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Analysis of differentially expressed genes in response to endogenous cytokinins during cotton leaf senescence

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Abstract

Cytokinins have been implicated in delaying leaf senescence. We previously generated transgenic cotton (*Gossypium hirsutum* L.) plants that harbor the *Agrobacterium* isopentenyl transferase gene (*ipt*) directed by a proteinase gene promoter. Here, we report that mRNAs were isolated from *ipt* cotton leaves and azygous leaves and were subsequently sequenced using *Illumina Solexa* technology. The sequence tags were searched against the *TIGR* database and the related gene expression profiles were compared resulting in the identification of 1 218 differentially expressed genes (DEGs): 719 up-regulated and 499 down-regulated. Analyzing the DEGs in the *ipt* cotton leaves showed that these genes belonged to four pathways: flavone biosynthesis, arginine and proline metabolism, glyoxylate and dicarboxylate metabolism, and RNA degradation. These pathways increased the activities of antioxidants, inhibited the effect of ethylene, and prevented degradation of macromolecules during senescence. The expression patterns of 17 genes were evaluated by real-time PCR and results were in agreement with the patterns of sequencing analysis. The identification of the DEGs may help us to understand a role of cytokinins in leaf senescence.

Additional key words: *Gossypium hirsutum,* metabolic pathways, *Solexa* sequencing, *TIGR* database, transgenic plants.

Introduction

Leaf senescence is not a simple degenerative process but plays an important role in the recycling of nutrients from old, non-functional leaves to young leaves and developing seeds (Liu *et al*. 2008). Senescence is influenced by the endogenous gene expression and by various environmental factors (Nooden *et al*. 1997, Hayano-Kanashiro *et al*. 2009). One strategy of delaying senescence involves the transgenic expression of an isopentenyl transferase gene (*ipt*) from *Agrobacterium tumefaciens* which encodes a rate-limiting enzyme in the cytokinin biosynthetic pathway (Gray 2004, Belintani *et al*. 2012). To date, the introduction of the *ipt* gene linked to senescence-associated gene promoters has been reported in many plant species, *e.g.*, lettuce (McCabe *et al.* 2001), rice (Lin *et al.* 2002), petunia (Chang *et al*. 2003), Italian ryegrass (Li *et al.* 2004), maize (Robson *et al.* 2004), medicago (Calderini *et al*. 2007), creeping bentgrass (Huang *et al.* 2009), wheat (Sykorova *et al.* 2008), and peanut (Qin *et al.* 2011). In these transformed plants, the activation of *ipt* transcription is triggered mainly in senescing leaves and plants develop normally. The expression of the *ipt* gene in plants results in increased content of endogenous cytokinins in senescing leaves.

Experiments have shown that cytokinins elevate activities of various antioxidant enzymes (Kurepa *et al*. 1997, Petit-Paly *et al*. 1999, Synkova *et al*. 2006) which eliminate the reactive oxygen species accumulation and so they preserve the light-harvesting chlorophyll *a*/*b* binding protein and the large and small subunits of Rubisco from degradation and maintain chloroplast structure during senescence (Miller 1992, Zavaleta-Mancera *et al*. 2007). Additionally, cytokinins have significant influence on other plant hormones, *e.g.*, they interact with methyl jasmonate and slowed the process of senescence in *Helianthus annuus* cotyledons (Naik *et al.*

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Abbreviations: AHK - *Arabidopsis* histidine kinase; DAE - days after emergence; DEG - differentially expressed gene; IPT - isopentenyl transferase; ODC - ornithine decarboxylase.
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2002, Zhang *et al.* 2011). AHK3 (*Arabidopsis* histidine kinase), one of the three receptors of cytokinins in *Arabidopsis*, plays a crucial role in delaying leaf senescence (Kim *et al.* 2006). Cytokinins, as a senescence-retarding plant hormone, has been identified for many years, however, to date, few cytokinin-responsive genes have been reported during leaf senescence.

We previously generated transgenic cotton plants that harbor the *Agrobacterium ipt* gene directed by a senescence specific promoter. The expression of *ipt* in cotton results in increased content of endogenous cytokinins and a significant delay in leaf senescence (Liu *et al.* 2012). In the present study, *Solexa* sequencing was used for genome-wide expression profiling of transgenic and azygous cotton lines to analyze the gene expression in response to endogenous cytokinins during leaf senescence.

Materials and methods

Three independent transgenic cotton (*Gossypium hirsutum* L.) lines (S1, S2, and S3) generated by introducing the *ipt* gene into cv. Zhongmian 10, and the corresponding azygous lines (non-transgenic segregants C1, C2, and C3) were used in this study. The content of cytokinins in leaves of transgenic lines was higher than in leaves of azygous lines at 50 - 60 d after emergence (DAE) (Liu *et al.* 2012). Cotton plants were grown in plastic pots containing nutrient-rich soil in a growth chamber under a 16-h photoperiod, irradiance of 450 μmol m^{-2} s^{-1}, day/night temperatures of 30/22 °C, and air humidity of 55 - 60 %. The first node main-stem leaves (counted after cotyledons) were targeted after emergence. At 50 DAE, leaves of 20 different plants were collected at the same position from all transgenic lines and their corresponding azygous lines, respectively. The mixture of 20 leaves was divided into two parts. One part was used to detect the expression of *ipt* gene and the second one for *Solexa* sequencing. Total RNA was extracted from frozen samples using the RNeasy plant mini kit (*Qiagen*, Fremont, CA, USA) according to the manufacturer's instructions.

Tag library construction was carried out in parallel using an *Illumina* gene expression sample preparation kit according to the manufacturer's instructions. Raw sequence reads were filtered according to the *Illumina* pipeline. For annotation, clean tags were mapped to cotton *TIGR* reference sequences.

To determine the genes differentially expressed between the two samples, a rigorous algorithm was used as described by Audic and Claverie (1997). The *P*-value determined by FDR (false discovery rate) in multiple tests corresponded to the differential gene expression test. A threshold (FDR $\leq$ 0.001 and the absolute value of $\log_2$ratio $\geq$ 1) was used to judge the significance of the difference in gene expression (Benjamini and Yekutieli 2001).

Pathway enrichment analysis was based on the Kyoto encyclopedia of genes and genomes (*KEGG*) database and identified significantly enriched metabolic pathways in DEGs compared with the whole genome background. Both the two-side Fisher's exact test with multiple testing and the χ^2 test were used to classify the pathway category. The *P*-value was determined by the FDR. A pathway (*Q* value $\leq$ 0.05) was defined as a significantly enriched pathway.

The first-strand cDNA synthesis and real-time PCR were performed using *AMV* first strand cDNA synthesis kits and hot start fluorescent PCR core reagent kits (*SYBR Green I*), respectively. The real-time PCR was carried out on a *Bio-Rad* (Hercules, USA) *iQ5* real-time PCR system with the following conditions: 95°C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 20 s and 72 °C for 31 s. The *GhACTI* (GenBank: AY305733) gene was selected as the internal control gene. The relative expressions of genes were calculated from the threshold cycle by the $2^{-\Delta\Delta Ct}$ method. All reactions were performed in biological triplicates and significant differences were calculated using *SigmaPlot v10.0* software.

Results and discussion

There were 6 183 347 and 5 766 375 raw tags obtained from transgenic and azygous cotton lines, respectively. After filtering the dirty tags (*i.e.* low quality tags), 224 617 and 200 071 distinct tags of clean tags were obtained from transgenic and azygous cotton lines, respectively (Table 1). The distribution of tags represented the expression level of genes and, in our sequencing, a minority of distinct tags had copy numbers > 100 and the majority had copy numbers within 2 - 20. The distributions of tags were similar for transgenic and azygous cotton lines. The distribution of the tags indicated, firstly, that in the two lines, most genes were expressed at low levels as in normal plants and secondly, that the result of sequencing was generally normal.

All clean tags were mapped to the cotton *TIGR* database (http://www.tigr.org) for gene annotation: 65.57 and 63.42 % of clean tags of transgenic and azygous cotton lines were mapped to the reference genes (Table 1). Of distinct tags in the transgenic cotton, 12.14 % were perfectly matched to sense reference genes (one tag matched to one gene) and 12.20 % in the azygous cotton. Some tags mapped to the complementary

Table 1. Classification and abundance of tags in transgenic (T) and azygous (C) cotton lines. Clean tags are raw tags after data-processing steps (filtering out dirty tags). Distinct tags represent all types of clean tags. Unambiguous tags are clean tags after elimination of tags mapped to reference sequences from multiple genes.

Summary		T	C
Raw data	total	6183347	5766375
	distinct tag	426904	417549
Clean tag	total number	5971958	5540065
	distinct tag number	224617	200071
All tag mapping to gene	total number	3916012	3513240
	total % of clean tag	65.57	63.42
	distinct tag number	83536	74714
	distinct tag % of clean tag	37.19	37.34
Unambiguous tag mapping to gene	total number	2153331	1941448
	total % of clean tag	36.06	35.04
	distinct tag number	56990	50939
	distinct tag % of clean tag	25.37	25.46
All tag-mapped genes	number	43625	41373
	percentage of reference genes	61.73	58.55
Unambiguous tag-mapped genes	number	24496	22860
	percentage of reference genes	34.66	32.35
Unknown tag	total number	2055946	2026825
	total % of clean tag	34.43	36.58
	distinct tag number	141081	125357
	distinct tag % of clean tag	62.81	62.66

strand which demonstrated that the antisense strands also had transcripts and performed sense-antisense regulation. However, more than 60 % of distinct clean tags could not be mapped to reference genes mainly because the cotton genome is not yet completely sequenced.

There were 719 up-regulated and 499 down-regulated DEGs identified between the two libraries and 75 and 40 DEGs only expressed in transgenic and azygous cotton, respectively. Several DEGs that responded to endogenous cytokinins were found during senescence (Table 2). Some DEGs had previously been detected and their function and structure were predicted, but most DEGs were novel and require further research for confirmation.

Increases in transcription of histidine kinases and type-B response regulator genes were detected in the transgenic cotton lines. Histidine kinases play a central role as sensors for transducing cytokinin signals across the plasma membrane (Yamada *et al.* 2001). Type-B response regulators are transcription factors that serve as positive regulators resulting in rapid induction of cytokinin-associated target genes (Yokoyama *et al.* 2007, Argyros *et al.* 2008). Early studies indicated that *Arabidopsis* histidine kinase 3 (AHK3) played a crucial role in controlling cytokinin-mediated leaf senescence through specific phosphorylation of *Arabidopsis* response regulator 2 (Kim *et al*. 2006). In addition, we identified several up-regulated genes in transgenic cotton that were directly activated by response regulator 1 and one kind of type B response regulator: including glutathione-S-transferase gene, RING-finger protein gene, and receptor protein kinase gene. They encoded proteins with diverse molecular functions (Taniguchi *et al.* 2007). Collectively, these data are consistent with earlier studies that showed two-component signalling systems were used to sense and respond to cytokinins.

Most transcription factor genes were strongly up-regulated in response to endogenous cytokinins in the transgenic cotton. They encoded zinc-finger protein, AP2 domain protein, basic helix-loop-helix family transcription factor, and MYB transcription factor. There was a gene encoding acetyltransferase that was 9-fold up-regulated in transgenic cotton and had not previously been linked to cytokinin action. Early studies suggested that acetyltransferase was responsible for the acetylation of histones which can improve transcriptional activity. One interesting known transcriptional regulator was NAC domain protein which was identified as cytokinin up-regulated (Brenner *et al.* 2005). However, in the present study, it was 8-fold down-regulated in transgenic cotton. This was due to close association of NAC domain protein with leaf senescence. Previous evidence provided from *Arabidopsis*, rice, and kidney bean plants showed that the NAC domain protein gene and its homologs were up-regulated during senescence (Guo and Gan 2006). Regulation of transcription factor genes is very important because they may activate the coordinated expression of many downstream target genes and by these means govern entire metabolic and developmental processes (Köllmer *et al.* 2011).

DEGs identified as associated with other plant hormones were those related to ethylene, abscisic acid (ABA), gibberellins, and auxins. Genes encoding ethylene-responsive transcriptional factors and ABA-responsive protein were down-regulated in the transgenic cotton. It has been postulated that ethylene, ABA, and

Table 2. DEGs responding to cytokinins during senescence between transgenic (T) and azygous (C) cotton line libraries. TPM represented number of transcripts per million clean tags.

Gene ID	$\log_2^{ratio(T/C)}$	TPM		Annotation
		T	C	
DW505104	1.84	51.74	14.44	type-B response regulator
TA35955	1.40	11.89	4.51	histidine kinase
TA26376	1.79	6.87	1.99	putative mitogen activated protein kinase
TA26769	8.92	4.86	0.01	RING-finger protein
DR459539	1.52	11.39	3.97	glutathione-S-transferase
DR458154	11.38	26.62	0.01	caltractin
TA34060	10.92	19.42	0.01	receptor protein kinase
TA26717	2.57	5.36	0.90	zinc-finger protein
TA37970	8.46	3.52	0.01	AP2 domain transcription factor
TA44021	2.05	9.04	2.17	basic helix-loop-helix family transcription factor
DW510210	9.45	7.03	0.01	acetyltransferase
TA39489	-8.08	0.01	2.71	NAC domain protein 1
CA992692	-3.20	68.65	633.03	NAC domain protein 2
DW236416	-3.71	0.33	4.33	WRKY transcription factor 2
TA21909	-2.73	1.17	7.75	WRKY transcription factor 1
TA27993	2.76	35.33	5.23	auxin-responsive protein
DW237759	8.92	4.86	0.01	GAI/RGA-like protein
TA25014	-3.46	9.38	103.25	ethylene-responsive transcription factor 5
TA25012	-3.23	23.44	220.58	ABA-responsive protein
DW501084	8.92	4.86	0.01	protein tyrosine kinase
DR458574	1.99	17.25	4.33	Ser-Thr protein kinase
TA22723	8.52	3.68	0.01	lactoylglutathione lyase
DN781087	10.67	16.24	0.01	cytochrome P_{450}
TA37583	6.74	231.41	2.17	carbonic anhydrase
TA25615	5.82	204.62	3.61	trypsin inhibitor
TA22292	4.98	39.69	1.26	UDP-glycosyltransferase
DW242854	8.06	2.68	0.01	ornithine decarboxylase
DW234782	-2.84	7.54	54.33	nitrate reductase
DW506177	-8.81	0.01	4.51	nitrate transporter
DN800630	-12.23	0.01	48.01	isocitrate lyase
TA34151	-6.07	1.84	124.01	malate synthase
DW498237	-10.46	0.01	14.08	phospholipase
TA43671	-9.08	0.01	5.42	CCR-associated factor
TA26103	-8.49	0.01	3.61	U-box protein
TA43016	-1.29	68.15	166.79	cysteine proteinase
TA29340	-1.45	6.87	18.77	ATP-sulfurylase

cytokinins exert antagonistic activities during the process of senescence. The accumulation of ethylene and ABA accelerate leaf senescence (Agarwal and Jha 2010) whereas cytokinins trigger responses to delay leaf senescence. *In planta* studies subsequently demonstrated that AHK2, AHK3, and AHK4 function as negative regulators in the ABA signaling pathway and the expression of ABA-responsive genes are strongly induced in cytokinin-deficient plants (Tran *et al.* 2007, Nishiyama *et al.* 2011). Genes encoding gibberellin-insensitive like proteins, negative regulators of gibberellin signal transduction, were up-regulated in the transgenic cotton which was in accordance with early predictions that cytokinins reduced gibberellin activity by up-regulation of suppressors of GA-signalling genes (Willige *et al.* 2007). Several auxin-responsive protein genes were up-regulated in the transgenic cotton consistently with earlier studies that found both auxin and cytokinins acted as growth regulators and could both induce the expression of auxin-responsive protein (Jones *et al*. 2010).

DEGs involved in lipid catabolism (including phospholipase, isocitrate lyase, and malate synthase genes) were down-regulated in the transgenic cotton. Early research had shown that isocitrate lyase and malate synthase, as the key enzymes of the glyoxylate cycle, were closely connected with lipid catabolism (Debellis *et al.* 1991, Graham *et al.* 1992); additionally, phospholipase was also responsible for lipid catabolism (Huang *et al.* 2001). It is well known that lipids of cell membranes are degraded and transferred to young tissues for reuse during senescence. Down-regulation of these genes could prevent lipids from being degraded. Genes associated with protein metabolism also expressed differently between transgenic and azygous cotton lines in response to endogenous cytokinins including those encoding U-box protein and trypsin inhibitor. U-box protein genes, encoding E4 ubiquitination factors, play

important roles in the degradation of aberrant proteins and are essential for cell development and cell programmed death (Azevedo *et al.* 2001). In contrast, trypsin inhibitor prevented protein degradation. Early studies suggested that leaf senescence was a complex and highly regulated process that involved the degradation of proteins (Liu *et al.* 2008), thus, down-regulated U-box protein genes and up-regulated trypsin inhibitor genes could protect proteins from degradation during senescence. Cytokinins were also suggested to play an important role in regulating nitrogen-utilization and were closely linked to N-signaling (Kiba *et al.* 2011). Several genes of N metabolism and transport were regulated by cytokinins. The nitrate reductase gene transcript abundance was increased whereas the nitrate transporter gene was strongly down-regulated in the transgenic cotton.

Some genes with a function in primary or secondary metabolism were found to respond to endogenous cytokinins: they included 4-coumarate-CoA ligase, lactoyl-glutathione lyase, and carbonic anhydrase genes. The up-regulated gene in the transgenic cotton encoding 4-coumarate-CoA ligase was associated with the phenylpropanoid metabolism pathway and was the key enzyme of this pathway. The up-regulated lactoyl-glutathione lyase gene in transgenic cotton is involved in the methylglyoxal (MG) metabolism pathway (Thornalley 1990). MG, as one type of cytotoxin, combines with proteins and nucleic acids through saccharification and forms end products which are harmful to cells. However, lactoylglutathione lyase catalyzes MG to form lactic acid and effectively prevents accumulation of MG. Additionally, lactoylglutathione lyase reduces the peroxidation of lipids and helps plants to increase their stress resistance (Singla-Pareek *et al.* 2004). One up-regulated gene in the transgenic cotton encodes carbonic anhydrase which mediates the rapid conversion of CO_2 to HCO_3^- and so plays an important role in carbon acquisition (Lapointe *et al.* 2008).

Seventeen DEGs identified by *Solexa* sequencing were selected randomly and detected by real-time PCR to evaluate the validity of *Solexa* sequencing. There were nine up-regulated genes (DW505104, TA26769, DR459539, DR458154, DW510210, DW501084, DN781087, TA25615, and DW242854) and eight down-regulated genes (TA39489, CA992692, TA21909, DW236416, DW234782, DW506177, DN800630, and DW498237). Expressions of these genes by real-time PCR (Table 3) were basically in agreement with the patterns of sequencing analysis. However, there were some differences between the results of real-time PCR and *Solexa* sequencing. The apparent differences may be due to different experimental methods and different algorithms (Ekman *et al.* 2003). In conclusion, the validity of the sequencing data was confirmed.

Fourteen significantly enriched pathways were obtained, involving 296 DEGs. Among them, four pathways were mainly associated with delaying senescence regulated by cytokinins involving 30 DEGs. These four pathways included flavone biosynthesis (http://www.genome.jp/kegg-bin/show_pathway?map 00944), arginine and proline metabolism (http://www.genome.jp/kegg-bin/show_pathway?map00330), glyoxylate and dicarboxylate metabolism (http://www.genome.jp/kegg-bin/show_pathway?map 00630), and eukaryotic RNA degradation pathways (http://www.genome.jp/kegg-bin/show_pathway?map 03018).

Table 3. Real-time PCR validation of differentially expressed genes from transgenic (T) and azygous (C) cotton lines.

Gene ID	Relative expression level		*P*-value
	T	C	
DW505104	1.65	0.92	0.010
TA26769	0.67	0.08	0.010
DR459539	5.47	1.31	0.001
DR458154	4.97	1.28	0.010
DW510210	1.15	0.06	0.001
DW501084	1.03	0.07	0.001
DN781087	6.48	0.06	0.001
TA25615	4.76	0.85	0.001
DW242854	2.45	0.07	0.001
TA39489	0.07	0.21	0.010
CA992692	12.35	38.27	0.001
TA21909	0.78	2.14	0.001
DW236416	1.32	6.71	0.001
DW234782	0.58	3.26	0.001
DW506177	2.45	0.07	0.001
DN800630	0.08	9.65	0.001
DW498237	0.07	10.08	0.001

Seven DEGs between transgenic and azygous cotton lines were involved in the flavone and flavonol biosynthesis pathways of which four genes were up-regulated and three down-regulated in the transgenic cotton.

Three genes encoding cytochrome P_{450} were significantly up-regulated in transgenic cotton. Cytochrome P_{450} participated in biosynthesis of many metabolites such as plant hormone precursors, aliphatic acids, and phenylpropanoids. Cytochrome P_{450} catalyzes the first committed step or the rate-limiting steps of these reactions. In the flavone and flavonol biosynthesis pathways, cytochrome P_{450} not only catalyzes conversion of apigenin into luteolin but also catalyzes concersion of kaempferol into quercetin and myricetin. Up-regulated genes of cytochrome P_{450} resulted in accumulation of luteolin, quercetin, and myricetin. The flavones in hickory leaf significantly increased the activity of superoxide dismutase (SOD) and decrease the MDA content (Chen *et al*. 2008). During plant senescence, the content of MDA continuously accumulated but antioxidant enzymes activity decreased. Thus, flavones as antioxidants might delay plant senescence.

The arginine and proline metabolism pathways involved 11 DEGs of which 5 genes were up-regulated and 6 down-regulated in the transgenic cotton. Among them, a gene encoding ornithine decarboxylase (ODC) was significantly up-regulated in the transgenic cotton.

ODC is the key enzyme in arginine metabolism and catalyzes conversion of ornithine into putrescine and then putrescine can transform into spermine after a series of reactions. The gene encoding ODC was up-regulated and the content of endogenous putrescine and spermine would likely increase. Early observations indicated that putrescine was an effective anti-senescence agent. Early study also demonstrated that polyamines (including putrescine, spermidine, and spermine) delayed the senescence of leaf discs of two diverse species of *Rosa* (Sood and Nagar 2003). The mechanism of senescence delay by polyamines is not clear, however, the putrescine might be involved in eliminating free radicals, retarding the decrease of chlorophyll content, inhibiting activities of peroxidase and cellulase, and preventing membranes from deterioration (Pandey *et al.* 2000). In addition, putrescine interacts with ethylene and so modulates plant senescence (Saftner and Baldi 1990). Putrescine is possibly related to inhibition of ethylene synthesis by inhibiting 1-amino-cyclopropane-1-carboxylic acid (ACC) synthesis. Additionally, ethylene and putrescine synthesis pathways compete for available S-adenosyl-methionine.

The glyoxylate and dicarboxylate metabolism pathway involved 9 DEGs of which 6 genes were up-regulated and 3 down-regulated in the transgenic cotton. Among them, two genes were significantly down-regulated and encoded isocitrate lyase and malate synthase in the transgenic cotton, respectively. As noted previously, isocitrate lyase and malate synthase are the key enzymes of the glyoxylate cycle. When genes which encode isocitrate lyase and malate synthase are significantly down-regulated, this may destroy the glyoxylate cycle. Thus, through this pathway, cytokinins might prevent degradation of lipids and so delay plant senescence.

The eukaryotic RNA degradation pathway included three DEGs among them one gene was up-regulated and two genes down-regulated in the transgenic cotton. During plant senescence, mRNA is largely degraded; however, mRNA degradation always starts with the shortening the poly (A) tail at the 3′-end by action of deadenylases and then the deadenylated RNA can enter one of two degradation pathways. Deadenylation is the first and possibly the rate-limiting step of mRNA degradation (Parker and Song 2004). One down-regulated gene encoded CCR4-associated factor (CAF) and was significantly down-regulated in the transgenic cotton. Early studies indicated that CAF was a subunit of the CCR4-NOT complex. CCR4-NOT is a conserved protein complex involved in mRNA deadenylation and its subsequent degradation (Liang *et al.* 2009, Walley *et al.* 2010). Gene encoding CAF was down-regulated and may influence the formation of CCR4-NOT complex and delay mRNA deadenylation. Thus, mRNA degradation might be delayed with effects on plant senescence.

The results suggest that the increase in endogenous cytokinins led to the increased content of flavones and polyamines. The former increase SOD activity and decrease MDA content, and the latter inhibit the effect of ethylene. Additionally, increase in endogenous cytokinins retards the degradation of lipids and mRNA. In conclusion, cytokinins delay senescence through flavone biosynthesis, arginine and proline metabolism, glyoxylate, and dicarboxylate metabolism and RNA degradation pathways.

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