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RESEARCH ARTICLE

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Identification of senescence-related genes by cDNA-AFLP in cotton (*Gossypium hirsutum* L.)

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Abstract Premature senescence at the late developmental stage occurs frequently in cotton (*Gossypium hirsutum* L.) production in North China. It is desirable to develop elite cotton cultivars with non-premature senescence and high photosynthetic capacity. In this study, cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis was employed to identify the genes that are related to senescence in cotton. Using 64 primer combinations, about 3000 cDNA fragments were generated, and among them 42 had a markedly up-regulated expression pattern with the leaf growth progression. Based on cloning, sequencing, and Blast search analysis, it was determined that 24 TDFs with putative known biologic functions could be classified into several major categories, such as signal transduction, transcription regulation, stress-responsive, primary and secondary metabolism, nutrients recycling, photosynthesis, cell wall biosynthesis, and senescence-related. TDF31, TDF32 and TDF33, with high similarity to the senescent-regulating genes MAP kinase 9 (*MKK9*) and non-yellowing protein 1(*NYE1*) from *Arabidopsis* and bean senescence-associated receptor-like kinase (*SARK*) could play possible roles in responding or modulating the leaf senescence in cotton. Therefore, leaf senescence in cotton is a complicated network involving many biological processes. Some putative genes with important modulation functions in regulating or responding to the senescence need to be further analyzed.

Keywords cotton (*Gossypium hirsutum* L.), senescence, cDNA-AFLP, gene expression, senescence-related transcript-derived fragments (TDFs)

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1 Introduction

As one of the most important fiber crops, cotton (*G. hirsutum* L.) has been widely planted in the world. In North China, increased fiber yield of cotton plays an important role in promoting the sustainable development of local agriculture. But, premature senescence of plants at the late developmental stage, resulting from the affects of cultivars, growth conditions, and unsuitable cultivations, has become a limiting factor for improvement of cotton fiber yield and cotton quality (Wright et al., 1999; Liu et al., 2009). Delaying of leaf senescence combined with a higher plant photosynthetic capacity at the late growth stage, will increase cotton fiber yield and improve cotton fiber quality.

For the past several years, studies have been conducted to explore the genetic biodiversity (Yu et al., 1994) and physiological and biochemical characterizations of cotton’s premature senescence (Liu et al., 2007). But little is known of the molecular mechanism that regulates the leaf or plant premature senescence in cotton. Thus, systematic identification of the senescence-related or responding genes in cotton will be helpful in exploring the molecular characteristics related to cotton premature senescence at the late developmental stage and provide a basis for genetic improvement of cotton cultivars with non-premature senescence and higher productivity.

In *Arabidopsis*, it was found that leaf senescence is a developmentally programmed degeneration process and is controlled by multiple developmental and environmental

signals (Lim et al., 2003). During senescence, leaf cells undergo dramatic changes in cellular metabolism and a sequential degeneration of cellular structures (Nam, 1997). At the molecular level, the leaf senescence begins with the identification of senescence-associated genes (*SAGs*). The *SAGs* showed an up-regulated expression pattern during leaf senescence. Functional classification analysis indicated that most *SAGs* are encoded with proteases, nucleases, lipases, hydrolases, enzymes involving nutrient recycling, stress-responsive proteins, and transcriptional regulators (Nam, 1997; Quirino et al., 2000; Buchanan-Wollaston et al., 2003; Gepstein et al., 2003; Lin et al., 2004). These results implied that some *SAGs* acted as key regulators during leaf senescence and may take active roles in regulating the plant senescence.

cDNA-amplified fragment length polymorphism (cDNA-AFLP) is an efficient, sensitive, and reproducible technology for the isolation of differentially expressed genes (Bachem et al., 1996). This approach does not require prior known genome or cDNA sequence information and has been used as a powerful tool for the identification of novel genes (Ditt et al., 2001; Jayaraman et al., 2008; Gupta et al., 2009; Li et al., 2009). In this study, cDNA-AFLP was employed to identify genes that exhibited a modulated expression following the leaf senescence in cotton. The identification and characterization of senescent-related genes may be helpful for a better understanding of the mechanisms of leaf senescence at the molecular level and provide a theoretical basis for generation of elite cotton lines with non-premature senescent traits and high photosynthetic capacity.

2 Materials and methods

2.1 Plant materials and culture conditions

Cotton (*G. hirsutum* L.) cv. 33B was selected in this study because previous work indicated this cultivar has potential premature senescence at the developmental stage under field cultivation condition. Seeds were rinsed with distilled water and incubated on soaked 3MM Whatman paper (Whatman, Maidstone, UK) in Petri dishes at 26°C for 5 d. Germinated seeds were then sown in pots filled with sand and vermiculite (1:1), with one plant in each pot. Plants were grown in a growth room under a diurnal photoperiod of 12 h light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The daily maximum and minimum temperatures were 28°C and 22°C, respectively. The relative humidity ranged from 65% to 85%. Hoagland solution with pH 5.7 was irrigated twice weekly to supply the nutrients. To identify the gene with transcriptional changes corresponding to leaf senescence, plants with the third leaf fully-expanded (0 d) were initiated for sampling. After 15 d, 30 d, and 45 d, the third leaves were harvested separately. The samples were quickly frozen in liquid nitrogen and stored at −80°C for RNA extraction.

2.2 Measurement of chlorophyll content

The chlorophyll content of samples harvested at various time points was measured non-destructively using a SPAD-502 Chlorophyll Meter (Minolta, Japan). Chlorophyll a and b contents were calculated from their absorption spectra as described by Lichtenthaler (1984). Mean SPAD readings for each leaf were plotted against total chlorophyll (a + b) content expressed on a fresh weight (FW) basis.

2.3 mRNA isolation and cDNA synthesis

Total RNA was extracted from the leaf samples harvested at various time periods using Trizol reagent (Gibco, Germany). Total RNA concentration was determined spectrophotometrically and adjusted to a final concentration of 1 $\mu g \cdot \mu L^{-1}$. Poly $(A)^+$ RNA fractions were isolated from 1 mg total RNA with the Oligotex mRNA Minikit (QIAGEN, Germany). First and second cDNA strands were synthesized by using M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan).

2.4 cDNA-AFLP analysis

The cDNA-AFLP-based transcript profiling procedure was performed according to the method described in Breyne et al. (2002). Double-stranded cDNA (500 ng) was used for cDNA-AFLP analysis. The double-stranded cDNA was digested by restriction enzymes of *Pst*I and *Mse*I (TaKaRa, Japan), and then ligated with the adaptors. The adaptors for *Pst*I were 5'-CTCGTAGACTGCGTACATGCA (forward) and 5'-TGTACGCAGTCTAC (reverse). The adaptors for *Mse*I were 5'-GACGATGAGTCCTGAG (forward) and 5'-TACTCAGGACTCAT (reverse). The pre-amplification with primer M00(5' -GATGAGTCCTGAGTA) and P00(5' -AGACTGCGTACATGCAG) was conducted according to following procedure: 95°C for 2 min, followed 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The PCR products were diluted 600-fold and 5 µL was used for final selective amplifications according to Breyne et al. (2002). A total of eight *Pst*I primers and eight *Mse*I primers with one or two selective nucleotide, respectively, were used for the cDNA-AFLP analysis (Table 1), and all 64 possible primer combinations were performed. The amplified products were separated on a 6% polyacrylamide gel run at 80 W for about 2 h. Gels underwent silver staining according to the standard protocol, and to capture the image by scanning.

2.5 Isolation and sequencing of fragments

The bands of interest in the gels, showing to be intensified with the leaf growth progression, were cut out with a razor blade, and incubated in 100 µL water at 94°C for 10 min. The DNA was eluted by centrifugation at 10000×g for

Table 1 Primers used in the selective amplification

M primer	P primer
M22:GAT GAG TCC TGA GTA GT	P19:AGA CTG CGT ACA TGC AGG A
M24:GAT GAG TCC TGA GTA TC	P25:AGA CTG CGT ACA TGC AGT G
M31:GAT GAG TCC TGA GTA AAA	P31:AGA CTG CGT ACA TGC AGA AA
M33:GAT GAG TCC TGA GTA AAG	P33:AGA CTG CGT ACA TGC AGA AG
M42:GAT GAG TCC TGA GTA AGT	P34:AGA CTG CGT ACA TGC AGA AT
M43:GAT GAG TCC TGA GTA ATA	P39:AGA CTG CGT ACA TGC AGA GA
M44:GAT GAG TCC TGA GTA ATC	P43:AGA CTG CGT ACA TGC AGA TA
M45:GAT GAG TCC TGA GTA ATG	P47:AGA CTG CGT ACA TGC AGC AA

5 min and was then used as the template for re-amplification by PCR with the same PCR conditions and primer combination as the selective amplification. The re-amplified products representing the senescence-related transcript-derived fragments (TDFs) were checked on 2% agarose gel and ligated to pUCm-T vector to transform competent *E. coli* strain DH5, and finally to be sequenced in Sangon (Shanghai, China) using the M13 forward primer.

2.6 Homology search of the differential TDFs

Database searches were performed using the BLAST Network service [NCBI (National Center for Biotechnology Information); http://www.ncbi.nlm.nih. gov/BLAST]. The sequence of each TDF was searched against all sequences in the databases using the BLASTN programs (Altschul et al., 1997). The putative biologic function of the identified TDFs was determined based on the BLAST results that the homologous had the highest similarity and the E-value was lower than 1e-07.

2.7 RT-PCR analysis

To verify the expression of candidate genes, first-strand cDNA from different leaf samples used in cDNA-AFLP analysis was generated from 3 µg RNA samples using the Superscript III RT (Invitrogen). The products were adjusted to initial RNA concentration of $2\ ng\cdot\mu L^{-1}$ for RT-PCR. Gene-specific primers were designed according to the TDFs sequences using the DNAStar Software and synthesized commercially (Shangon Biotechnology, Shanghai, China). The TDFs used for transcript detection were TDF31, TDF32, and TDF33, with high similarity to *Arabidopsis* MAP kinase kinase 9 (*MKK9*), *Arabidopsis* non-yellowing protein 1(*NYE1*), and Arabidopsis *SAG12* (SENESCENCE-ASSOCIATED GENE 12), respectively. The PCR was conducted in a 20-L volume containing $1.6\ mmol\cdot L^{-1}$ dNTP mix, 20 ng plasmid DNA harboring the respective TDFs cDNA, $0.5\ \mu mol\cdot L^{-1}$ forward primer and reverse primer each, and 1 unit of Taq DNA polymerase (TaKaRa). The PCR reactions were performed under the following conditions: 94°C for 3 min, followed 25 cycles of 94°C for 30 s, 53°C for 30 s, and 30 s for 72°C. Products (8 µL) for each sample were then electrophoresed in a 1% ethidium bromide agarose gel and recorded under DNA gel analyzer. For comparison of the gene expression levels at various growth phases, the constitutive expressed gene tubulin in wheat was used as the internal standard, the primers for RT-PCR analysis of the wheat tubulin gene were as follows: 5'-AGAA-CACTGTTGTAAGGCTCAAC (forward) and 5'-GAGCTTTACTGCCTCGAACATGG (reverse).

3 Results

3.1 Changes of chlorophyll content in the third leaf with the growth progression

From the leaf full expansion (0 d) to 45 d after leaf expansion (45 d), the contents of chlorophyll a (Chla), b (Chlb) and Chla + Chlb were all gradually decreased with the growth progression. There was a little difference at the dropping rate of above parameters, in which the Chla and Chla + Chlb dropped a little faster than Chlb (Fig.1). The gradually degradation of chlorophyll indicated that the leaf was gradually senescent after the leaf's full expansion.

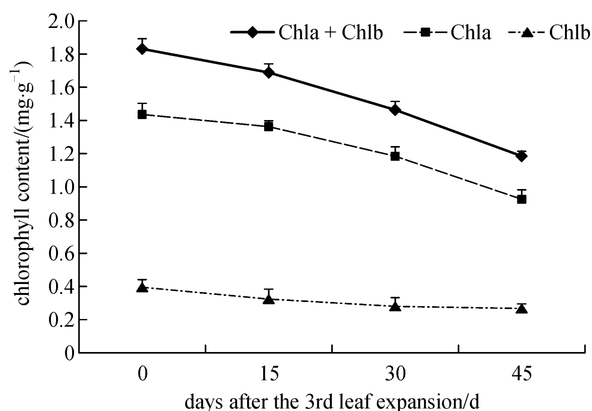

Fig. 1 The changes of contents of chlorophyll a (Chla), chlorophyll b (Chlb), and total chlorophyll (Chla + Chlb) during the leaf growth progression

3.2 Analysis of cDNA-AFLP

cDNA-AFLP analysis was performed to identify the genes during the leaf senescence. The representative selected-PCR amplification results at different leaf growth phase were showed in Fig. 2. Using 64 primer combinations, about 3000 cDNA fragments were counted and all bands longer than 80 bp were compared in the four leaf samples with different growth phases.

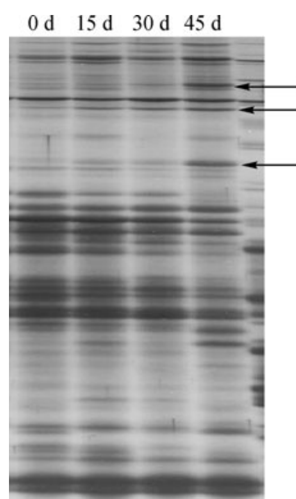

Fig. 2 cDNA-AFLP image showing one primer combination (M24 × P19) of the TDFs with changeable expression patterns along with the leaf growth progression. The arrow heads show the representative upregulated TDFs.

3.3 Identification of differential expressed TDFs

In total, 43 upregulated gene fragments (TDFs) were identified as senescence modulated. The upregulated gene fragments were re-amplified, cloned and sequenced. Blast search analysis revealed that 33 of the total differential expressed genes have putative functions. The putative gene functions of the differentially expressed ESTs (TDFs) are listed in Table 2. Similar to *Arabidopsis SAG12* (SENESCENCE-ASSOCIATED GENE 12, GenBank accession number XM_002519335), a gene fragment (TDF30) with high similarity to *SAG12*, has also been markedly upregulated with the leaf growth progression, suggesting that the gene corresponding to TDF30 should have been senescence-associated in cotton. In the meantime, several TDFs with high similarity to the important senescent-regulated or senescence-responding genes, such as TDF31, TDF32 and TDF33, with high similarity to *Arabidopsis* MAP kinase kinase 9 (*MKK9*, GenBank accession number NM_106009), *Arabidopsis* non-yellowing protein 1(*NYE1*, GenBank accession number NM_118421), and bean senescence-associated receptor-like kinase (*SARK*) respectively, showed markedly upregulated expression pattern. Therefore, the genes corresponding to TDF31, TDF32, and TDF33 could play important roles in responding to or modulating the leaf senescence in cotton.

3.4 RT-PCR analysis of several senescence-regulated genes

To validate the cDNA-AFLP expression patterns, several TDFs were selected for further semiquantitative PCR analysis. Four TDFs with putative important senescent-responding or regulated functions, including *Arabidopsis* senescence-associated gene 12 (*SAG12*, TDF30), *Arabidopsis* motigen-activaten kinase kinase 9 (*MKK9*, TDF31), the bean (*Phaseolus vulgaris*) senescence-associated receptor-like kinase (*SARK*, TDF32), and *Arabidopsis* non-yellowing protein 1(*NYE1*, TDF33), were selected as the targets for further RT-PCR analysis.

The RT-PCR results at the growth phase of 0 d (leaf expansion), 15 d (15 d after leaf expansion), 30 d (30 d after leaf expansion), and 45 d (45 d after leaf expansion) were shown in Fig. 3. The results of RT-PCR in the four tested TDFs were all similar to those in cDNA-AFLP analysis, suggesting that the TDFs identified in this study based on cDNA-AFLP approach were reliable and could be confirmed. Thus, the cDNA-AFLP technique is powerful in isolation of differentially expressed genes of senescence-responding and modulation in cotton, and is feasible for exploring the differential expressed genes responding to biotic/abiotic stresses in other crops.

3.5 Functional classification of the cotton senescent-responsive genes

Of the 43 sequenced senescence-modulated genes, 24 had known or putative functions, 9 had high hits in the GenBank without known biologic functions, and 10 had no homology in the GenBank (Table 3). Thus, the ratio of the upregulated genes with the known biologic functions to the total was 55.81% in this study.

Based on the functional classification of the genes in *Arabidopsis*, the 24 TDFs with known functions could be grouped into eight major functional categories, including signal transduction, transcriptional regulation, stress-responsive, primary and secondary metabolism, nutrients recycling, photosynthesis, cell wall biosynthesis, and senescence-related. The gene numbers varied in each category. Signal transduction and transcriptional regulation both had three numbers; nutrients recycling, photosynthesis, and senescence-related all had two numbers; stress response had five numbers; primary and second metabolism had six numbers; and cell wall biosynthesis had one number (Table 3). Therefore, the leaf senescence in cotton is a complicated network involving many biologic processes, such as signal transduction, transcription regulation, and biochemical metabolism.

Table 2 Homologies of sequences of cDNA-AFLP fragments to sequences in the databases

TDF number	length/bp	accession number	homology	blast score	expression pattern
TDF1	177	EF643509.1	*G. hirsutum* S-adenosylmethionine synthetase (SAMS) mRNA	5e-64	15d, 30d
TDF2	229	CU229683.1	*P. tremula* × *P. alba* EST from mild drought-stressed leaves	5e-15	15d, 30d
TDF3	229	NM_117129.1	*A. thaliana* CID12; RNA binding (CID12) mRNA	3e-11	15d, 30d
TDF4	264	NM_118987.4	*A. thaliana* transducin family protein /WD-40 repeat family protein (AT4G28450) mRNA	2e-57	15d, 30d
TDF5	264	AY062623.1	*A. thaliana* SOF1 protein-like protein (F20O9.130) mRNA	2e-57	15d, 30d
TDF6	206	AF139469.2	*V. radiata* ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) mRNA	1e-08	15d
TDF7	135	EF470293.1	*G. hirsutum* aquaporin PIP1-2 mRNA	3e-46	15d
TDF8	243	AM434998.2	*V. vinifera* contig VV79X009893.2, whole genome shotgun sequence	3e-12	15d, 30d
TDF9	206	AM487181.2	*V. vinifera* contig VV78X103755.56, whole genome shotgun sequence	1e-14	15d, 30d
TDF10	183	EF691473.1	*T. repens* clone CloverSSR-5_G01_002_1 microsatellite sequence	1e-08	15d, 30d, 45d
TDF11	205	EF415945.1	*S. rostrata* cDNA-AFLP fragment 026BT11M41-177.5 genomic sequence	1e-14	15d, 30d
TDF12	142	AB158409.1	*T. aestivum* GRP mRNA for putative glycine-rich protein	9e-15	15d, 30d, 45d
TDF13	125	AY463623.1	*A. thaliana* TAF8 (TAF8) mRNA, partial cds	9e-08	15d, 30d, 45d
TDF14	142	AB066265.2	*T. aestivum* WCSP1 mRNA for cold shock protein-1	5e-50	15d, 30d, 45d
TDF15	229	NM117129.2	*A. thaliana* CID12; RNA binding (CID12) mRNA	3e-11	15d, 30d, 45d
TDF16	279	AF511408.1	*G. hirsutum* endo-1,4-beta-glucanase mRNA	2e-91	15d, 30d, 45d
TDF17	282	EF379384.1	*A. chinensis* VTC2-like protein mRNA	2e-27	15d
TDF18	282	CU229541.1	*Populus* EST from mild drought-stressed leaves	1e-16	15d, 30d, 45d
TDF19	181	NM_104604.3	*A. thaliana* WD-40 repeat family protein /beige-related (AT1G58230) mRNA	1e-15	15d, 30d, 45d
TDF20	166	AF186183.1	*G. max* retrovirus-like element Calypso2-1	1e-08	15d
TDF21	126	AY436777.1	*P. communis* ferredoxin-dependent glutamate synthase-like mRNA	1e-8	15d, 30d, 45d
TDF22	155	AC216405.1	*P. trichocarpa* clone ACSB1217-K12	6e-11	15d, 30d, 45d
TDF23	142	EF691459.1	*T. repens* clone CloverSSR-5_E09_036_1 microsatellite sequence	1e-08	15d, 30d, 45d
TDF24	223	M86213.1	*G. hirsutum* 2S albumin storage protein (Mat5-A) gene	3e-08	15d, 30d, 45d
TDF25	223	EF457754.1	*G. hirsutum* putative calcium binding protein gene	2e-07	15d, 30d, 45d
TDF26	223	AJ132636.1	*G. hirsutum* sad1 gene, exons 1-3	1e-16	15d
TDF27	302	DQ245361.1	*Zea mays* clone 14758 mRNA sequence	1e-106	15d, 30d
TDF28	183	EU417410.1	*T. repens* clone CloverSSR-077_G02_002_1 microsatellite sequence	1e-08	15d, 30d
TDF29	230	NM001060850.	*O. sativa* Os04 g0685100 mRNA	7e-13	15d, 30d
TDF30	222	NM_123957	*A. thaliana* SAG12 (SENESCENCE-ASSOCIATED GENE 12)	5e-46	15d, 30d, 45d
TDF31	266	NM_106009	*A. thaliana* MKK9	2e-26	15d, 30d, 45d
TDF32	478	NM_118421	*A. thaliana* non-yellowing protein 1(NYE1)	1e-27	15d, 30d, 45d
TDF33	173	AF285172	*P. vulgaris* leaf senescence-associated receptor-like protein (SARK)	1e-24	15d, 30d, 45d
TDF34	136	No homology			
TDF35	170	No homology			
TDF36	144	No homology			
TDF37	233	No homology			
TDF38	245	No homology			
TDF39	268	No homology			
TDF40	424	No homology			
TDF41	245	No homology			
TDF42	532	No homology			
TDF43	378	No homology			

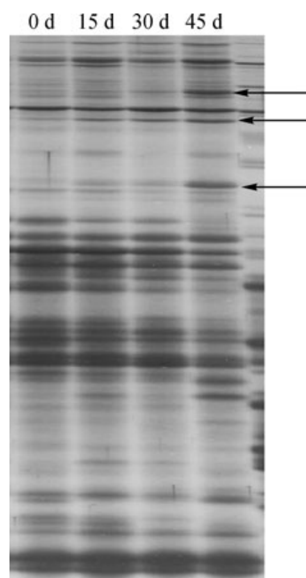

Fig. 3 Semiquantitative RT-PCR analysis of several upregulated TDFS along with the leaf growth progression

4 Discussion

Leaf senescence is a developmentally programmed degeneration process that constitutes the final step of leaf development and is controlled by multiple developmental and environmental signals (Lim et al., 2003). It is clearly noted that the leaf cells undergo dramatic changes in cellular metabolism and a sequential degeneration of cellular structures during senescence (Nam, 1997). Using suppression subtractive hybridization, Gepstein et al. (2003) isolated approximately 800 cDNA clones representing senescence-associated genes (*SAGs*) expressed in *Arabidopsis* senescing leaves. Differential expression was confirmed for the 130 non-redundant genes and over 70 of the identified genes have been shown to participate in the senescence process. Analyses of expression profiles of those SAGs indicated a complex regulatory network of functions in leaf senescence processes (Park et al., 1998; Weaver et al., 1998; Quirino et al., 1999; He et al., 2001; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005). In our study, we have identified 43 TDFs that markedly upregulated during the leaf senescence in cotton cultivar 33B. Among the 36 TDFs with putative known functions, various functional categories could be classified, including signal transduction, transcriptional regulation, stress-responsive, primary and secondary metabolism, nutrients recycling, photosynthesis, cell wall biosynthesis, and senescence-associated. Therefore, similar to that in *Arabidopsis*, the leaf senescence in cotton leaf is a complex regulatory network modulated by a large number of genes with different biologic functions.

It is suggested that some key regulators may take active roles in regulating senescence among the whole differential expressed genes which are responded to or regulated by senescence. Several important senescence-associated regulatory genes have recently been identified and functionally analyzed, such as *Arabidopsis* transcription factors *WRKY6* (WRKY DNA binding protein 6; Robatzek and Somssich, 2001), *WRKY53* (Hinderhofer et al., 2001; Miao et al., 2004), and rice Nuclear-Localized CCCH-Type Zinc Finger Protein, *OsDOS* (Kong et al., 2006), *Arabidopsis* receptor-like kinases *SIRK* (senescence-induced receptor-like kinase; Robatzek and Somssich, 2002), *Arabidopsis* motigen-activaten kinase kinase 9 (*MKK9*, Zhou et al., 2009), the bean (*Phaseolus vulgaris*) *SARK* (senescence-associated receptor-like kinase; Hajouj et al., 2000), and Arabidopsis non-yellowing protein 1(*NYE1*) (Ren et al., 2007).

Similar to other known developmental programs in plants, the senescence program may also express components of signal transduction pathways. During the senescence process, the cell first perceives a signal(s), and then triggers the transduction cascade by mediating the induction, or increasing the levels of certain

Table 3 Functional classification of senescence-related gene product in cotton

function category	TDFs	total number	%
signal transduction	4, 25, 31	3	6.98
transcriptional regulation	3, 15, 19	3	6.98
stress-responsive	2, 14, 16, 18, 20	5	11.63
primary and secondary metabolism	1, 5, 7, 13, 17, 26	6	13.95
nutrients recycling	21, 24	2	4.65
photosynthesis	6, 32	2	4.65
cell wall biosynthesis	12	1	2.33
senescence-related	30, 33	2	4.65
unknown	8, 9, 10, 11, 22, 23, 27, 28, 29	9	20.93
no homology	34, 35, 36. 37, 38, 39, 40, 41, 42, 43	10	23.26
total		43	100

proteins encoded by SAGs. This eventually leads to the biochemical, physiologic, and morphological changes of senescence (Smart, 1994; Gan and Amasino, 1995; Buchanan-Wollaston, 1997; Nam, 1997; Nooden et al., 1997). The MAPK cascade is widely involved in various biotic and abiotic stress responses, hormone responses, cell proliferation, differentiation, and developmental processes in plants (Nakagami et al., 2005). In *Arabidopsis*, 20 MAPKs, 10 MAPKKs, and more than 60 MAPKKKs have been identified (Ichimura et al., 2002). One member of *Arabidopsis* MAPKK subgroup, MKK9, plays several roles in the signal transductions, such as to be the negative regulator of abiotic stress responses (Alzwiya and Morris, 2007), transduction of the ethylene signaling via a cascade of MKK9-MPK3/MPK6 (Yoo et al., 2008). Recently, an important role of MKK9 has been figured out in the regulation of leaf senescence by a cascade of MKK9-MPK6 in *Arabidopsis*. Thus, the TDF31, with high similarity to *Arabidopsis* MKK9 identified in this study, is possibly involved in regulating the leaf senescence in cotton by adopting similar molecular mechanism.

Chlorophyll (Chl) catabolism has been known as a separable event from other senescence processes in senescing vegetative plant organs and ripening fruits (Thomas et al., 1975; Thomas et al., 2000). Extensive chemical and biochemical analyses have led to the construction of a major Chl catabolism pathway, i.e. pheophorbide a oxygenase (PaO) pathway (Hörtensteiner, 2006; Tanaka et al., 2006). An alternative way to explore the regulatory mechanism of Chl catabolism is to identify genes directly regulating Chl degradation during senescence through molecular techniques. Ren et al. (2007) isolated the causal gene *AtNYE1* by positional cloning and confirmed its identity by genomic complementation, based on a nonyellowing mutant (nye1-1) from a fast-neutron mutagenized M2 population of *Arabidopsis*. Overexpression of *AtNYE1* caused either pale-yellow true leaves or even albino seedlings. We have identified an upregulated expressed gene (TDF32) with high similarity to *AtNYE1*, suggesting that this gene responds to the senescence cue and is involved in the modulation of chloroplast degradation during the leaf senescence in cotton. The distinct molecular characterization of the gene corresponding to TDF32 needs to be studied further.

Genes encoding proteins involved in signal transduction of senescence have not yet been identified, probably due in part to their low transcript abundance relative to transcripts of genes that participate in the metabolic processes themselves (Buchanan-Wollaston, 1997; Nam, 1997; Nooden et al., 1997). In bean, a senescence-associated receptor-like kinase (*SARK*) showed an induced expression prior to the loss of chlorophyll and the decrease of chlorophyll a/b binding protein mRNA. Detached mature bean leaves, which senesce at an accelerated rate compared with leaves on intact plants, showed a similar temporal pattern of *SARK* message accumulation. In the meantime, light and cytokinin, which delayed the initiation of leaf senescence, also delayed *SARK* gene expression; in contrast, darkness and ethylene, which accelerated senescence, advanced the initial appearance of the SARK transcript and the protein accumulation (Hajouj et al., 2000). This suggests that the SARK acts as an important senescence signal transducer in bean. Here, we identified that one of the TDFs (TDF33) showed a high similarity to the bean *SARK*, with an upregulated expression pattern following the leaf senescence progression. It suggests that the TDF33 gene in cotton may also act as one of the important signal transducers in leaf senescence in cotton.

In conclusion, this study verifies that cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis is a powerful tool for identification of differential expression genes on a large scale in cotton. During the leaf senescence, lots of genes showed a changeable transcription pattern. Some upregulated genes with putative known biologic functions could be functionally classified into various categories, including signal transduction, transcriptional regulation, stress response, primary and secondary metabolism, photosynthesis, cell wall biosynthesis, nutrients recycling, and senescence-related. Several differential expressed genes with high similarity to those functionally analyzed previously, such as TDF31, TDF32, and TDF33, could play various roles by mediation of signal transduction and regulation of chloroplast degradation in cotton. The distinct biologic functions of the above putative important senescent-regulating genes need to be further elucidated in the future.

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