measured with a spectrometer, and the concentrations of Chl and Car were calculated as described previously (Lichtenthaler 1987). Malondialdehyde (MDA) and soluble sugar contents were measured according to the procedure described previously in our laboratory (Li et al. 2014). For MDA analysis, leaf samples (0.5 g) were homogenized in 10 ml 10% trichloroacetic acid (TCA) and centrifuged at 12,000 g for 20 min. Then, 2 ml 10% TCA (with 0.6% thiobarbituric acid) was added to 2 ml supernatant. The mixture was heated and then quickly cooled in an ice bath. After centrifugation, the absorbance of the mixture at 450, 532, and 600 nm was determined. The concentration of MDA was calculated using MDA's extinction coefficient of $155 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. For soluble sugar analysis, about 0.5 g of fresh sample with 10 ml distilled water were placed in a test tube, heated at 100 °C for 1 h, and then filtered into 25 ml volumetric flasks. Reaction mixture (7.5 ml) contained 0.5 ml extracts, 0.5 ml mixed reagent (0.01 g anthrone + 0.5 ml ethyl acetate) and 5 ml H_2SO_4 (98%), plus 1.5 ml distilled water. The mixture was heated at 100 °C for 1 min and absorbance was read at 630 nm to calculate soluble sugar. All measurements had three technical replicates.

Isolation of mRNA, preparation of libraries and Illumina sequencing

Total RNA from mature and old leaves of three different lines was isolated using a Plant RNA kit (Omega bio-Tech, USA) and then treated with RNase-free DNase I (NEB) to remove contaminating genomic DNA. RNA degradation and contamination was monitored on 1% agarose gels and RNA purity was checked using the NanoPhotometer[®] spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit[®] RNA Assay Kit in Qubit[®] 2.0 Fluorometer (Life Technologies, CA, USA) and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 3 µg RNA per sample was used as input material for RNA sample preparations. Sequencing libraries were generated using NEBNext[®] Ultra[™] RNA Library Prep Kit for Illumina[®] (NEB, USA) following the instruction manual, and index codes were added to attribute sequences to each sample. Library quality was assessed on the Agilent Bioanalyzer 2100 system, and the library preparations were sequenced on an Illumina Hiseq platform. Clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N and low quality reads from raw data. The Q20, Q30 (meaning the probability that the base was miscalled is 1 and 0.1%, respectively) and GC content

of the clean data were calculated, and all downstream analyses were based on high quality clean data.

Readings mapping and DEGs analyses

The genome assembly and annotation of red clover were downloaded (<http://dx.doi.org/10.5281/zenodo.17232>) (De Vega et al. 2015). The index of the reference genome was built using Bowtie (version 2.2.3) (Langmead and Salzberg 2012), and clean reads were aligned to the reference genome using TopHat (version 2.0.12) (Trapnell et al. 2009). HTSeq (version 0.61) software was used to count the read numbers mapped to each gene (Anders et al. 2015). Then, the FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) of each gene was calculated based on the length of the gene, and read counts were mapped to this gene (Trapnell et al. 2010). Differential expression analysis of mature and senescent samples (three biological replicates for each) was performed using the DESeq R package (version 1.18.0) (Robinson et al. 2010). The resulting *P* values were adjusted using Benjamini and Hochberg's approach to control the false discovery rate (FDR). The Differential expression genes (DEGs) were obtained after filtering using a $FDR < 0.05$ and an absolute value of $\log_2(\text{FoldChange}) > 1$.

Quantitative RT-PCR

To verify the results of the DEG analyses, 20 genes with differential expression were randomly selected and measured by qRT-PCR, as described previously (Chao et al. 2014). Leaves at mature and old stages were harvested and total RNA from each was isolated using a Plant RNA kit. Genomic DNA was removed at a volume of 10 μL , containing 2 μL 5 $\times$ gDNA Eraser Buffer (TaKaRa, Dalian, China), 1 μL gDNA Eraser (TaKaRa, Dalian, China) and 7 μL total RNA, under the condition of 42 $^{\circ}\text{C}$ for 2 min. The cDNA was synthesized according to the manual of PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The reaction solution consisted of 5 μL gDNA-free RNA, 1 μL PrimeScript RT Enzyme Mix I, 1 μL RT Primer Mix (containing 2 primers, Oligo d(T) and random 6 mers), 4 μL 5 $\times$ PrimeScript Buffer 2 and 9 μL RNase Free dH_2O , and the reverse transcript was performed under the conditions of 37 $^{\circ}\text{C}$ for 15 min and then 85 $^{\circ}\text{C}$ for 5 s. Each cDNA was diluted 2 times with dH_2O and 1 μL diluted product was used for qRT-PCR. The qRT-PCR was performed in 96-well blocks with an RT-PCR system (CFX Connect, BIO-RAD, Hercules, California, USA) using an UltraSYBR Mixture (CWbio, Beijing, China) at a volume of 25 μL , which contained 12.5 μL 2 $\times$ UltraSYBR Mixture, 1 μL forward primer, 1 μL reverse primer, 1 μL

cDNA and 9.5 μL dH_2O . A two-step amplification protocol included initial denaturation at 95 $^{\circ}\text{C}$ for 10 min; 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 1 min; and a melt curve step at the end of the PCR (95 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 1 min, 95 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 15 s). The relative expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. All results are presented as the means of three independent RNA extractions (three biological replicates with three technical replicates) with corresponding standard deviations (SD). Based on the Illumina sequencing results, the red clover UBQ gene (TGAC_v2_mRNA12221) was used as an internal control, and all primers used in the qRT-PCR analyses are reported in Supplementary Table 3.

GO and pathway enrichment analysis

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the Goseq R package (version: Release 2.12) (Young et al. 2010). GO terms with a corrected *P* value of less than 0.05 were considered significantly enriched by DEGs. KOBAS software (version: 2.0) was used to test the statistical enrichment of DEGs in KEGG pathways, as described previously (Mao et al. 2005).

Assays of TFs and hormone-related genes

A comprehensive plant TF database was downloaded from PlnTFDB (<http://plntfdb.bio.uni-potsdam.de/v3.0/>) (Perez-Rodriguez et al. 2010), and all sequences of DEGs were compared with the sequence of the plant TF database using Blastx with an E-value $\leq 10^{-5}$. For analysis and classification of hormone-related genes, all DEGs were compared with protein sequences from the Arabidopsis Hormone Database (<http://ahd.cbi.pku.edu.cn/>) using Blastx (E-value $\leq 10^{-5}$).

Results

Biochemical changes and hormone levels

After growing in soil for about 2 months, some leaves of red clover entered senescence and some remained green. We harvested leaf samples of mature (green) and early senescence stages (old) for further experiments (Fig. 1a). We measured chl, carotenoids, soluble sugar and MDA contents, which were used as markers for the progression of senescence. The results showed that chlorophyll and carotenoid levels in senescent leaves declined to 37.54 and 11.00% of those in mature leaves, respectively. The MDA was elevated to 141.77% of mature leaves and soluble sugar displayed 430.99% elevated level (Fig. 1b–e). The

Fig. 1 Phenotypes, physiology and biochemical measurements. **a** Phenotypes of red clover at senescence and mature stages. **b–e** MDA (**b**), soluble sugar (**c**), chlorophyll (**d**) and carotenoid (**e**) contents were measured in leaf samples at the mature and senescence stages. **f** The ratio of Chl a/b was calculated in leaf

samples at mature and senescence stages. Error bars represent the SD from three independent experiments. ** indicates significant differences of the means at $p < 0.01$ between mature and old leaves for each parameter measured ($n = 3$) (color figure online)

ratio of Chl a/b is an important parameter to assess leaf senescence, and the Chl a/b ratio in senescent leaves was reduced to 56% of mature leaves, indicating a higher degradation rate of Chl a than Chl b in leaf senescence (Fig. 1f). In conclusion, leaf senescence in red clover affects chl, car, soluble sugar and MDA contents.

RNA-seq analysis and DEGs identification

The RNA of leaf samples harvested at two stages, i.e. mature and old, was isolated and RNA libraries were prepared. These libraries were sequenced with Illumina HiSeq 4000 instruments. Approximately 51–55 million raw reads with high Q20 (> 96%) and Q30 (> 90%) in each library were generated (SRA submission number: SUB2425623), and the GC content of each library ranged from 40.63% to 41.17% (Table 1). The pairwise correlation analysis of RNA-Seq data between samples was shown

in Online Resource 1. The total number of clean reads per library was about 48–50 million after filtering out low quality reads (Table 1). A total of 35,067 genes (FPKM > 0) were detected in leaves of the two developmental stages (Table S1).

By DEGs analysis, there were 481 DEGs identified, with 95 genes up-regulated and 386 genes down-regulated (Fig. 2; Table S2). To verify the DEG results, 20 genes (including 10 up-regulated and 10 down-regulated genes) were randomly selected to design primers for qRT-PCR (Table S3). The qRT-PCR results showed similar expression patterns as the DEGs analysis (Fig. 3), suggesting that RNA-Seq was an efficient, accurate and high-throughput tool for DEGs analyses of leaf senescence in red clover.

Table 1 Data quality of RNA-Seq

Sample name	Raw reads	Clean reads	Error rate (%)	Q20 (%)	Q30 (%)	GC content
Old sample 1	51,470,150	49,201,096	0.02	96.06	90.35	40.63
Old sample 2	52,074,244	49,705,156	0.02	96.03	90.28	40.74
Old sample 3	51,390,604	49,091,248	0.02	96.2	90.59	40.86
Mature sample 1	52,909,440	50,138,634	0.02	96.3	90.81	41.17
Mature sample 2	51,135,388	48,794,966	0.02	96.05	90.31	41.16
Mature sample 3	55,063,838	52,643,740	0.02	96.14	90.49	41.05

Fig. 2 Volcano plot of differentially expressed genes. Each plot represents one gene with three colors, including red (up-regulated), green (down-regulated) and blue (unchanged). The X-axis represents the value of $\log_2(\text{Fold Change})$ in the two samples, and the Y-axis indicates the negative value of $\log_{10}(\text{FDR})$ (color figure online)

SAGs analyses

The DEGs were compared with those in the Leaf Senescence Database (LSD, <http://psd.cbi.pku.edu.cn/>) to analyze the sequence conservation of SAGs between red clover and other plants. These genes were grouped into several categories according to the function and classification of the most closely related homolog. A total of 207 out of the 481 DEGs had homologous genes in other plants

in the LSD, and these orthologs were mainly involved in plant hormone responses, chlorophyll degradation, lipid/carbohydrate metabolism, nutrient recycling and others (Table S4).

GO and KEGG analyses

GO analysis of DEGs showed that the enrichment of SAGs could be divided into three groups, including biological processes, molecular functions and cellular components (Fig. 4). Genes involved in biological processes mainly consisted of metabolic, biosynthetic, transport, response to stimulus, developmental and cell cycle processes. Genes in the molecular functions were enriched for catalytic activity, transferase activity and transporter activity. For the category, cellular component, genes were mainly involved in external encapsulating structures, apoplast, cell walls, and others. More detailed GO terms are available in Table S5.

All DEGs were analyzed to identify metabolic pathways, and the KEGG results demonstrated that 386 downregulated genes were mapped to 40 KEGG pathways and 95 upregulated genes were mapped to 20 KEGG pathways (Tables S6 and S7). There were 5 significantly downregulated and 1 significantly upregulated pathways with corrected P value < 0.05 in the senescent leaf of red clover (Table 2). The pathway playing a role in

Fig. 3 The qRT-PCR validation of 20 DEGs selected during leaf senescence. Columns represent the value of $\log_2(\text{Fold Change})$ (X-axis). The right columns represent the expression levels of transcripts that are up-regulated in leaf senescence, and left columns represent the expression levels of transcripts that are down-regulated in leaf senescence. Blue columns represent value from qRT-PCR, and yellow columns represent the value from RNA-Seq. Error bars represent the SD from three independent experiments (color figure online)

Fig. 4 Distribution of GO terms for all annotated unigenes. GO including biological process (black), cellular component (green) and molecular function (blue). The X-axis represents GO terms, and the Y-axis represents the number of differentially expressed genes (color figure online)

phenylpropanoid biosynthesis was significantly up-regulated, and the pathways involved in plant hormone signal transduction; metabolism of cyanoamino acid, cysteine and methionine, starch and sucrose; and biosynthesis of secondary metabolites were significantly down-regulated (Table 2). For example, auxin and cytokinin play important roles in delaying leaf senescence, and the pathways involved with auxin and cytokinin biosynthesis were largely down-regulated (Fig. 5). These results suggested that the DEGs involved in those pathways play important roles in leaf senescence.

DEGs involved in plant hormones

Plant hormones play important roles in the regulation of leaf senescence, so we identified the plant hormone related genes by searching DEGs in the Arabidopsis Hormone

Database. The results showed that 90 DEGs were identified as being associated with hormone biosynthesis, response, signaling, receptors and metabolism (Table 3), and more detailed results are shown in Table S8.

ABA is an important senescence promoting factor. TGAC_v2_mRNA16268, a homologous gene of *IP5PI* in Arabidopsis, which plays an important role in the abscisic acid-activated signaling pathway, was only detected in senescent leaves in red clover. Cytokinins involved in delaying leaf senescence and five DEGs involved in hormone transportation and signal transduction were found (Table S8). The TGAC_v2_mRNA17931 gene (homolog of *ARR9* in Arabidopsis) involved in hormone signal transduction of cytokinin was drastically decreased in old samples. Auxin plays a negative role in regulating leaf senescence, and 15 DEGs related to auxin transportation and signal transduction were significantly down-regulated.

Table 2 Significantly enriched pathways in red clover during leaf senescence

#Term	ID	Input number	Background number	Corrected <i>P</i> value	Up- or Down-regulated
Phenylpropanoid biosynthesis	mtr00940	16	188	2.89E−05	Up-regulated
Cyanoamino acid metabolism	mtr00460	8	51	0.000196912	Down-regulated
Plant hormone signal transduction	mtr04075	13	272	0.020930402	Down-regulated
Cysteine and methionine metabolism	mtr00270	6	67	0.020930402	Down-regulated
Biosynthesis of secondary metabolites	mtr01110	27	873	0.03902305	Down-regulated
Starch and sucrose metabolism	mtr00500	10	206	0.03902305	Down-regulated

Fig. 5 DEGs in auxin and cytokinin signal transduction of KEGG pathways. Green boxes represent key enzymes in plant hormone signal transduction, and gene IDs in black represent homologous genes in *Medicago truncatula*. Gene IDs in green represent down-

regulated DEGs in red clover, and the numbers in parentheses are $\log_2$ (Fold Change) in leaf senescence. Auxin and cytokinin signal transduction was simplified from the pathway in *Medicago truncatula* (color figure online)

Table 3 Hormone-related DEGs in red clover during leaf senescence

Plant hormone	Number of genes	Up-regulated	Down-regulated
Brassinosteroid	23	8	15
Auxin	19	4	15
ABA	14	2	12
SA	10	2	8
Ethylene	10	1	9
GA	7	5	2
CK	5	1	4
JA	1	1	0

The auxin signal transduction-related gene of senescent leaves, TGAC_v2_mRNA40943 (homolog of *GLP4* in *Arabidopsis*), declined to 3.9% of the level in mature leaves (Table S8).

DEGs of TFs in leaf senescence

TFs are key regulatory proteins involved in regulating leaf senescence. There were 63 TF transcripts from 23 TF families identified (Table S9), and the major 6 were ERF (14), WRKY (7), bHLH (6), NAC (6), MYB (6) and ARF (4) families (Fig. 6). Seventeen transcription factors, including 3 WRKY and 3 NAC proteins, were significantly upregulated in senescent leaves. The gene TGAC_v2_mRNA12943, encoding a WRKY protein, showed a more than sevenfolds increase in level of expression. The NAC gene TGAC_v2_mRNA29036 was only detected in senescent leaves. Moreover, an ERF transcription factor gene, TGAC_v2_mRNA21328 was up-regulated more than 30 times in senescent leaves. In addition, 46 transcription factors were down-regulated in leaf senescence. TGAC_v2_mRNA25879 (EIL family), TGAC_v2_mRNA25879 (ERF family) and TGAC_v2_mRNA37166 (WRKY family) were only

Fig. 6 Differentially expressed transcription factor genes in leaf senescence. The X-axis represents transcription factor family, and the Y-axis represents the number of transcription factor genes. The red columns represent up-regulated transcription factor genes, and the blue columns represent down-regulated transcription factor genes (color figure online)

expressed in mature leaves. A bZip transcription factor gene, TGAC_v2_mRNA15436 was down-regulated to less than 1/300 times in senescence leaves when compared with mature leaves.

Discussion

Before the publication of the whole genome sequence of red clover, *de novo* assembly of red clover transcriptome was used (Chakrabarti et al. 2016; Yates et al. 2014). As the second genome assembly of a forage legume, the genome of red clover was sequenced in 2015 (De Vega et al. 2015). This transcriptome analysis of red clover is the first work to study senescence in the leaves of a legume species with next-generation sequencing (NGS). A transcriptome analysis of leaf senescence is presented in the legume model *Medicago truncatula* with the cDNA-amplification fragment length polymorphism (AFLP) technique, and over 500 genes were identified in 2009 (De Michele et al. 2009b).

We determined that the Chl and the Car contents were both decreased in leaf senescence (Fig. 1d, e). The findings were consistent with previous reports. During senescence, leaf cells undergo dramatic changes in cellular metabolism and a sequential degeneration of cellular structures, especially the chloroplast (Nam 1997). Leaf senescence is manifestly characterized by a progressive yellowing due to chlorophyll degradation and loss of photosynthetic activity (De Michele et al. 2009a; Gan and Amasino 1997). During leaf senescence, the contents of Chl a, Chl b and Car were significantly reduced (Gut et al. 1987; Tang et al. 2015). In addition, Chl a is relatively more sensitive to leaf senescence than Chl b, suggesting that the ratio of Chl a/b is an effective indicator of leaf senescence (Grover et al. 1986; Young et al. 1991). In our experiment, the Chl a/b levels in senescent leaves were decreased (Fig. 1f).

We used high-throughput Illumina sequencing technology to explore the DEGs between senescent and non-senescent leaves and analyze regulatory mechanisms of leaf

senescence in red clover. A total of 35,067 genes were detected (FPKM > 0) in the 6 samples (Table S1). During the leaf senescence process, multiple genes are up- or down-regulated, and these genes were involved in different signaling pathways regulating leaf senescence. In this work, 481 DEGs were identified in leaf senescence of red clover, and the DEGs of leaf senescence were also identified in other plants by transcriptome analysis. The transcriptome profiling of developmental leaf senescence in sorghum (*Sorghum bicolor*) was analyzed, and 4293 DEGs were identified (Wu et al. 2016). The senescence process in cotton (*Gossypium hirsutum* L.) leaves was investigated from different senescence stages using RNA-Seq, and 3624 genes were determined to be differentially expressed during leaf senescence (Lin et al. 2015).

A total of 207 SAGs were identified by searching the leaf senescence database by BLASTx. SAGs were mainly involved in plant hormone responses, protein degradation/modification, lipid/carbohydrate metabolism, nutrient recycling and others (Table S4). The Arabidopsis inorganic phosphate (Pi) transporter gene, *Pht1;5* plays a critical role in mobilizing Pi from P source to sink organs. Overexpression of the *Pht1;5* gene in transgenic Arabidopsis altered Pi remobilization and promoted premature senescence by ethylene signaling (Nagarajan et al. 2011). A novel gene, Novel00333 from red clover, a homologue of the *Pht1;5* gene, was greatly up-regulated more than 45 times in senescent leaves. A total of 45 nutrient recycling-related genes were changed during leaf senescence (Table S4). Leaf senescence is a nutrient mining and recycling process, and the released nutrients are remobilized to actively growing regions, such as young leaves. In a senescing leaf, nitrogen is released primarily from protein degradation and nucleic acid catabolism (Hörtensteiner and Feller 2002), and 36 protein degradation and 2 nucleic acid degradation genes are shown in Supplementary Table 4. Notably, 5 genes involved in nutrient recycling of Mg^{2+} were down-regulated in unison, demonstrating that the ability to recycle Mg^{2+} was affected by senescence. Many of the genes are predicted to encode transporters for ions,

amino acids and sugars, consistent with substantial nutrient recycling during leaf senescence. The findings in red clover were consistent with those in *Arabidopsis* (Guo et al. 2004), which showed our transcriptome data were accurate.

We identified 63 differentially expressed TFs from 23 TF families (Table S9; Fig. 6), and 38 of those were also displayed in SAGs analysis (Table S4). TFs play important roles in regulating leaf senescence. Expression of *AtWRKY6* gene resulted in triggering senescence processes (Robatzek and Somssich 2001). Over-expressed *MYBRI* in *Arabidopsis* delayed leaf senescence, and loss-of-function *mybr1* plants showed more rapid chlorophyll loss and senescence (Jaradat et al. 2013). The NAC family is important in modulating plant senescence, and many NAC genes were reported as senescence promoters in many plant species. Expression of a NAC transcription factor gene, *BeNAC1* resulted in various senescence phenotypes in *Arabidopsis* (Chen et al. 2011). TGAC_v2_mRNA12324 was identified as a NAC transcription factor gene, which showed enhanced expression levels in leaf senescence, and a homologue gene *ORS1* plays a positive role in dark/starvation/H₂O₂-induced senescence in *Arabidopsis* (Balazadeh et al. 2011; Buchanan-Wollaston et al. 2005). The abiotic stress-induced *NAC016* in *Arabidopsis* encoded a NAC protein, playing a role in promoting senescence by binding to the promoters of *NAP* and *ORS1* (Kim et al. 2013). *ERF* genes were reported to be involved in transcriptional regulation of senescence processes (Liu et al. 2011). There were 10 ERF genes, including 9 down-regulated and 1 up-regulated, in leaf senescence (Table S8). Two ERF genes, TGAC_v2_mRNA6885 and TGAC_v2_mRNA38928, were down-regulated in leaf senescence, and their homologue gene in *Arabidopsis*, *CBF2*, was involved in delaying leaf senescence (Sharabi-Schwager et al. 2010). Those results demonstrated that TFs played different roles in regulating leaf senescence in red clover. Interestingly, some TFs exhibit different expression patterns. The bZIP gene (AT3G58120) and C2H2-type zinc finger protein gene (AT1G75710) in *Arabidopsis* were up-regulated in senescent leaves (Balazadeh et al. 2008), while homologous genes in red clover were down-regulated. The differences in TF expression might be caused by different roles of genes and different regulatory pathways between red clover and other plant species. TFs play important roles in the regulatory networks of senescence, and TFs of DEGs can be key factors involved in triggering of the senescence processes.

Multiple developmental and environmental signals control senescence, and plant hormones are thought to play important roles in the regulation of senescence (Khan et al. 2014). Ethylene and jasmonic acid play roles in accelerating plant senescence (Burg 1968; He et al. 2002; Kim et al. 2015; Thompson et al. 1982). Transgenic lines with

low expression levels of *LOX2*, reduced JA accumulation, demonstrating that *LOX2* promotes leaf senescence by controlling biosynthesis of JA (Seltmann et al. 2010). The expression level of TGAC_v2_mRNA8174 (a homologue gene of *LOX2*) was increased more than 4 times in leaf senescence in red clover. An ethylene signal transduction gene, TGAC_v2_mRNA4338, showed up-regulated expression levels, and the homologous gene, *CEV1* in *Arabidopsis* plays a positive role in ethylene accumulation or stimulation of the ethylene signal pathway (Ellis et al. 2002; Ellis and Turner 2001). ABA is well known to promote plant senescence (Colquhoun and Hillman 1972; Kumar et al. 2014; Mayak and Dilley 1976; Mayak and Halevy 1972; Samet and Sinclair 1980). In our work, two ABA-related genes (TGAC_v2_mRNA23747 and TGAC_v2_mRNA16268) showed enhanced expression levels (Table S8). TGAC_v2_mRNA16268 was only detected in senescent leaves, and TGAC_v2_mRNA23747 was increased more than 10-fold. *IP5PI*, homologue gene of TGAC_v2_mRNA16268 in *Arabidopsis* was up-regulated slightly, and the homologue gene (*LECRKA4.2*) of TGAC_v2_mRNA23747 was up-regulated over 6.9-fold in senescent leaves (Buchanan-Wollaston et al. 2005; Gunsekera et al. 2007). Cytokinin can delay leaf senescence (Mayak and Halevy 1970; Zwack and Rashotte 2013), and regulated expression of the cytokinin biosynthesis gene, *IPT* delays leaf senescence (Kant et al. 2015). Four cytokinin-related genes, involved in signal transduction and transportation, showed down-regulated levels in leaf senescence, indicating the delayed effects on senescence processes by cytokinin pathways in red clover (Table S8). Auxin is another important plant hormone in delaying leaf senescence (Kim et al. 2011). In this study, genes taking part in the auxin signaling pathway presented declined levels in leaf senescence (Fig. 5). There were four auxin biosynthesis-related genes (TGAC_v2_mRNA29126, TGAC_v2_mRNA33699, TGAC_v2_mRNA37362 and TGAC_v2_mRNA6387) that were up-regulated in leaf senescence. As a homologue gene of TGAC_v2_mRNA29126, *SUR1* showed a similar expression pattern in *Arabidopsis* and the *sur1* mutant exhibited a “high-auxin” phenotype (Mikkelsen et al. 2004). The plant microRNA, miR10515 promotes IAA biosynthesis by suppressing *SUR1* (Kong et al. 2015). *SUR2* gene (a homologue of TGAC_v2_mRNA33699 and TGAC_v2_mRNA37362) plays similar roles in regulating the biosynthesis of IAA (Delarue et al. 1998). It can be inferred that TGAC_v2_mRNA29126, TGAC_v2_mRNA33699 and TGAC_v2_mRNA37362 play negative roles in regulating IAA biosynthesis in red clover. However, the function of *CYP79B2* in *Arabidopsis* (a homologue of TGAC_v2_mRNA6387) is proved as an IAA biosynthesis enhancer. *CYP79B2* plays a role in

converting tryptophan (Trp) to indole-3-acetaldoxime (IAOx), which is an important intermediate in auxin biosynthesis, and overexpression of *CYP79B2* gene in *Arabidopsis* leads to phenotypes of IAA overproduction (Mikkelsen et al. 2009). One possibility on the elevated expression in leaf senescence is that there is some feedback regulation in the IAA biosynthesis pathway. High levels of IAA can reduce transcriptional activity of *CYP79B2* or TGAC_v2_mRNA6387, and low levels of IAA promote the transcriptional expression. This assumption was supported by down-regulated expression of *CYP79B2* induced by IAA with *Arabidopsis* eFP Browser (http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi). Another possibility is that other hormones play a role in increasing the expression of *CYP79B2*. In leaf senescence, JA biosynthesis genes are up-regulated, resulting in high levels of JA. The expression of *CYP79B2* was up-regulated by the addition of JA (Jost et al. 2005). Similar hormone functions and regulatory pathways in red clover might cause high expression levels of TGAC_v2_mRNA6387 during the senescence process. In summary, genes involved in hormone biosynthesis play important roles in leaf senescence and function in complicated regulatory mechanisms.

In red clover, many genes involved in different biological processes and pathways were effected during leaf senescence. We used NGS technology to analyze the senescence processes in red clover, and a total of 481 genes were confirmed as DEGs. Furthermore, candidate SAGs, TFs and hormone-related genes were identified in leaf senescence. This study provides the basis for characterization of SAGs and new insights to understand the regulatory mechanism of leaf senescence in red clover. Understanding plant senescence will help in designing strategies to delay this process and improve the yields of legume plants.

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Authors' contribution LH conceived and designed the research. YC, JY, TG and FL conducted experiments. YC and TG analyzed data. YC wrote the manuscript. LX provided suggestion and revised the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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