A sugarcane R2R3-MYB transcription factor gene is alternatively spliced during drought stress

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MYB transcription factors of the R2R3-MYB family have been shown to play important roles in many plant processes. A sugarcane R2R3-MYB gene (ScMYB2) and its two alternative forms of transcript (ScMYB2S1 and ScMYB2S2) were identified in this study. The deduced protein of ScMYB2S1 is a typical plant R2R3-MYB protein, while ScMYB2S2 encodes a truncated protein. Real-time qPCR analysis revealed that ScMYB2S1 is suppressed under PEG-simulated drought stress in sugarcane, while ScMYB2S2 is induced at later treatment stage. A senescence symptom was observed when ScMYB2S1 was injected into tobacco leaves mediated by Agrobacterium, but no symptom for ScMYB2S2. Further investigation showed that the expression levels of 4 senescence-associated genes, NtPR-1a, NtNYC1, NtCAT3 and NtABRE, were markedly induced in tobacco leaves after ScMYB2S1-injection, while they were not sensitive to ScMYB2S2-injection. Moreover, MDA and proline were also investigated after injection. Similarly, MDA and proline levels were induced by ABA and ScMYB2S1, while inhibited by ScMYB2S2. We propose that ScMYB2, by alternatively splicing two transcripts (ScMYB2S1 and ScMYB2S2), is involved in an ABA-mediated leaf senescence signaling pathway and play positive role in respond to drought-induced senescence in sugarcane. The results of this study provide information for further research in sugarcane stress processes.
MYB proteins are elaborately regulated at multiple steps\(^{14,28}\). In particular, accumulating evidence illustrates that post-transcriptional control of mRNA modulates the transcription factor activities during plant response to environmental stimuli\(^{28}\). Alternative splicing (AS), to regulate gene expression, is one subset of post-transcriptional processes\(^{29}\). Through effecting the production of mRNA isoforms with different exonic composition from a single gene, alternative splicing creates multiple mRNA transcripts\(^{30}\). Alternative splicing can affect protein function and influence protein diversity when AS events occur within the translated regions of mRNAs\(^{30}\). It is also common for AS to result in isoforms that contain a premature termination codon, which subsequently become targets for nonsense mediated decay\(^{30}\). By this way, instead of a truncated polypeptide, no protein is produced.

Plant leaf senescence is an age-dependent deterioration process and is also triggered by environmental stresses and phytohormones\(^{31,32}\). It has been recognized that senescence associated genes (SAGs) are induced by senescence\(^{32,33}\). Abscisic acid (ABA) is an important phytohormone and plays a critical role in regulating plant development and responses to various stress signals, such as drought\(^{32}\).

In the present work, the function of a sugarcane (\textit{Saccharum officinarum}) R2R3-MYB gene, \textit{ScMYB2}, with its two alternative splicing transcripts, \textit{ScMYB2S1} and \textit{ScMYB2S2}, were investigated. We utilized real-time qPCR to evaluate the response of the alternative splicing transcripts to drought stress induced by PEG. A visible symptom of leaf senescence, i.e. de-greening, was observed when one alternatively spliced transcript of \textit{ScMYB2S1} was transiently over-expressed in tobacco (\textit{Nicotiana tabacum}) leaves. To further investigate the role of \textit{ScMYB2S1} and \textit{ScMYB2S2} in senescence, the contents of malonaldehyde (MDA) and proline, and then the expression profiles of 4 previously reported senescence-associated genes (SAGs) are checked in tobacco leaves after \textit{ScMYB2S1}-or/and \textit{ScMYB2S2}-transiently-transformed. Results suggest that \textit{ScMYB2} is involved in the response to drought.

The differential expression of two alternatively spliced transcripts during PEG stress could be one kind of drought tolerance molecular mechanism in sugarcane.

Results

Cloning and sequence analysis of \textit{ScMYB2S1}. Two rounds of PCR were performed, and gel electrophoresis of the inner PCR products showed one fragment (Supplementary Fig. S1). The amplicons of about 1,100 bp were separated and recovered from the agarose gel, which were subsequently used for T-A cloning and transformed into \textit{Escherichia coli}. Ten randomly positive clones were picked and sequenced, and two R2R3-MYB-like cDNA sequences were obtained, designated as \textit{ScMYB2S1} (GenBank Accession Number KM387410) and \textit{ScMYB2S2} (GenBank Accession Number KM387411), respectively. \textit{ScMYB2S1} had a full length of 1,066 bp, with an ORF of 687 bp, 5′ UTR (untranslated region) of 40 bp, and 3′ UTR of 339 bp (Fig. 1). The deduced protein of \textit{ScMYB2S1} was a typical plant R2R3-MYB protein, containing two MYB DNA-binding domains (R2 and R3 repeats) at the N-terminal. Within the R2 and R3 repeats, the highly conserved tryptophan (W) residues...
Implicated in DNA-binding were spaced by the 19 or 18 amino acid residues, respectively. The first W of R3 repeat in ScMYB2S1 protein was replaced by a methionine (M) (Fig. 1).

Overlapping the full-length sequences of ScMYB2S1, 968-bp long ScMYB2S2 transcript contained an additional 29-bp-sequence inserting into the corresponding location of the ORF, which interrupted the reading frame of a subsequent region behind the start codon and caused frameshift mutation of the sequence. Thus, compared with the amino acid sequence of ScMYB2S1, the first MYB DNA-binding domain (R2) in the amino acid sequence of ScMYB2S2 was missing, which resulted in the residue part starting with the first methionine (M) of R3 repeat, thereafter sharing 100% homology to the ScMYB2S1. Cloning a genomic sequence of the gene was also performed to identify whether the two transcripts, ScMYB2S1 and ScMYB2S2, were produced by alternative splicing of the same gene. The genomic sequence of the ScMYB2 gene (GenBank Accession Number KM387409) displayed at least two alternatively spliced isoforms (Fig. 2): a typical plant R2R3-MYB transcription factor gene ScMYB2S1 and ScMYB2S2 encoding a truncated protein starting at a methionine in the R3 repeat.

The genomic sequence of the ScMYB2 had a highly conserved splicing arrangement with three exons and two introns (126 bp and 76 bp). The 126 bp intron appeared to consist of two short tandem “GT-AG” structures, which produced the structural basis for alternative splicing. Further sequence analysis revealed that ScMYB2S1 was a member of the genomic sequence of the ScMYB2S1.

Expression profiles of ScMYB2S1 and ScMYB2S2 under drought stress. To further examine the function of the alternatively spliced transcripts of ScMYB2, their responses to drought stress were performed. When treated with 25.0% PEG, the expression level of ScMYB2 rapidly decreased at 3 h (Fig. 4) and stayed at the relatively low level during the periods from 3 h to 24 h, but increased in the later periods (48 h and 72 h). By using primers specific for each splice variant, the level of ScMYB2S2-injection and mixed-injection (with ScMYB2S2 and control was recorded at 24 h after injection.

To gain insights into the role of ScMYB2 in sugarcane, transient expression of pGreenII0229-ScMYB2S1, pGreenII0229-ScMYB2S2 and pGreenII0229 (control) were tested for their effect on tissue-cultured tobacco (N. tabacum) leaves via A. tumefaciens injection. The effect of over-expressing ScMYB2S1, ScMYB2S2 or control was recorded at 24 h after injection. The whole leaf over-expressing ScMYB2S1 changed color from green to yellow (Fig. 5a), when compared with control (Fig. 5c). Meanwhile, there was no obvious change in leaf color when the ScMYB2S2 was over-expressed (Fig. 5b).

Physiological measurement. MDA and proline content in tobacco leaf samples were detected 48 h after ABA-treatment or injection. Figure 6 showed that ScMYB2S1-injection induced both MDA and proline levels obviously, while ScMYB2S2-injection and mixed-injection (with ScMYB2S1 and ScMYB2S2 volume ratio of 1:1) had limited effect on MDA or proline levels. Predictably, both MDA and proline contents were increased significantly after ABA-treatment (Fig. 6).

Expression profiles of SAGs in tobacco leaves. Real-time qPCR was used to examine the expression profiles of four SAGs, NtNYC1, NtPR-1a, NtCAT3 and NtABRE, in tobacco leaves under different treatments. All these genes were induced after ScMYB2S1 injection, with an expression level about 2.17, 3.80, 5.14 and 2.48 times higher than that of the control, respectively (Fig. 7). Conversely, after injection of ScMYB2S2, the expression of NtNYC1 and NtPR-1a were decreased with 0.81 and 0.75 times, respectively, lower than that of the control (Fig. 7). While at the same time, the expression level of NtCAT3 and NtABRE were about 1.54 and 1.12 times higher than control (Fig. 6). Overall, however, the expression levels of these genes were between the two former situations after mix-injection of ScMYB2S1 and ScMYB2S2 (Fig. 7).

Discussion
In the present study, a R2R3-MYB gene was isolated from sugarcane, designated as ScMYB2, showing to produce two alternatively spliced transcripts: ScMYB2S1 and ScMYB2S2. Sequence analysis showed that the 126-bp-intron of the genomic sequence of the ScMYB2 gene consisted of two short tandem “GT-AG” structures, which provided the structural basis for alternative splicing. Further sequence analysis revealed that ScMYB2S1 was a member of the ScMYB2S group along with other Myb family genes from Arabidopsis. The results of this study suggest that ScMYB2 may play a role in drought stress response in sugarcane, possibly by influencing the expression of SAGs involved in stress response.
Figure 3. Phylogenetic relationships between *Arabidopsis* MYB transcription factors and ScMYB2S.

Figure 4. The expression profiles of ScMYB2 and its two transcript versions under PEG-simulated drought stress. ScMYB2 represents a pool of both variants. The other two are splice variant specific. Each value is the average of three replicate experiments ± standard error (n = 3). The different lowercase showed the significance difference of ScMYB2, ScMYB2S1 and ScMYB2S2 expression levels under p-value < 0.01 level.
functional protein, containing two complete MYB DNA-binding domains (R2 and R3 repeats). ScMYB2S2, with the first MYB DNA-binding domain (R2 repeat) missing in the N-terminal amino acid sequence, might limited its function on directly involved in transcriptional regulation. Similarly, two Arabidopsis R2R3-type MYB genes, AtMYB59 and AtMYB48, both were found to have four distinctively spliced transcripts that encoded either MYB-related proteins or R2R3-MYB proteins. Interestingly, ScMYB2, AtMYB59 and AtMYB48 were found to be within the same phylogenetic subgroup in this study (Fig. 3).

Alternative splicing occurs widely in eukaryote and provides the main source of transcriptome and proteome diversity in an organism. Recent studies suggested that the signal transduction related genes associated with

Figure 5. Phenotypic change of tobacco leaves after injection with different transcripts of ScMYB2. (a) pGreenII0229-ScMYB2S1; (b) pGreenII0229-ScMYB2S2; (c) Control: pGreenII0229.

Figure 6. MDA and proline contents in tobacco leaves. 1: ABA-treatment; 2: Injected with recombinational Agrobacterium containing empty vector; 3. Injected with recombinational Agrobacterium containing the target gene of ScMYB2S1; 4. Injected with recombinational Agrobacterium containing the target gene of ScMYB2S2; 5. Injected with the mixture of the two recombinants containing ScMYB2S1 and ScMYB2S2. (A) MDA level (nmol/g); (B) Proline contents (μg/g). The different lowercase indicated the significance difference of MDA and proline contents under p-value < 0.01 level.
various stress responses seemed to be particularly prone to alternative splicing in plants and animals\(^{35,37}\). The R2R3-MYB transcription factor superfamily has been shown to play an important role in many plant processes including abiotic stress responses\(^{18}\). It had been demonstrated that several MYB genes were alternatively spliced, and encoded proteins with regulatory functions\(^{35,38,39}\).

Previous studies have shown that alternative splicing also presents in sugarcane MYB transcription factor genes. Prabu et al.\(^{40,41}\) identified an inducible alternatively spliced R2R3-MYB transcription factor gene \(ScMYBAS1\) (EU670236) in \(S.\ officinarum\) based on a cDNA suppression subtractive hybridization library. Subsequently, semi-quantitative RT-PCR analysis revealed that the alternatively spliced transcripts, \(ScMYBAS1\)-2 and \(ScMYBAS1\)-3, were constitutively expressed at high level when subjected to water deficit and salt stress treatments, however no sequence information was provided\(^{40,41}\). In GenBank the genomic DNA of \(MYBAS1\) (HM136779) from \(Saccharum\) hybrid cultivar Co740, and three alternatively spliced variants: \(MYBAS1V1\) (HM136780), \(MYBAS1V2\) (HM136781) and \(MYBAS1V3\) (HM136782), are noted as being induced by water deficit stress based on semi-quantitative RT-PCR analysis. Sequence analysis shows that \(MYBAS1V1\) is only 2 bases different from \(ScMYBAS1\) (Data not showed).

Many members in plant R2R3-MYB family have been reported as abiotic-stress-induced transcription factors\(^{40–43}\). Moreover, over-expressing the R2R3-MYB genes improved the resistance to freezing, drought, and salt stresses in transgenic plants\(^{44–46}\). In this study, \(ScMYB2\), one alternatively spliced transcript of \(ScMYB2\), was induced at the late periods of PEG-simulated drought stress. Contrary to expectation, the expression of \(ScMYB2S1\), the other alternatively spliced transcript of \(ScMYB2\), was suppressed constitutively by PEG-simulated drought stress. This shows that highly specific probes or primers are crucial to identify the expression pattern of a certain gene for alternatively spliced transcripts or allelic genes which are highly homologous to each other.

It was reported that leaf senescence was affected by drought stress\(^{47}\). In this study, the de-greening symptom displayed when one alternatively spliced transcript of \(ScMYB2S1\) was transiently over-expressed in tobacco leaves, suggesting the function of \(ScMYB2S1\) might relate to leaf senescence regulation. The stress hormone ABA promotes leaf senescence in an ethylene-independent pathway and induces compatible solutes (e.g. malondialdehyde and proline) accumulation in leaf\(^{50,69}\). Within those compatible solutes, MDA and proline levels are commonly known as markers of stress\(^{50}\). To further investigate the role of \(ScMYB2S1\) and \(ScMYB2S2\) in leaf

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**Figure 7. The expression profiles of 4 SAGs in tobacco leaves after injection.** 1: Injected with recombinational \(Agrobacterium\) containing empty vector; 2: Injected with recombinational \(Agrobacterium\) containing the target gene of \(ScMYB2S1\); 3: Injected with recombinational \(Agrobacterium\) containing the target gene of \(ScMYB2S2\); 4: Injected with the mixture of the two recombinants containing \(ScMYB2S1\) and \(ScMYB2S2\). (A) \(NtNYC\); (B) \(NtPR-1a\); (C) \(NtCAT3\); (D) \(NtABRE\). The different lowercase indicated the significance difference of 4 SAGs expression levels under p-value < 0.01 level.
Plant senescence is regulated by senescence associated genes (SAGs)\textsuperscript{31,32}. Stress-induced senescence signals are perceived and then transferred via SAGs\textsuperscript{33}. To date, a series of SAGs have been identified from various plant species\textsuperscript{33,35}. In this study, 4 SAGs genes were selected randomly to investigate whether they were involved in the co-expression network regulating by ScMYB2. Among these four SAGs, pathogenesis-related 1a (PR1a) and catalase (CAT) have been identified as marker gene of biotic stress\textsuperscript{51}. Non-yellow coloring (NYC1) gene, encoding the membrane-spanning isoform of Chl b reductase was expressed during leaf senescence in rice\textsuperscript{53}, and its function on chlorophyll degradation also has been proved in Arabidopsis\textsuperscript{53}. Recent studies have shown that abscisic acid responsive element (ABRE)-binding factor gene (ABRE) also involved in responding to ABA-induced leaf senescence\textsuperscript{25,34}. An ABRE-binding factor has been identified as a positive regulator of abiotic stress and ABA signaling both in Arabidopsis and in rice\textsuperscript{55}. Collectively, the function of all these four genes was identified to accelerate leaf senescence\textsuperscript{33,36}. Previous studies have shown that both endogenous ABA and exogenously applied ABA can induce the expression of SAGs and promote leaf de-greening and senescence in general\textsuperscript{53}. The present study showed that these 4 SAGs were significantly induced by ScMYB2S1-injection, while ScMYB2S2-injection didn't have much effect (Fig. 7). We concluded that when transiently over-expressing in tobacco leaf, it is ScMYB2S1 rather than ScMYB2S2 playing an ABA-like function, induced the expression of the four SAGs.

ScMYB2S1 was down-regulated in responding to drought stress during the whole processing period in sugarcane. Combined analysis of its performance in different samples and the expression levels of SAGs in tobacco (Figs 5, 6 and 7) suggested that ScMYB2S1 could act as a negative regulator and play a positive role in response to drought-induced senescence process. Meanwhile, the expression level of ScMYB2S2 in sugarcane was up-regulated by drought stress at the later stage. It seems that ScMYB2 tends to generate the isoform of ScMYB2S2 by alternative splicing during the later stage. Different with ScMYB2S1, ScMYB2S2 has limited effect on leaf de-greening (Fig. 5), MDA and proline contents (Fig. 6) and the expression of SAGs. It needs to further investigate whether this limited influence is due to its incomplete functional domain. And, recent studies have shown that alternative splicing of some transcription factor genes generates small interfering peptides (siPEPs), which negatively regulates the target transcription factors\textsuperscript{28}. Further studies are also needed to substantiate whether the two transcripts are existed an interaction at nucleic acid level or protein level.

In conclusion, a hypothesis is proposed here that ScMYB2, by alternatively splicing two transcripts (ScMYB2S1 and ScMYB2S2), might involve in an ABA-mediated leaf senescence signaling pathway and play positive role in respond to drought-induced senescence in sugarcane. The differential expression of two alternatively spliced transcripts during PEG stress could be one kind of drought tolerance molecular mechanism in sugarcane. The alternative splicing of the transcription factor gene ScMYB2 may be pivotal to the molecular defense mechanism of sugarcane during drought-induced senescence processes.

**Methods**

**Materials and treatments.** 3'-Full RACE Core Set Ver.2.0 Kit, TaKaRa LA PCR\textsuperscript{TM} in vitro Cloning Kit, PrimeScript RT-PCR Kit, DNA markers were purchased from TaKaRa (Dalian, China). RQ1 RNase-Free DNase was obtained from Promega Corporation (USA), SYBR\textsuperscript{®} Green PCR Master Mix Kit was purchased from Applied Biosystems TM (USA), and the instrument used in the real-time qPCR analysis was the ABI PRISM7500 realtime PCR system (USA). Plant Malondialdehyde (MDA) assay Kit and Proline assay Kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Sugarcane cultivar Badila (S. officinarum), tobacco (N. tabacum) variety K326 seedlings and tissue culture plantlets used in this study was provided by the Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture, Fuzhou, China. According to Guo et al.\textsuperscript{37}, uniform plantlets of an elite sugarcane cultivar Badila were grown in 1/4 Hongland nutrient solution for one week and then subjected to PEG8000 (25.0%) treatment. The sampling times were 0h, 3h, 6h, 12h, 24h, 48h and 72h after the start of treatment.

Agrobacterium-infiltrated transient transformation of tobacco was carried out according to Lin-Wang described\textsuperscript{24}. For phenotype observation assay, tobacco tissue culture plantlets were used for transiently transformed assay. Approximately 150 μL of each recombinant Agrobacterium culture was infiltrated at four points into a tender leaf. Tobacco seedlings grown in a greenhouse with environmental control systems were used for physiological measurement and gene expression assay. Two kind of stress treatments were applied to 8-week-old plants before flowering: ABA treatment (sprayed 100μM ABA on leaf at 48h) and Agrobacterium injection (using a suspension of recombinational Agrobacterium introduced into tobacco leaf by direct injection). As for the latter, approximately 300 μL of Agrobacterium containing the target gene or empty vector control were infiltrated at four points into the leaf. After 48h treatment, all the leaf samples were collected and divided into three groups and assayed, respectively. One group was fixed in liquid nitrogen immediately and stored in a refrigerator at -85°C until RNA extraction. According to the methods from Plant Malondialdehyde (MDA) assay Kit and Proline assay Kit, the other two groups were grind into buffer solution immediately and then measured, respectively. All of the treatments were repeated independently three times. The data were analyzed with DPS v7.05 directly and the significance difference of the MDA and proline contents were marked using different lowercase in figure.

**Molecular cloning, sequencing and bioinformatics analysis.** A sugarcane R2R3-MYB EST was obtained based on the bioinformatics analysis using the data from the previous RNA-Seq experiments (not yet published). Two nested gene-specific 3’ RACE primers was designed according to the EST sequence information,
and one pair of gene-specific primer was designed to amplify the given target regions using genomic DNA as a template. Primer sequences are as follows:

3′ RACE GSP1: 5′-ACATAGTGCTTCTCCGTCC-3′;
3′ RACE GSP2: 5′-TATCCAAGTGAGCCAGCA-3′;
ScMybF: 5′-TAGCTGGTGAAGCGGAC-3′ (same to 3′ RACE GSP2);
ScMybR: 5′-CCTATTG GTGGGTGAACAATCC-3′.

The method used in 3′ RACE was followed to the specifications of the 3′-Full RACE Core Set Ver.2.0 Kit (Takara). A full-length R2R3-MYB homolog gene of sugarcane (named ScMYB2) was identified by Blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with two Myb-like DNA-binding domains (pam00249). The open reading frame (ORF) of the full-length cDNA sequence of ScMYB2 was predicted using the ORF Finder online tool from NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Sequence alignment was performed using DNAMAN 5.2.2 software.

Expression profiles of ScMYB2 under PEG-simulated drought stress. Total RNA isolation was performed using the TRizol® Reagent (Invitrogen, USA). The removal of DNA from RNA samples was realized by RQ1 RNase-Free DNase (Promega, USA). The reverse transcription was realized by following the specifications of the PrimeScript® RT reagent Kit (Takara). Finally, the SYBR® Green PCR Master Mix (AB, USA) was employed in real-time qPCR reaction.

The 25 S rRNA (BQ536525) gene was chosen as an internal control in real-time qPCR analysis and the forward and reverse primers for 25 S rRNA were 5′-GCACGCAAGGGTTTCAGACG-3′ and 5′-CATTATTGGTGAACAATCC-3′. Based on the common sequences of ScMYB2S1 and ScMYB2S2, a pair of real-time qPCR primers was designed using the Primer Express 3.0 software and the forward and reverse primers for ScMYB2S1 were 5′-ACCCTGGTTGGACCTTCATT-3′ (MYB2qF) and 5′-CAGGCCGGTATGTGAGGAGA-3′ (MYB2qR), respectively (Fig. S2). Moreover, pairing with common reverse primer (MYB2qR), two sequence-specific forward primers were designed for ScMYB2S1 and ScMYB2S2 with the sequences 5′-CATTGGCCCAAGTCTAGGCC-3′ (MYB2S1qF) and 5′-CATTGGCCCAAGTCTAGGCC-3′ (MYB2S2qF), respectively (Supplementary Fig. S2).

The real-time qPCR reaction was realized with following conditions: 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 94 °C for 15 s, and 60 °C for 60 s. Each assay was repeated by three times. When the reaction was completed, a melting curve was obtained. The 2−ΔΔ method was adopted to analyze the real-time qPCR results. The data were analyzed with DPS v7.05 directly and the significance difference of the gene expression were marked using different lowercase in figure.

Binary vectors construction. To study the function of ScMYB2S1 and ScMYB2S2 in tobacco, PCR was performed using the clone containing ScMYB2S1 or ScMYB2S2 as template to obtain the ScMYB2S1 or ScMYB2S2 ORF with matched sites. The primer sequences were MYB2S1F: 5′-CGCAAGGGTTTCATTCATT-3′; ScMYB2S2F: 5′-CGCAAGGGTTTCATTCATT-3′; MYB2S2R: 5′-CAGGCCGGTATGTGAGGAGA-3′ and MYB2S2R: 5′-CAGGCCGGTATGTGAGGAGA-3′. A full-length R2R3-MYB homolog gene of sugarcane (named ScMYB2) was identified by Blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with two Myb-like DNA-binding domains (pam00249). The open reading frame (ORF) of the full-length cDNA sequence of ScMYB2 was predicted using the ORF Finder online tool from NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Sequence alignment was performed using DNAMAN 5.2.2 software.

Expression profiles of SAGs in tobacco leaves. Four verified senescence-associated genes (SAGs), NtPR-1a (ACC. No. X12737.1)35, NtCAT3 (ACC. No. Z36977.1)35, NYC1 (XM016652882)35 and NtABRE (KF736850)35, were selected to verify their expression profiles in tobacco leaves after ABA treatment or ScMYB2s transiently-transformed, respectively. The forward and reverse primers for NtPR-1a...
were 5′-ATATCCACCTTGGCGGTGCCCAA-3′ and 5′-GCTACCTTGTCCTCCAGGTCA-3′. The forward and reverse primers for NtCAT3 were forward 5′-CTGCAAGCTCAGTTAAC-3′ and 5′-GACATCAACCCCACTTGCA-3′. Using the Primer Express 3.0 software, real-time qPCR primers were designed according to NtNYC1 (Accession No. XM016652882.1) and NtABRE (Accession No. KF736850) sequences, respectively. The forward primers for NtNYC1 and NtABRE were 5′-TGACATTTGGTAAACAAACGCT-3′ and 5′-CTAATAGAACACGGGTGAAACTG-3′, respectively. The reverse primers for NtNYC1 and NtABRE were 5′-ATCCATAGACAGCCTGTAGAGG-3′ and 5′-ATCCATAGACAGCCTGTAGAGG-3′, respectively. The elongation factor 1α (ELF-1α) (AF120093) was chosen as an internal reference gene for normalization of real-time RT-PCR in tobacco and the forward and reverse primers for EF-1α were 5′-TGAGATGCCCACACAGGCTTCTC-3′ and 5′-CACACATTGTCCACCAGGAAGTG-3′. The real-time qPCR reaction was realized with following conditions: 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 94 °C for 15 s, and 60 °C for 60 s. Each assay was repeated by three times. When the reaction was completed, a melting curve was obtained. The 2^−ΔΔCT method was adopted to analyze the RT-qPCR results. The data were analyzed with DPS v7.05 directly and the significance difference of the gene expression were marked without using lower case in figure.

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