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Identification of candidate genes associated with senescence in durum wheat (*Triticum turgidum* subsp. *durum*) using cDNA-AFLP

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Abstract Senescence is an integrated response of plants to various internal (developmental) and external (environmental) signals. It is a highly regulated process leading eventually to the death of cells, single organs such as leaves, or even whole plants. In cereals, which are monocarpic plants, senescence represents the final stage of development. In order to study senescence in durum wheat (*Triticum turgidum* subsp. *durum*), a cDNA-AFLP analysis was performed. The transcription profiles of plants at different developmental stages (flowering and senescent) were compared. About 2000 cDNA fragments, ranging in size from 160 to 1900 bp, were reproducibly detected. This allowed the identification of 57 differentially expressed cDNAs corresponding to genes belonging to different functional categories related to cellular metabolism, transcription, maintenance of DNA structure, transport and signal transduction. This paper reports the identification of novel durum wheat candidate genes involved in the senescence process, and provides new information about the senescence programme of this important crop species.

Keywords Candidate genes · cDNA-AFLP · Gene expression · Senescence · *Triticum turgidum* subsp. *durum*

Abbreviations

CAB	Chlorophyll a/b binding protein gene
cDNA-AFLP	cDNA-Amplified fragment length polymorphism
DAF	Days after flowering
EST	Expressed sequence tag
QRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RBCS	Rubisco small subunit gene
Rubisco	Ribulose bisphosphate carboxylase/oxygenase
TdLFC	*T. durum* leaf flowering clone
TdLSC	*T. durum* leaf senescence clone

Introduction

Senescence is the age-dependent sequence of biochemical events associated with deterioration processes leading to the programmed death of cells, tissues, organs, and whole plants.

In cereals, which are monocarpic plants, senescence represents the final stage of development and is a highly regulated process involving a sequence of molecular steps that are programmed at the genetic level. This actively ordered process implies the synthesis of new RNAs and proteins, which are responsible for metabolic changes and the disassembly of the cells. The most significant event at the cellular level is the dismantling of chloroplasts followed by the disruption of the photosynthetic apparatus. At the molecular level the assimilation of carbon is replaced by metabolic activities aimed at the conversion of stored assimilates to the developing grains. Senescence is important for plant life in general and in crop plants, such as

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durum wheat, can be considered of utmost relevance for agricultural productivity related to nutrient use efficiency [1].

Recently, the molecular basis of senescence has been investigated to identify genes involved in both the initiation and regulation of the genetic programme. Genomic approaches in *Arabidopsis* [2–4], and in rice [5], together with the use of mutants [6] have allowed the identification and characterization of many senescence-regulated (up- and down-regulated) genes. Among these, many genes that participate in a complex molecular network of regulation have been identified [7]. These include transcription factors, hormone receptors, hormone signalling components, regulators of metabolism, and components of responses to various stresses. The latter indicates that senescence is not only developmentally regulated, but is also induced by environmental conditions. In fact many studies have shown that various stresses (i.e. drought, heat, UVB radiation, etc.) induce premature senescence [2, 6]. Currently, most of the molecular events regulating senescence are still unknown. Molecular studies of senescence have been carried out on *Arabidopsis*, a dicot plant model that has limited relevance to monocots such as cereals. In recent years studies on monocot plant models such as barley, bread wheat, and rice [8] have begun to approach this problem; however the gene expression profiles of these species are still under investigation [4, 5, 9]. Understanding the regulation of senescence in cereals will enrich our knowledge about this fundamental biological process, and may also be useful for managing the senescence process in important agricultural crops such as durum wheat.

With the aim of identifying genes that are up- and down-regulated during the senescence of durum wheat, the transcription profile of senescent flag leaves was compared with that of non-senescent ones. This analysis was performed by cDNA–AFLP, which is an efficient and sensitive method for sorting out differentially expressed genes, and is often used as a gene discovery tool [10–14]. The differentially expressed transcripts were sequenced, classified, and their expression pattern was analysed. On the basis of these data the involvement of some of them in the senescence process is discussed.

Materials and methods

Plant material and chlorophyll content evaluation

Durum wheat plants (*Triticum turgidum* subsp. *durum*) cv. Trinakria were grown in a glasshouse as already reported [15, 16]. Briefly: plants were grown in glasshouse in 25 cm pots (5 plants/pot) in soil containing slow release fertilizer (3.5 kg Osmocote m^{-3} with 15-11-13 NPK plus micronutrients, under irradiance of ca. 750 μmol $m^{-2}s^{-1}$ (400 W sodium lamps) with 16 h light period, at 18–20°C under lighting and 14–16°C under darkness. At 0, 7, 14, 19, 25 and 29 days after flowering (DAF), the relative chlorophyll content of six flag leaves was determined using the Chlorophyll Meter SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan). Measurements were taken of the apical, central and basal parts of each leaf, and each measurement was repeated three times. After chlorophyll determination, the leaves were detached and separately frozen before RNA extraction.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from flag leaves using the SV Total RNA Isolation System (Promega, Madison, WI) according to the supplier's instruction, and was quantified spectrophotometrically. Semi-quantitative RT-PCR was performed as previously described [17]. In particular amplifications were performed in a thermal cycler (MJ PTC-100; MJ Research, Sierra Point, CA, USA) using one step of 2 min at 94°C, and then a variable number of cycles (21–40) each of 30 s at the annealing temperature optimized for each primer pair, and 60 s at 72°C followed by a final step of 7 min at 72°C. The range at which the amount of the PCR products was exponentially increasing was determined for each gene using a fixed quantity of cDNA and serial numbers of cycles [18]. All the primer sequences are listed in Table S1.

PCR products were separated on 1% (w/v) agarose gels, stained with ethidium bromide, analysed under UV light, and a relative estimate of the amount of cDNA was obtained by the 1D Image Analysis software of Kodak EDAS 290 (Eastman Kodak Company, Rochester, NY).

cDNA–AFLP analysis

First and second strand cDNAs were synthesized on 5 μg of total RNA using a biotinylated oligo-dT primer in streptavidin-coated PCR tubes as described by Feron and co-workers [19] using SuperScript™ III RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA), DNA Polymerase I (Invitrogen), *E. coli* DNA Ligase (Invitrogen), and an mRNA Capture Kit (Roche Diagnostics, Indianapolis, IN). The cDNAs were digested with *Eco*RI (Invitrogen) and released fragments were removed by washing. A second digestion was performed with *Mse*I (Invitrogen), and the cDNA fragments released were collected and subsequently ligated to *Eco*RI and *Mse*I adapters according to the procedure described in the AFLP® Analysis System II (Invitrogen). The pre-amplification reaction was carried out using 30 cycles (94°C, 30 s; 56°C, 1 min; 72°C, 1 min) with

primers corresponding to *Eco*RI and *Mse*I adapters without extension and 1/10 of template volume. Following the pre-amplification step, the product was diluted (25×) with TE buffer and 5 µl were used for selective amplification, using 46 cycles including 14 touchdown cycles comprising a reduction of the annealing temperature from 65°C to 56°C, in 0.7°C steps, which was then maintained for 32 cycles. Sixty-four primer combinations were used for selective amplification; the primers were *Eco*RI-N_2 and *Mse*I-N_3, where N represents the selective nucleotides (A/T/C/G). Selective amplification products were denatured in formamide (50%) at 95°C, separated by electrophoresis in polyacrylamide gel (6%) containing urea and TBE according to standard procedures [20], and visualized by silver staining as previously described [21].

For DNA elution, the bands of interest were excised with a razor blade, incubated in 100 µl of water at 65°C for 15 min and then left overnight at room temperature. The eluted DNA was re-amplified using the same primers as described for the selective amplification using 40 cycles (94°C, 30 s; 56°C, 30 s; 72°C, 1 min). The re-amplified products were checked on 2% (w/v) agarose gels, cloned, and sequenced using standard procedures [20]. The DNA sequences obtained were compared with public databases using the BLAST programme [22].

Quantitative (Real-Time) PCR (QRT-PCR)

QRT-PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), using the v. 1.1 software for data analysis. For each reaction 20 ng of first strand cDNA were used with 1× TaqMan Universal Mastermix (Applied Biosystems), 250 nM probes and 200 nM primers. Primer and probe sequences are reported in Supplementary Table S1. Reaction conditions were: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was amplified in triplicate, and each experiment was repeated at least twice with cDNAs obtained from independent experiments. Transcript levels were normalized with respect to the *a-tubulin* gene. All calculations and statistical analyses were performed as described in the ABI 7500 Sequence Detection System User Bulletin 2 (Applied Biosystems), using the Microsoft Excel programme. The specificity of the amplicons was determined by sequencing.

Results

Assay of leaf senescence

In order to identify the best sample to be used for the cDNA-AFLP, a preliminary evaluation of the senescence stage of the plants was performed. Analysis of senescence was performed using the base of the flag leaf detached from plants grown in a greenhouse, starting from flowering until 29 DAF. Analysis was performed at the physiological level, by evaluating chlorophyll degradation, and at the molecular level, by RT-PCR, to estimate differences in the expression of some senescence-modulated genes.

The chlorophyll content was measured at 0, 7, 14, 19, 25 and 29 DAF; the results obtained are shown in Fig. 1. The chlorophyll content decreased slowly starting from flowering until 25 DAF, when it was 73% of that measured at flowering, and afterwards it fell more rapidly to 40%.

Molecular analysis was performed on genes known to be down-regulated by senescence, i.e. *RBCS* and *CAB* from durum wheat [15], as well as genes that are known to be up-regulated by this process in other plant species, i.e. barley *HvS40* coding for a nuclear protein, *Brassica napus LSC650* coding for a catalase, and *T. aestivum W-ER1* coding for an ethylene receptor. In particular, barley *HvS40* and *B. napus LSC650* sequences were compared with an EST collection of *T. aestivum* by BLASTN. This search identified an EST (GenBank accession no. BF473337) that was 90% homologous to *HvS4*0, allowing the S40f/r primers to be designed. Similarly, a *CATA* sequence of *T. aestivum* (GenBank accession no. X94352), which was 75% homologous to the *LSC650* of *B. napus*, was identified, allowing the design of the primers 650f/r.

The specificity of all the primers was verified by RT-PCR performed on cDNAs obtained from the flag leaves collected at 0 and 29 DAF. Amplicons were cloned and sequenced. The new sequences were registered in GenBank, named *TdER1* (GenBank accession no. AM941021), *TdS40* (GenBank accession no. AM939937), and *Td650* (GenBank accession no. AM939938), and compared to the databases

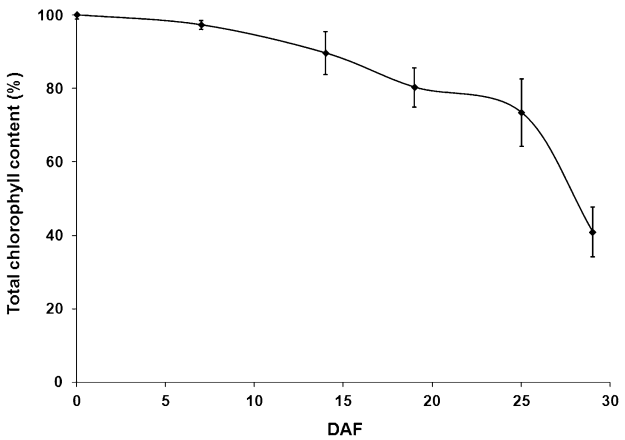

Fig. 1 Chlorophyll content of flag leaves at 0, 7, 14, 19, 25 and 29 DAF. Each value represents the mean of six independent measurements performed as described in the Materials and Methods. Data are presented as the means ± S.E. *DAF* days after flowering

(Table S2). The primers were then used for the RT-PCR analysis to determine the expression of the target genes. As shown in Fig. 2, the level of *TdRBCS* transcripts remained constant until 19 DAF, but at 25 DAF decreased to almost 50%, dropping to 30% at 29 DAF. The *TdCAB* transcripts rapidly decreased at 19 DAF to around 50% and then slowly decreased to 25% at 29 DAF. In contrast, the *TdER1*, *TdS40* and *Td650* transcripts accumulated from 0 to 29 DAF when they reached a maximum.

Analysis of cDNA-AFLP

Differentially expressed cDNAs were detected by selective amplification using 64 primer combinations. About 2000 cDNA fragments, ranging in size from 160 to 1900 bp, were reproducibly detected. Among these, we focused on the 57 most differentially expressed cDNAs. These cDNAs were cloned, sequenced, registered in the database and named *TdLFC (T. durum* Leaf Flowering Clone) if they were expressed at flowering (down-regulated during senescence) or *TdLSC (T. durum* Leaf Senescence Clone) if they were expressed at 29 DAF (up-regulated during senescence); the similarity of the sequences was determined using the BLAST programme against the NCBI, TIGR and RAP-DB databases, as reported in Table 1.

Comparison with sequences available in GenBank allowed the identification of 24 cDNAs exhibiting a high level of similarity with genes of known or putative function; 31 were homologous to uncharacterized genes, such as ESTs expressed under different stress conditions, at flowering, or after flowering, or to genes with an unknown role. Two cDNAs showed no significant matches. The names and characteristics of these cDNAs are reported in Table 1, grouped into different functional categories according to gene annotations from the Gene Ontology database: carbohydrate metabolism, ubiquitin-dependent protein catabolic process, sulphur metabolic process, translation, proteolysis, protein biosynthesis, biosynthesis process, transport, stress response, regulation of transcription, protein amino acid phosphorylation, DNA recombination, DNA repair and chromosome organization, and unknown role. The graphic representation of the distribution of the putative genes belonging to different functional categories is reported in Fig. 3. Listed in Table 1, there are also two cDNAs that did not show significant matches in the database, and thus correspond to new sequences identified as being modulated during senescence in durum wheat.

RT-PCR was carried out on the selected cDNAs (marked with * in Table 1) to confirm the differential expression of the corresponding genes (Fig. S1). This showed that the transcripts were senescence-modulated, with 21 being up-regulated (*Td*LSC) and 12 down-regulated (*Td*LFC).

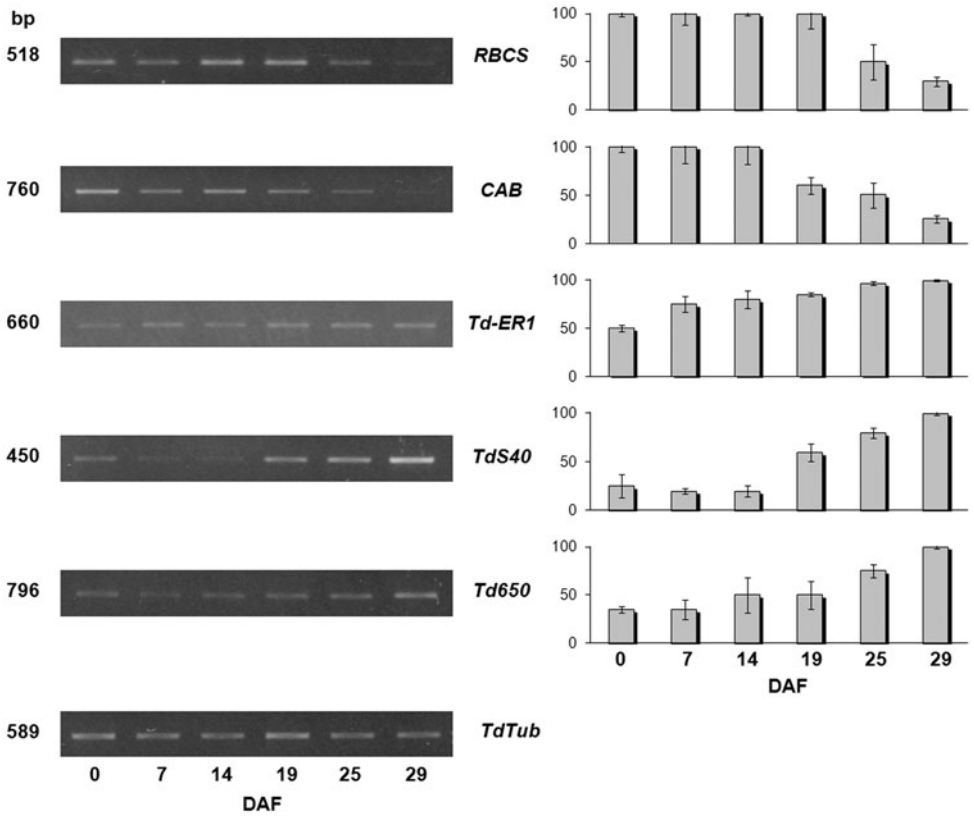

Fig. 2 Expression analysis of *TdRBCS, TdCAB*, *TdER1*, *TdS40* and *Td650* genes. Levels of mRNA were analysed by RT-PCR with specific primers (Supplementary Table S1) for target genes and for α-*tubulin* (*TaTub*), used as an internal control. Band intensities (columns), expressed in pixels, were measured as described in the Materials and Methods, and normalized to α-*tubulin*. Five independent samples for each DAF were analysed in triplicate; each value is the mean ± S.E. The quantified gels are shown on the left. *DAF* days after flowering

Table 1 List of differentially expressed cDNAs, isolated by cDNA-AFLP analysis

Clone name	Accession number	Length (bp)	Homology	BLAST E-value
EST homology				
*Td*LSC1*	AM939939	221	Developing seeds, 5 days after anthesis, from *T. aestivum* (BQ237994)[a]	5e-91
*Td*LSC3*	AM939941	677	Heading stage top three leaves from *H. vulgare* (BJ471515)[a]	3e-62
*Td*LSC5*	AM939943	517	Anthers undergoing meiosis from *T. aestivum* (CA600403)[a]	1e-122
*Td*LSC6*	AM939944	375	Seedling subjected to powdery mildew infection from *T. aestivum* (CJ935708)[a]	0.0
*Td*LSC8*	AM939945	403	Seedling subjected to powdery mildew infection from *T. aestivum* (CJ941094)[a]	1e-137
*Td*LSC16*	AM939947	518	Spikelet at late flowering from *T. aestivum* (BJ300557)[a]	0.0
*Td*LSC21*	AM939952	637	Developing kernel, 7 days after anthesis from *T. aestivum* (CA707847)[a]	8e-152
*Td*LSC22*	AM939953	486	Roots of desiccated seed from *T. aestivum* (CJ583051)[a]	0.0
*Td*LSC26	AM939956	456	Plant cold treated from *T. aestivum* (DY742055)[a]	6e-142
*Td*LFC39*	AM939968	407	Plant subjected to aluminium treatments from *T. aestivum* (CJ785521)[a]	0.0
*Td*LFC55	AM939979	235	Roots of plants subjected to aluminium treatments from *T.* aestivum (CJ846630)[a]	6e-114
*Td*LFC60	AM939983	218	Etiolated seedling from *T. turgidum* subsp. *durum* (FM208338)[a]	2e-83
*Td*LFC61*	AM939984	750	Grain (608 degrees per day after pollination) from *T. aestivum* (CD918953)[a]	0-0
*Td*LFC63	AM939986	167	Pre-anthesis spike cDNA library from *T. aestivum* (BQ170480)[a]	3e-54
*Td*LFC70	AM939992	179	Leaf 7 day old seedling from *T. aestivum* (CA625065)[a]	1e-85
*Td*LFC73	AM939995	232	Heading stage top three leaves from *H. vulgare* (BJ485020)[a]	2e-77
*Td*LFC74	AM939996	235	Anther at meiosis from *T. aestivum* (CJ655820)[a]	4e-87
*Td*LFC80	AM939998	400	Seedling subjected to powdery mildew infection from *T. aestivum* (CJ936387)[a]	3e-09
Carbohydrate metabolism				
*Td*LSC2*	AM939940	187	Xylose isomerase from *H. vulgare* (X95257)[b]	3e-80
*Td*LSC34*	AM939963	464	Putative alkaline alpha-galactosidase seed imbibition protein from *O. sativa* (BAD10122)[c]	1e-44
*Td*LFC56*	AM939980	469	RuBisCO small subunits from *T. aestivum* (AB042069)[b]	2e-155
Ubiquitin-dependent protein catabolic process				
*Td*LSC35	AM939964	473	UBX domain containing protein from *O. sativa* (Os08t0546400-01)[e]	1e-10
*Td*LFC72	AM939994	272	Ariadne from *O. sativa* (BAD34421)[c]	6e-30
Sulfur metabolic process				
*Td*LFC44	AM939970	736	Diphosphonucleotide phosphatase 1 from *T. aestivum* (TA58417_4565)[d]	8.6e-140
Translation				
*Td*LFC45*	AM939971	416	Ribosomal protein L7 from *T. aestivum* (AAW50989)[c]	7e-55
Proteolysis				
*Td*LFC59	AM939982	561	Peptidase C14, caspase catalytic domain containing protein from *O. sativa* (Os03t0388900-01)[e]	1e-53
*Td*LFC67	AM939989	330	Peptidase aspartic, catalytic domain containing protein from *O. sativa* (Os12t0583300-01)[e]	2e-17

Table 1 continued

Clone name	Accession number	Length (bp)	Homology	BLAST E-value
Protein biosynthesis				
*Td*LSC25	AM939955	320	Valyl-tRNA synthetase from *O. sativa* (Os3t0694900-01)[e]	1e-95
Biosynthesis process				
*Td*LSC30	AM939960	341	Putative ethylene-inducible protein from *T. aestivum* (TA50091_4565)[d]	2e-51
*Td*LFC69*	AM939991	391	Carbamoyl-phosphate synthetase small subunit from *O. sativa* (BAD08107)[c]	5e-20
Transport				
*Td*LSC31*	AM939961	461	Potential copper-transporting ATPase from *O. sativa* (BAD09318)[c]	1e-13
*Td*LSC33*	AM939962	423	Nucleobase-ascorbate transporter 11 from *A. thaliana* (NP_195518)[c]	2e-27
*Td*LFC71	AM939993	1221	Ferric reductase from *Z. mays* (NP_001147916)[c]	1e-151
Stress response				
*Td*LFC51	AM939976	410	Cp31 nucleic acid-binding protein from *T. aestivum* (ACO71288)[c]	2e-17
Regulation of transcription				
*Td*LSC11	AM939946	448	Putative cell differentiation protein from *O. sativa* (AK063769)[d]	1e-85
*Td*LSC24*	AM939954	477	NAC2 protein-like from *O.sativa* (TA58381_4565)[d]	1.9e-54
*Td*LFC65*	AM939988	1223	Putative LHY protein from *Z. mays* (NP_001131529[c]	3e-118
Protein amino acid phosphorylation				
*Td*LFC62*	AM939985	168	Protein kinase, core domain containing protein from *O. sativa* (Os05t0481100-01)[e]	1e-26
*Td*LFC64	AM939987	258	SERK1 from *O. sativa* (Os04t0457800-01)[e]	1e-58
DNA recombination, DNA repair, Chromosome organization				
*Td*LSC28*	AM939958	780	SMC6-protein from *O. sativa* (Os09t0121050-00)[e]	1e-54
*Td*LSC29*	AM939959	239	Histone H2A.7 from *T. aestivum* (D38089)[b]	3e-108
*Td*LFC43*	AM939969	261	Putative helicase from *T. aestivum* (TA90056_4565)[d]	5.5e-34
Unknown role				
*Td*LSC17*	AM939948	522	Putative Pi-b protein from *O. sativa* (BAD33147)[c]	1e-13
*Td*LSC18*	AM939949	551	REF/SRPP-like protein from *T. aestivum* (TA52392_4565)[e]	3.2e-100
*Td*LSC19*	AM939950	216	Putative RNA apurinic site specific lyase from *T. turgidum* (AJ612352)[e]	1.1e-40
*Td*LSC20	AM939951	370	CP12 precursor from *T. aestivum* (CA662359)[e]	6.8e-40
*Td*LSC27*	AM939957	407	O-methyltransferase, N-terminal domain containing protein, from *O. sativa* (Os7T0247100-01)[e]	2e-21
*Td*LSC37*	AM939966	928	Retrotransposon protein from *O. sativa* (AAX95804)[c]	4e-50
*Td*LFC38*	AM939967	637	Leucine zipper protein-like from *S. bicolour* (XM_002453334)[b]	7e-64
*Td*LFC49	AM939974	687	HcrVf1 protein-like from *O. sativa* (BAD53108)[c]	2e-56
*Td*LFC50	AM939975	332	AvrRpt2-induced protein 2-like from *T. aestivum* (TA85731_4565)[e]	3.8e-63
*Td*LFC54	AM939978	238	Putative protein from *T. aestivum* (TA473_4565)[d]	3.1e-21
*Td*LFC57*	AM939981	308	Cyclin-like F-box domain containing protein from *O. sativa* (Os01t0550200-00)[e]	1e-92

Table 1 continued

Clone name	Accession number	Length (bp)	Homology	BLAST E-value
*Td*LFC68	AM939990	212	WD40-like domain containing protein from *O. sativa* (Os10t0498700-01)[e]	1e-60
*Td*LSC82*	AM939999	675	*T. aestivum* chromosome 3B-specific BAC library, ctg0954b (FN564434.1)[b]	0.0
No match				
*Td*LFC75*	AM939997	378	No match	
*Td*LFC36*	AM939965	558	No match	

TdLFC T. durum leaf flowering clone (down-regulated during senescence), *TdLSC T. durum* leaf senescent clone (up-regulated during senescence). Confirmation of gene expression performed by semi-quantitative RT-PCR is noted and marked with (*)

[a] Analyses of clone sequences using BLASTN on GenBank NCBI, database est_others

[b] Analyses of clone sequences using BLASTN on GenBank NCBI, database nr

[c] Analyses of clone sequences using BLASTX on GenBank NCBI, database nr

[d] Analyses of clone sequences using BLASTN on TIGR Plant Transcript Assemblies database

[e] Analyses of clone sequences using BLASTN on RAP- DB database

QRT-PCR analysis

The expression of the genes corresponding to three *TdLSC* and one *TdLFC* was further analysed by quantitative RT-PCR. In particular, *TdLFC*75 was chosen as a representative of a gene of novel function, which was down-regulated during senescence; *TdLSC8* and *TdLSC22* were chosen based on their homology to stress-induced ESTs and *TdLSC24* was chosen based on its putative role as a transcription factor. The kinetics of transcript accumulation during senescence are shown in Fig. 4. The quantitative RT-PCR results confirm in general the data obtained by cDNA-AFLP, indicating that expression of the *TdLFC*75 transcripts was inhibited during senescence while the expression of *TdLSC8* increased gradually throughout the senescence process. The expression of *TdLSC22* increased at 7 DAF and remained almost the same until 25 DAF, although a further increase was evident at 29 DAF, whereas *TdLSC24* transcripts dramatically increased during senescence.

Discussion

Senescence can be considered as an integrated response of plants to various internal (developmental) and external (environmental) signals. Therefore, studying the molecular basis of leaf senescence will enhance our understanding of this fundamental biological process. Many different parameters (i.e. chlorophyll content, photochemical efficiency, senescence-associated enzyme activities, change in protein levels and gene expression, etc.) can be measured in order to follow the various physiological and molecular changes that occur during senescence. When approaching the study of this process in cereals leaf senescence has to be measured on a single leaf basis, because the individual leaves of cereals have different ages. In this context we decided to use two parameters: the measurement of chlorophyll content as a physiological marker, and the evaluation of the expression of already known senescence-related genes as a molecular marker. We performed our analyses starting from the flowering stage, which is the stage in the life cycle at which the plant has reached full maturity, until plant death. As far as the chlorophyll content is concerned, it is well known that chloroplast degeneration, which occurs during senescence, is paralleled by chlorophyll degradation and the progressive loss of chloroplast proteins such as Rubisco and CAB. According to the results reported here, the timing of the decrease in the flag leaf chlorophyll content indicates that at 29 DAF plants were definitely senescent. The molecular parameter utilized was the expression of two *T. durum* genes that are related to the photosynthetic process and are

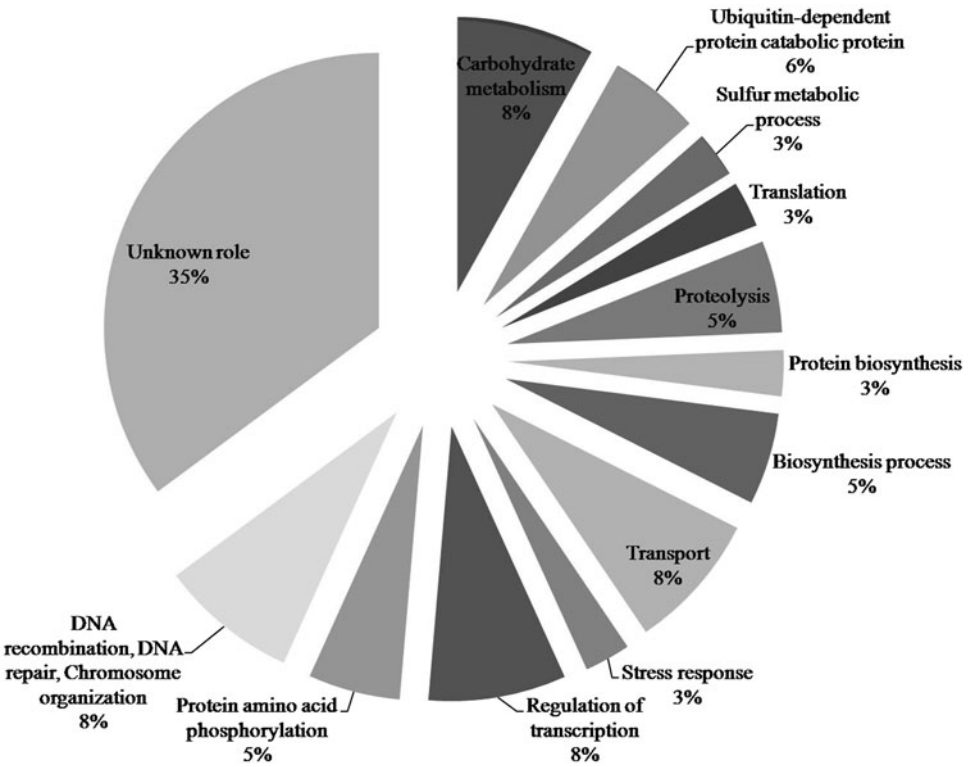

Fig. 3 Functional classification of senescence modulated transcripts from cDNA-AFLP analysis of *T. durum*

inhibited during senescence [15]. Other genes that were chosen because they are up-regulated during senescence in other plant species [23–25] were also used as molecular markers. This analysis was essential to establish the starting material for the subsequent differential gene expression study since it allowed us to define a senescent (29 DAF) and a control (0 DAF) stage.

To date, plant senescence has been studied in the monocot rice model using several approaches (molecular, genetic and genomic); the completion of genome sequencing and transcriptomic studies on rice has allowed the identification of many senescence-related genes [5]. In contrast, in the case of durum wheat there is still a lack of sequence information, and thus differential expression analysis is a valid approach for identifying differentially expressed genes because it does not require prior sequence knowledge. In this context we decided to use cDNA-AFLP technology because it is an extremely powerful and reliable tool for quantitative transcription profiling and for the identification of differentially expressed genes, even if they are only present as a single copy, as previously reported [12, 26, 27]. Using this approach we identified a number of differentially expressed sequences relative to putative genes involved in the senescence process, many of which are putative new genes in durum wheat, and which have been deposited in GenBank (Table 1).

A detailed description of all the senescence-modulated genes is not given here; however some differentially expressed genes are discussed. The first group of cDNAs that are up- and down-regulated during senescence exhibit homology with EST sequences mainly from *T. aestivum* and *H. vulgare* that are related to diverse physiological plant states, such as life stages and/or stress situations. The presence of senescence up-regulated genes that are homologous to ESTs related to stress is consistent with the observation that senescence can also be precociously induced by environmental stresses such as energy deprivation, darkness, excess light, drought, salinity, nutrient limitation and wounding [28–32].

The presence of cDNAs homologous to genes participating in various metabolic pathways indicates that they could be involved in the reduction of normal cellular activities, and/or the start of new activities related to the senescence process. Some of these correspond to genes that have been identified by different approaches and are known to be either down- or up-regulated by senescence in other plant species: i.e. the *Rubisco* gene in bread wheat [4], the *xylose isomerase* gene in *Arabidopsis* [3], and an *alkaline α-galactosidase* gene that is involved in rice leaf senescence [33]. In particular, the *Rubisco* gene is down-regulated and both the *xylose isomerase* and the *alkaline α-galactosidase* genes are up-regulated. The enzymes coded by the latter genes are involved in modification of the cell wall, acting on degradation of plant cell wall galactomannans [34], processes that in many plant species are known to occur during senescence.

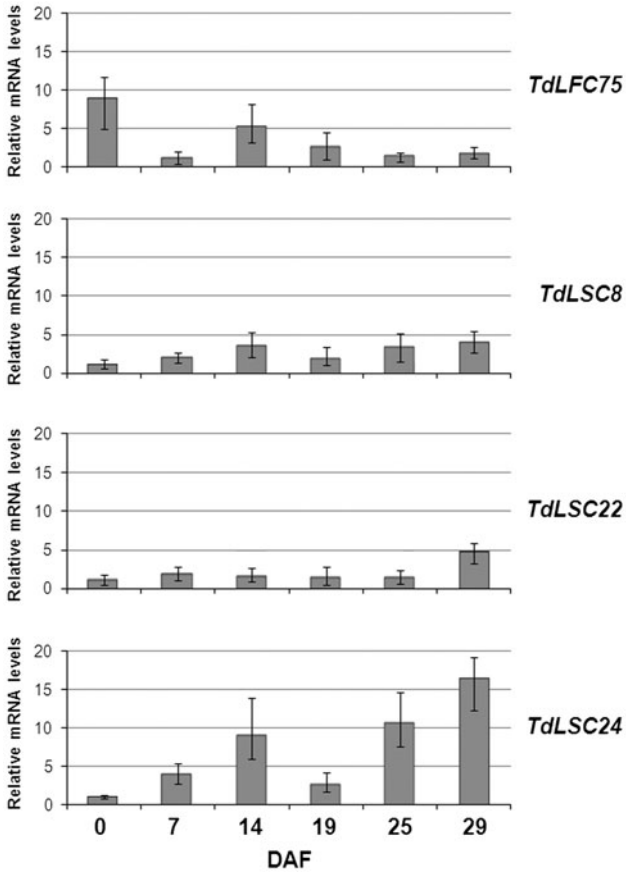

Fig. 4 QRT-PCR of *TdLFC75*, *TdLSC8*, *TdLSC22* and *TDLSC24* at 0, 7, 14, 19, 25 and 29 DAF. The error bar represents ±S.E. from six biological replicates for each sample

Some of the cDNAs identified are related to genes involved in defence and/or resistance mechanisms. Among these, *TdLSC19*, which is induced in senescent leaves, corresponds to a putative RNA apurinic site-specific lyase (RALyase), supporting the data indicating that this enzyme, and/or a ribosome inactivating protein (RIP), could be responsible for the termination of ribosomal activity when, as in plant senescence, programmed cell death occurs [35]. Another is *TdLSC37*, a cDNA that is highly homologous to a rice gene coding a retro-transposon protein, a type of protein that is considered to be a sensitive marker of plant stress [36]. This further confirms the suggestion that there are points of similarity between stress induced events and natural/developmental senescence [1]. The cDNA named *TdLSC17* encodes a putative Pi-b protein, belonging to the plant disease resistance gene (R genes) family. R proteins contain a central nucleotide binding domain, the so called NB-ARC domain; this is a signalling motif that is shared by plant R gene products and animal regulators associated with programmed cell death [37], further confirming the idea that leaf senescence can be considered a form of programmed cell death [38, 39].

Other sequences, namely *TdLFC62*, *TdLFC38* and *TdLSC24*, correspond to genes whose products are implicated in the regulation of transcription such as kinase-like, leucine zipper-like and NAC-like proteins. Our findings are consistent with the data reported in other cereals indicating a functional role for NAC transcription factors in relation to senescence, as already reported in wheat [4, 40, 41].

Two of the differentially expressed cDNAs identified are related to genes that are involved in the maintenance of DNA structure. *TdLSC29* shares homology with a *H2A.7* histone gene, and *TdLSC28* codes a protein sharing high identity with the SMC6 protein from *O. sativa*, a member of the SMC (structural maintenance of chromosome) protein family [42], a group of ubiquitous proteins that play crucial roles in mitotic chromosome dynamics, the regulation of gene expression, and DNA repair [43]. The up-regulation of these genes during senescence could reflect the fact that plants must continue their transcriptional and translational activities during senescence, and therefore need to maintain genome stability for as long as possible.

Two up-regulated cDNAs (*TdLSC31* and *TdLSC33*) correspond to genes related to cell transport mechanisms. Thus *TdLSC31* is homologous to a sequence encoding a putative copper-transporting ATPase, a class of enzymes known to be involved in the mobilization of nutrients to seeds during leaf senescence in *Arabidopsis* [44]. To our knowledge this is the first reported gene coding copper-transporting ATPase in durum wheat. Meanwhile, *TdLSC33* is homologous to a gene encoding a member (NAT11) of the nucleobase-ascorbate transporter (NAT) family, proteins that are involved in nucleobase transport during the cellular activities related to nucleic acid turnover [45]. However, the functions of these genes are still unclear [46]. Although 12 genes encoding for this type of protein were identified in *Arabidopsis* as well as in rice genomes, this is the first time that a NAT coding sequence has been associated with the senescence process.

Taken together the data show that cDNA-AFLP analysis resulted in the identification of new gene functions involved in the senescence process, many of which still do not have a known function. Moreover it is interesting to note that two cDNAs (*TdLFC75*, *TdLSC36*) showed no significant similarity with any sequences in the public databases. These sequences might represent yet uncharacterized genes or sequences corresponding to the less conserved 3' end region of transcripts. This finding is in accordance with data reported for *Brassica* and *Arabidopsis,* in terms of sequences identified by cDNA-AFLP that do not show any correspondence with the annotated *Arabidopsis* sequence databases [47, 48]. Comparison of our data with those obtained in bread wheat [4] identified only three overlapping sequences, namely the down-regulated *Rubisco*, the up-regulated α-*galactosidase*, and a member

of the *NAC* family transcription factors. All the other sequences reported here were associated with senescence for the first time.

During this study we also determined the kinetics of transcript accumulation of some of the genes that are differentially expressed during senescence. The results of this analysis confirmed the modulation of gene expression during senescence for both down- and up-regulated cDNAs. In particular, the strong induction of a homologue of the senescence-associated transcription factors belonging to the NAC family (*TdLSC24*), as also reported for many other species [4, 29, 40, 49, 50], implies conservation of regulatory mechanisms involved in senescence in distantly related species. It also indicates that understanding the regulatory network by which transcription factors, in particular the NACs, are coordinated may shed new light on the regulation of senescence.

In conclusion, this paper describes the first large-scale investigation into the molecular basis of senescence in durum wheat. A significant proportion of the differentially expressed cDNAs were not represented in durum wheat sequence databases. Some of the novel transcripts identified had never been reported as being associated with the senescence process. Although further analyses are required to completely characterize the newly identified durum wheat genes, this work adds new information about the senescence programme in this important crop plant.

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