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Potential interaction between autophagy and auxin during maize leaf senescence

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Abstract

Leaf senescence is important for crop yield as delaying it can increase the average yield. In this study, population genetics and transcriptomic profiling were combined to dissect its genetic basis in maize. To do this, the progenies of an elite maize hybrid Jidan27 and its parental lines Si-287 (early senescence) and Si-144 (stay-green), as well as 173 maize inbred lines were used. We identified two novel loci and their candidate genes, Stg3 (ZmATG18b) and Stg7 (ZmGH3.8), which are predicted to be members of autophagy and auxin pathways, respectively. Genomic variations in the promoter regions of these two genes were detected, and four allelic combinations existed in the examined maize inbred lines. The Stg3Si-144/Stg7Si-144 allelic combination with lower ZmATG18b expression and higher ZmGH3.8 expression could distinctively delay leaf senescence, increase ear weight and the improved hybrid of NIL-Stg3Si-144/Stg7Si-144 × Si-144 significantly reduced ear weight loss under drought stress, while opposite effects were observed in the Stg3Si-287/Stg7Si-287 combination with a higher ZmATG18b expression and lower ZmGH3.8 expression. Thus, we identify a potential interaction between autophagy and auxin which could modulate the timing of maize leaf senescence.

Keywords: Autophagy and auxin pathways, interaction, leaf senescence, maize yield, population genetics, transcriptomic profiling.

Introduction

Senescence, an indispensable process for nutrient remobilisation at the final stage of leaf development, plays a crucial role in crop yield (Wu et al., 2012; Thomas and Ougham, 2014). The onset of leaf senescence depends on the developmental stages of a leaf, which are known as age factors or age-related changes (Jing et al., 2003; Schippers, 2015). Leaf senescence can also be induced by various biotic or abiotic stresses, such as pathogen infection, drought stress and darkness (Guo and Gan,
2012; Liang et al., 2014). During leaf senescence, many physiological, biochemical and molecular processes are initiated, such as breakdown of chlorophyll, destruction of photosynthetic membrane systems, changes in source-sink communication, and expression of thousands of senescence-associated genes (SAGs), which are generally referred to as the senescence syndrome (Bleecker and Patterson, 1997; Schippers et al., 2015; Yolcu et al., 2018). The timely delaying of senescence or prolonging of stay-green stage are beneficial to extend the leaf functional period, which can facilitate the source strength and thus improve crop yield (Jordan et al., 2012; Gregersen et al., 2013). For example, delaying leaf senescence can increase the average yield by 0.29 tonne ha⁻¹ in maize (Zhang et al., 2019), 10% in rice (Mao et al., 2017), and 6–28% in wheat (Christopher et al., 2008). Therefore, it is of great importance to illustrate the underlying molecular mechanisms of leaf senescence for crop breeding.

Maize (Zea mays) is one of the most important multi-purpose plants, functioning not only as food, but also as feed, fuel and industrial raw materials. Selection and breeding of stay-green or senescence-delayed cultivars have been considered as one of the effective strategies to tackle the challenges of yield stability caused by the frequent occurrence of climatic disasters, especially drought stress. Inbred lines with distinct leaf senescence differences such as Qi-319, Mo17, Zheng58, CML444 and A150–3–2, have been selected as the parental lines in maize. By using various populations including recombinant inbred lines (Zheng et al., 2009; Messner et al., 2011; Wang et al., 2012; Almeida et al., 2014; Khanal et al., 2015; Yang et al., 2017), backcrossed lines (Belicuas et al., 2014; Trachsel et al., 2016), top-cross progenies (Beavis et al., 1994) and double haploids (Liu et al., 2020), a total of 268 major quantitative trait loci (QTL) for this trait have been mapped onto the 10 chromosomes, accounting for 0.5–24.3% of phenotypic variation. In addition, transcriptomic analyses have identified a large number of differentially expressed genes (DEGs) for leaf senescence in maize, which are significantly enriched in hormone biosynthesis, photosynthesis, carbohydrate metabolism and amino acid transport (Sekhon et al., 2012; Zhang et al., 2014; Chai et al., 2019; Sekhon et al., 2019).

However, so far, only a small number of genes controlling leaf senescence have been characterised at the molecular or cellular level in maize. Some of them were characterised by reverse genetic approaches. For instance, ACC (aminocyclopropane-1-carboxylic acid) synthases (ACS) are the enzymes at the first step of ethylene biosynthesis, and the Mu-insertion lines of ACS6 in maize showed a reduction in ethylene synthesis and delayed leaf senescence (Young et al., 2004). ZmVQ52 is a VQ family transcription factor (proteins containing a unique and conserved VQ motif). Overexpression of ZmVQ52 in Arabidopsis showed premature leaf senescence, and the transcriptomic data revealed that this function was mainly through photosynthesis and circadian rhythm pathways. Additionally, the overexpression lines exhibited enhanced sensitivities to jasmonic acid (JA) and salicylic acid (SA), but were tolerant to abscisic acid (ABA), suggesting a potential connection between ZmVQ52 and hormone signalling in the regulation of leaf senescence (Yu et al., 2019). SnRK1s (sucrose non-fermenting-1-related protein kinase 1 genes) encode enzymes that are important members in sugar signalling and carbohydrate metabolism, and the ectopic expression of ZmSnRK1 in Arabidopsis distinctly delayed leaf senescence, suggesting that ZmSnRK1 may have a similar function in maize (Wang et al., 2019). Furthermore, some genes have been identified by forward genetic approaches. Through genome-wide association study (GWAS), two genes were analysed. Mir3 encodes a cysteine protease, and the knockout mutant of its ortholog RD21A in Arabidopsis exhibited loss of protease activity and a delayed leaf senescence phenotype. In the senescent line, an earlier onset of β-glucosidase activity was detected, while the stay-green line showed a delayed β-glucosidase activity, potentially caused by the differential post-transcriptional regulation of BGLU42 (β-glucosidase-42; Sekhon et al., 2019). By QTL mapping, a gene encoding a NAC [No apical meristem (NAM), Arabidopsis transcription activation factor (ATAF) and cup-shaped cotyledon (CUC)]-domain transcription factor, named NAC7, was characterised. RNA-sequencing (RNA-seq) analysis revealed that genes encoding photosynthesis-associated enzymes exhibited higher expression but those for chlorophyll degradation showed lower expression in nac7 RNAi lines; these enzymes are associated with a delayed leaf senescence phenotype and increased grain yield, demonstrating that NAC7 acts as a negative regulator of photosynthetic activities, promotes chlorophyll degradation, and accelerates leaf senescence (Zhang et al., 2019).

Recently, a transcriptomic analysis reported that ZmNAC126 was a positive regulator in chlorophyll degradation by affecting the expression of CCGs (CHLOROPHYLL CATABOLIC GENES) to promote leaf senescence. Meanwhile, expression of ZmNAC126 was up-regulated in response to ethylene treatment, which accelerated leaf senescence in maize, suggesting the molecular link between chlorophyll degradation and ethylene pathway (Yang et al., 2020).

Information from breeders revealed that Jidan27 is an elite maize hybrid, which is specifically bred for North-eastern China Corn Belt with cumulative temperatures between 2300–2500 °C in Heilongjiang Province. The hybrid usually suffers from premature leaf senescence at the adult plant stage, presumably from the female parent Si-287, which displays a prominently accelerated senescence phenotype compared with the male parent Si-144. In-depth investigation of leaf senescence will facilitate targeted maize improvement.

Here, through an integrated analysis of population genetics and transcriptome profiling, we identified two candidate genes, ZmATG18b and ZmGH3.8, which are important components in autophagy and auxin pathways, respectively. Four combinations were found for the natural allelic variations of these two genes in maize inbred lines. The subtle balance between their expression in different allelic combinations is closely related
to leaf senescence and yield trait, indicating that the potential interaction between autophagy and auxin at a transcriptional level might be involved in regulating the timing of maize leaf senescence.

Materials and methods

Plant materials and field experiments

Two maize inbred lines, Si-287 and Si-144 with distinctive senescence phenotypes were obtained from Jilin Academy of Agricultural Sciences (Changchun, China). Si-287 (carrying \(\text{Stg}_3^{\text{Si}-287}/\text{Stg}_7^{\text{Si}-287}\) alleles) is derived from a cross between the maize inbred line 444 and Jm-03, and Si-144 (carrying \(\text{Stg}_3^{\text{Si}-144}/\text{Stg}_7^{\text{Si}-144}\) alleles) is derived from a Thai hybrid.

An \(F_2\) population with 207 individuals was developed from a cross between Si-287 (female parent) and Si-144 (male parent). Moreover, 3725 individuals of \(BC_2F_2\), \(BC_3F_2\) and \(BC_4F_2\) generations were generated from repetitive back-crossed progeny from a cross between Si-287 (recipient) and \(F_2\) individuals (donor). The near-isogenic lines (NILs), which contained one [NIL-\(\text{Stg}_3^{\text{Si}-287}/\text{Stg}_7^{\text{Si}-287}\) alleles], \(\text{NIL-}\text{Stg}_3^{\text{Si}-144}/\text{Stg}_7^{\text{Si}-287}\) (carrying \(\text{Stg}_3^{\text{Si}-287}/\text{Stg}_7^{\text{Si}-287}\) alleles), or two [NIL-\(\text{Stg}_3^{\text{Si}-287}/\text{Stg}_7^{\text{Si}-287}\) alleles] donor segments, were screened from the progeny in the \(BC_3F_4\) generation. In addition, a total of 173 maize inbred lines collected from Jilin Academy of Agricultural Sciences (Changchun, China) were used in this study (Supplementary Table S1).

During the natural growing season, maize plants were planted in the experimental fields in Beijing, China (39°55’ N, 108°42’ E) in 2015 and 2016, Sanya, Hainan Province, China (18°36’ N, 107°42’ E) in 2016, and Jinta, Gansu Province, China (39°57’ N, 98°22’ E) in 2018 and 2019. Plant materials were grown in the normal conditions in accordance with the local agricultural practices. In the summer of 2017, the improved hybrids and Jidan27 (as hybrid control) for drought tolerance analysis were grown at the experimental fields in Jinta, Gansu Province and Bayan Nur, Inner Mongolia, China (40°75’ N, 107°42’ E), respectively. These two locations are all with low annual rainfall and large evaporation during the growth period. For the well-watered conditions, the management followed the local agricultural practices. For the drought stress conditions, water was withheld from seven days days before anthesis (DBA) to 45 days after anthesis (DAA), and other management was the same as the well-watered conditions.

Phenotypic data collection

The maize population varied substantially in flowering time, spanning from 68 to 110 days after sowing. To accurately record and calculate the phenotype of leaf senescence, we firstly marked the fifth and tenth leaves from 68 to 110 days after sowing. To accurately record and calculate the phenotype of leaf senescence, we firstly marked the fifth and tenth leaves from 68 to 110 days after sowing. To accurately record and calculate the phenotype of leaf senescence, we firstly marked the fifth and tenth leaves from 68 to 110 days after sowing.

Chlorophyll measurement

Chlorophyll of the collected samples was extracted using 80% (v/v) acetone at 25–26 °C for 24 h in the dark. Before quantification, the mixture was centrifuged at 6000 \(\times\) g for 10 min. The absorbance of the supernatant was measured at 645 nm and 663 nm, and total chlorophyll content was calculated by the formula: C (mg g\(^{-1}\)) = 20.2 \(\times\) A\(_{645}\)+8.02 \(\times\) A\(_{663}\), and the volume of extracted solution and the fresh weights of tissues were used to calculate the concentration of chlorophyll in leaves (Arnon, 1949).

Soluble protein measurement

Plant materials were extracted in double-distilled water. The mixture was centrifuged at 5000 \(\times\) g for 10 min at 4 °C. Then the supernatant was collected, after which 100 µl of the collected supernatant was taken, and 900 µl double distilled water and 5 ml Coomassie brilliant blue G250 were added for mixing [for the standard curve, supernatant and double distilled water were replaced by Bovine serum albumin (BSA) standard solution of different concentrations]. After 2 min standing, the absorbance at 595 nm was measured. The soluble protein concentration of different samples was calculated according to the equation (C \(\times\) Vt) / (FW \(\times\) Vs \(\times\)1000). C represents the value from a standard curve, Vt and Vs represent the volume of extracted solution and the volume of test solution, respectively, and FW represents the fresh weight of leaf samples.

RNA extraction and RT–qPCR analysis

Leaf samples were collected in the field at seven DBA and 30 DAA, then quickly frozen in liquid nitrogen and stored at −80°C for RNA extraction. Three biological replicates were carried out for each sample. Total RNA was extracted by Quick RNA isolation Kit [Huayueyang Biotechnology (Beijing) Co., Ltd., China] and treated with RNase-free DNase I to remove residual genomic DNA, following which OD\(_{260/280}\) of the extracted RNA was measured. A ReverTra Ace reverse transcriptase kit (Toyobo, Japan) was used to synthesize cDNA. RT–qPCR analysis was performed with an Eco system (Illumina, USA) using UltraSYBR Mixture (Cwbio, China), and at least three replicates were performed for each sample. After stability evaluation of six candidate reference genes under all experimental conditions by NormFinder and geNorm software tools (Andersen et al., 2004; Vandesompele et al., 2002), Zm00001d036201, which encodes a hypothetical protein, was used as the internal control for normalization of gene expression values (Lin et al., 2014; Bertels et al., 2020; Supplementary Fig S1). The relative expression of amplified genes was calculated by the 2\(^ {-\Delta\Delta CT}\) method (Livak and Schmittgen, 2001). Primers used for RT–qPCR analysis are listed in Supplementary Table S2. Detailed information about RT–qPCR is listed in Supplementary Table S3 (Bustin et al., 2009).

Molecular marker development and genotyping

Genomic DNA of each individual was extracted by the CTAB method (Murray and Thompson, 1980; Causse et al., 1994). Kompetitive Allele-Specific PCR (KASP) markers were developed [China Golden Marker (Beijing) Biotech Co., Ltd., China] on the basis of the B73 genome, and a total of 127 markers were used after validation in Si-287 and Si-144. In addition, the InDel and SNP (single nucleotide polymorphism) markers were developed for fine mapping of the target loci. Primers were designed with the Primer Premier 5.0 software. The polymorphisms of designed markers were screened between the parental lines using agarose gel electrophoresis or Sanger sequencing. All the molecular markers developed for genotyping are listed in Supplementary Table S4.
QTL analysis of the F2 population was conducted using the QTL IciMapping software (version 4.1) via inclusive composite interval mapping (ICIM-ADD; Meng et al., 2015). The threshold of LOD (logarithm of odds) for a quantitative trait locus was determined by 1000 permutation tests at a significance level of $P = 0.05$, and the additive effects were also displayed.

QTL-seq approach (whole-genome sequencing-based QTL mapping; Takagi et al., 2013) was performed in the BC1xF2 population. Two bulked DNA pools with extreme phenotypes (stay-green pool, C2 and senescence pool, C1) were prepared by mixing DNA from 30 individuals in an equal ratio. Furthermore, whole-genome resequencing of the selected DNAs was carried out, and the high-quality reads obtained from the DNA bulks were aligned to the reference sequence to identify SNPs (Supplementary Table S5). SNP-index was calculated for all the detected SNP positions, and those with sequencing depth <7 and SNP-index <0.3 were excluded from the two extreme bulks. After filtering, 4 817 475 SNPs were obtained, and $\Delta$(SNP-index) was calculated for all these positions by the following formula: $\Delta$(SNP-index) = SNP-index (C2) $-$ SNP-index (C1). With 10 000 replications of the permutation test, the threshold values were selected at the 95% and 99% levels. The candidate loci were obtained in the continuous and evenly distributed regions that exceeded the 99% confidence threshold. Furthermore, recombinant individuals were screened from the successive generations of BC1xF2 and BC2xF1 (1106 individuals) with senescent phenotype segregation.

**Transcriptome data analysis**

Clean reads were obtained by filtering the raw reads (removing the adapters and low-quality sequences) and then mapped to the B73 reference genome (ftp://ftpensemblgenomes.org/pub/plants/release-39/fasta/zea_mays/). The HISAT2 software (Version 2.1.0) was used to count the total mapped reads and uniquely mapped rate (Kim et al., 2015). FPKM (Fragments Per Kilobases per Million reads) values were used to calculate gene expression with RSEM software (Version 1.2.31; Li and Dewey, 2011). DEGs were obtained by DEseq2 software (Version 1.22.1; Love et al., 2014) with the condition of $|\log_{2}$(fold change) $|$ ≥1, FDR <0.05 (false discovery rate). Gene clustering and pathway enrichment were conducted by R (Version 3.5.3).

**Sequence analysis and natural variations of the candidate genes**

Genomic sequences of the candidate genes were amplified from Si-287 and Si-144 by KOD-Plus-Neo (Toyobo, China). Primers used for the amplification are listed in Supplementary Table S6. PCR was performed in a 25 µl volume containing 2.5 µl PCR buffer, 2.5 µl dNTPs (2 mM), 1.5 µl MgSO4 (25 mM), 1 µl forward primer (10 µM) and 1 reverse primer (10 µM), 1 µl DNA template (100 ng µl$^{-1}$), 0.5 µl KOD-Plus-Neo (1 U µl$^{-1}$) and 15 µl sterile water. All the amplifications were carried out on an Applied Biosystems Veriti Thermal Cycler (ABI, USA), and the cycling program was 94 °C for 2 min, followed by 38 cycles at 98 °C for 10 s, 58 °C for 30 s and 68 °C for 1–2 min 30 s, then an extension at 68 °C for 7 min. The PCR products were separated and purified by a 1.5% agarose gel, and the DNA was extracted from the fragments and sequenced by Sanger sequencing. The alignments of the obtained sequences were conducted by DNAMAN software (version 7.0), and variations between the parental lines were detected. The gene structure in this study was drawn by IBS software (Liu et al., 2015).

Natural variations of the candidate genes were amplified from 173 maize inbred lines by PCR using Goldstar Best MasterMix (Cwbio). The genotype of each line was distinguished by agarose gel electrophoresis or sequenced by Sanger sequencing, and aligned to identify variations.
and leaf senescence phenotypes of 1904 plants were measured. To confirm the loci (Stg3 and Stg7) detected by the classical QTL mapping, a QTL-seq approach which is based on next-generation sequencing (NGS) technology and widely used in many plant species (Takagi et al., 2013; Lu et al., 2014; Zheng et al., 2020), was performed on respective 30 individuals with extreme phenotypes (senescence and stay-green). At the 99% level of significance, two major QTL were detected on Chr3 (145.66–161.01 Mb) and Chr7 (167.12–170.68 Mb), which overlapped with the major regions of Stg3 and Stg7, respectively, identified by classical QTL mapping (Fig. 2C, D). To further narrow down the targeted intervals, 30 pairs of polymorphic molecular markers were developed to dissect the BC3F3 and BC3F4 populations (Supplementary Table S4), and 1106 individuals were genotyped (Fig. 2A). Finally, Stg3 was mapped to a 5.86 Mb interval, and Stg7 to a 380 kb interval (Fig. 2C, D). These data suggest that these two loci might play vital roles in delaying leaf senescence, and genes in the targeted regions deserve further analysis.

To validate the function of Stg3 and Stg7, we identified the background of homozygous recombinants from the BC3F4 population, and a total of six individuals possessing the Stg3 [NIL–Stg3Si-144 (carrying Stg3Si-144/Stg7Si-144 alleles)], Stg7 [NIL–Stg7Si-144 (carrying Stg3Si-287/Stg7Si-144 alleles)] and both the two loci [NIL–Stg3Si-144/Stg7Si-144 (carrying Stg3Si-144/Stg7Si-144 alleles)] from the donor parent (Si-144) were selected (Supplementary Fig. S2). The genetic background recovery rates of all these NILs ranged from 90.4% to 98.1% (Supplementary Table S8). To further determine the function of these two major loci, the characteristics of leaf senescence were analysed. Compared with Si-287, which was senescent at 30 DAA, delayed leaf senescence was observed in all the NILs (Supplementary Fig. S3A), as shown by the significantly (P<0.05) higher percentage of green leaves and the concentration of chlorophyll in NILs compared with those in Si-287 (Fig. 3A, B). Soluble protein concentrations in NIL–Stg7Si-144 was higher than that in Si-287, but no significant difference (P>0.05) was detected amongst NIL–Stg3Si-144, NIL–Stg3Si-144/Stg7Si-144 and Si-287 (Fig. 3C). We further measured the $F_v/F_m$ values of different lines and found that the photosynthetic activity of Si-287 was significantly (P<0.01) lower than that of NILs (Supplementary Fig. S3B; Fig. 3D). At 45 DAA, significant (P<0.01) phenotypic differences were observed between Si-287 and NILs. Si-287 was almost completely yellow, while NILs remained green and the percentage of green leaves was significantly (P<0.01) higher than that of Si-287 (Fig. 3E, F).

**SAGs contributing to phenotypic variation of leaf senescence identified by transcriptomic analysis**

Leaf senescence is driven by thousands of so-called SAGs. To understand the differences in leaf senescence in the parental lines at the transcriptional level, we performed RNA-seq analysis of Si-287 and Si-144. Raw data were acquired with Q30 >95%, and 596 490 046 reads were obtained after filtering and quality control (Supplementary Table S9). Here, we mainly focused on the unique up-regulated genes in Si-287 and those shared by Si-287 and Si-144 with $\Delta FC \geq 2$. Therefore, a three-step data analysis protocol was used to further identify SAGs: (i) by
Fig. 2. Identification of two major loci for leaf senescence via QTL mapping. (A) The scheme of the construction of mapping population for the detection of candidate loci controlling leaf senescence. Si-287 was used as the recurrent parent backcrossed for three generations. (B) Frequency distribution of leaf senescence in F₂ population. (C) Identification of leaf senescence locus Stg3 on Chr3. PVE represents phenotypic variation identified from classical QTL mapping in F₂ population. Delta SNP Index graph from QTL-seq further identified Stg3 in the region of 145.66–161.01 Mb on Chr3. Linkage analysis with molecular markers on the mapping population delimited the region to an interval between markers M36 and A-3–11. (D) Identification of leaf senescence locus Stg7 on Chr7. Delta SNP Index graph from QTL-seq further identified Stg7 in the region of 167.12–170.68 Mb on Chr7. Linkage analysis with molecular markers on the mapping population refined the location to an interval defined by molecular markers A-7-6 and A-7-7.

comparing the gene expression at 30 DAA with seven DBA, DEGs of Si-287 and Si-144 were detected. A total of 3577 up-regulated and 1424 down-regulated genes were identified in Si-287, and 3958 up-regulated and 1424 down-regulated genes were identified in Si-144 (Fig. 4A); (ii) we then compared the up-regulated genes between Si-287 and Si-144, and removed DEGs between Si-287_7 DBA and Si-144_7 DBA to avoid the effects of developmental stages and genetic background (Fig. 4B), because the phenotype of leaf senescence was not different between Si-287 and Si-144 at the initial stage (7 DBA). Then we obtained 1671 specific up-regulated DEGs in Si-287 (Fig. 4B); (iii) genes in the shared part between Si-287 and Si-144 in the up-regulated DEGs were also important for us to detect SAGs. Amongst them, the 461 genes in U2 set with |ΔFC|≥2 were of more interest to us (Fig. 4B). Finally, we obtained the SAGs set of 2132 genes, including 1671 up-regulated DEGs and 461 genes in U2 set with |ΔFC|≥2 (Fig. 4B). We further compared our results (SAGs_Si-287) with a previous transcriptome study (SAGs_B73) of maize developmental leaf senescence (Sekhon et al., 2019). Although different plant materials were used (Si-287 and B73), 1655 common genes were still identified. Gene ontology (GO) enrichment analysis revealed that these common genes were significantly enriched in sugar catabolism, autophagy, amino acid
Fig. 3. Alleles of Stg$_{3/144}^{3560}$ and Stg$_{7/144}^{3560}$ delayed leaf senescence. (A) Statistic analysis of green leaves ratio of Si-287 and NILs (NIL-Stg$_{3/144}^{3560}$, NIL-Stg$_{7/144}^{3560}$ and NIL-Stg$_{3/144}^{3560}$/Stg$_{7/144}^{3560}$). Si-287 harboured the combination of alleles of Stg$_{3/144}^{3560}$/Stg$_{7/144}^{3560}$. NIL-Stg$_{3/144}^{3560}$ = near-isogenic line with the Stg$_{3/144}^{3560}$ allele in Si-287 background; NIL-Stg$_{7/144}^{3560}$ = near-isogenic line with the Stg$_{7/144}^{3560}$ allele in Si-287 background; NIL-Stg$_{3/144}^{3560}$/Stg$_{7/144}^{3560}$ = near-isogenic line with the stacking of Stg$_{3/144}^{3560}$ allele and Stg$_{7/144}^{3560}$ allele in Si-287 background. (B, C) Concentration of...
transport, nutrient transport and catalytic activity categories (Fig. 4C). These results indicate that the identified SAGs, especially the 1655 common genes and related GO categories, might be significant regulators of maize leaf senescence.

**Putative genes in Stg3 and Stg7**

Through QTL analysis, we obtained 108 and 13 candidate genes in Stg3 and Stg7, respectively (Supplementary Tables S10; S11). Compared with the SAGs set from the transcriptome analysis, three genes were identified. Amongst them, Zm00001d042215 and Zm00001d042241 were the candidate genes for Stg3. For Zm00001d042215, the expression was significantly (P<0.01) higher in Si-287 than that in Si-144 at 30 DAA. However, there was no significant difference (P>0.05) in the expression of Zm00001d042241 between the parental lines at 30 DAA. Moreover, candidate region association analysis detected a significant SNP [−log10 (P) > 4.63] in Zm00001d042215, while no significant [−log10 (P)<4.63] signal was detected in Zm00001d042241 (Supplementary Fig. S4). Therefore,
Zm00001d042215 was selected as the causal gene for Stg3. For Stg7, Zm00001d022017 was the only candidate gene (Fig. 5A, B). Furthermore, we analysed the functional annotations of these two genes. Zm00001d042215 (also named ZmATG18b) participates in the formation of autophagosomes and plays a vital role in the autophagy pathway (Li et al., 2015). Zm00001d022017 (ZmGH3.8) encodes an indole-3-acetic acid-amido synthetase (GH3) which is involved in auxin metabolism (Du et al., 2012). It deserves attention that both autophagy and auxin pathways have been reported to be involved in plant development and stress responses (Du et al., 2012; Ustun et al., 2017). Thus, we speculated that these two pathways were significant components in maize leaf senescence. To validate this, we analysed the related genes in these two pathways, which fit at least one of the following criteria: (i) genes in the SA4s set identified in this study; (ii) genes in both the reported leaf senescence QTL in maize (Zheng et al., 2009; Almeida et al., 2014; Belicuas et al., 2014; Khanal et al., 2015; Trachsel et al., 2016; Yang et al., 2017) and DEGs identified by transcriptome analysis in this study (Fig. 5C). In the main auxin biosynthesis pathway, two genes, ZmGH3.8 and ZmYUC5 (Zm00001d019527) were found. In the autophagy pathway, a total of 14 genes met the criteria.

Through Sanger sequencing and sequence alignment, we detected the genomic variations of ZmATG18b and ZmGH3.8 between Si-287 and Si-144 (Fig. 5D, E). For ZmATG18b, a 6106 bp DNA fragment containing the whole genomic region and the 1600 bp upstream region of 5’UTR were amplified and sequenced. In the genomic region, there was only one variation (A to G) in the first exon, which caused a non-synonymous mutation, but it was not located in the vital motifs of this gene. Considering the differences in expression mentioned above, we compared the variations in the promoter region. A total of 52 variations including SNPs and InDels were detected, two of which were detected in major cis-elements (TATA-box and AE-box), which are important elements in stress and light responses. Compared with Si-144, there was a deletion of the TATA-box at −1498 bp (1498 bp upstream of the 5’UTR region) in Si-287. Mutation of A to C was also found in the AE-box at −1439 bp (1439 bp upstream of the 5’UTR region) in Si-287. Thus, the corresponding alleles were designated as Stg3Si-287 type (senescent allele) and Stg3Si-144 type (stay-green or non-senescent allele), respectively (Fig. 5D). For ZmGH3.8, 16 and 31 variations were exhibited in the whole genomic region (2492 bp) and the 1600 bp region upstream of 5’UTR, respectively. Amongst them, only two variations existed in the exons, which caused non-synonymous mutations, but they were also not located in the important motifs. Similarly, we speculated that variations in the promoter region might be the causative sites responsible for phenotype differences in leaf senescence. To validate this speculation, we analysed the cis-elements in the promoter region, as several auxin-responsive cis-elements, such as ARFAT, ASF1MOTIFCAMV (TGACG) and AUXREPSIAA4, which were functionally important, have been identified in the promoter region of GH3 in maize (D. Zhang et al., 2016). By comparing the sequences of these cis-elements in the parental lines, differences in ASFI MOTIFCAMV (TGACG) were detected. There was one copy in Si-287, but two copies were found in Si-144. Amongst them, one copy, both in Si-287 and Si-144, was located at −857 bp (857 bp upstream of the 5’UTR region), another copy which existed only in Si-144, was located at −1288 bp (1288 bp upstream of the 5’UTR region), and there existed a 211 bp deletion in Si-287. We then designated the corresponding alleles as Stg7Si-287 type (senescent allele) and Stg7Si-144 type (stay-green or non-senescent allele), respectively (Fig. 5E). In-depth investigation of these allelic differences will facilitate understanding of the regulatory mechanisms of leaf senescence.

**Population genetics of the effects of Stg3 and Stg7 on leaf senescence and yield**

To further analyse the functions of the vital alleles in ZmATG18b and ZmGH3.8, we detected the natural allelic variations of these sites in a total of 173 maize inbred lines (Supplementary Table S1). Intriguingly, we found that according to the variations identified, genotypes of these inbred lines can be classified into four combinations, including Stg3Si-144/Stg7Si-144 type (n=51), Stg3Si-144/Stg7Si-287 type (n=98), Stg3Si-287/Stg7Si-144 type (n=5), and Stg3Si-287/Stg7Si-287 type (n=19) (Fig. 6A, B). Statistically, lines with Stg3Si-144/Stg7Si-144 type had a significantly (P<0.01) delayed leaf senescence (stay-green) phenotype than those with Stg3Si-287/Stg7Si-287 type. For lines with Stg3Si-144/Stg7Si-287 type and Stg3Si-287/Stg7Si-144 type, there were no significant differences (P>0.05) compared with Stg3Si-144/Stg7Si-144 type or Stg3Si-287/Stg7Si-287 type (Fig. 6B). In addition, we compared the ear weight of lines with different combinations of the Stg3Si-144 and Stg7Si-144 alleles and similar trends were observed (Fig. 6C). These results suggest that the stacking of Stg3Si-144 and Stg7Si-144 alleles could delay leaf senescence and increase ear weight in maize.

Additionally, we also explored the implications of identified alleles on maize breeding. We used Si-144 as the donor parent and NILs as the receptor parent for cross combination. The improved hybrids and Jidan27 were planted under two conditions: normal condition and drought stress condition. At 45 DAA, we calculated the ratio of green leaves of the improved hybrids and Jidan27 in the field. Compared with normal growth condition, the percentage of green leaves ratio reduction and ear weight loss traits in NIL-Stg3Si-144/Stg7Si-144 × Si-144 (Stg3Si-144/Stg7Si-144) were both significantly (P < 0.05) lower than that of Jidan27 under drought stress (Fig. 6D, E). Considering the two putative genes obtained and the performance of the stacking combination in inbred lines or improved hybrids, we suggested that the stacking effect might be controlled by the interaction between autophagy and auxin pathways.
Fig. 5. Candidate genes revealed by the integrated analysis of QTL mapping and transcriptome analysis. (A) Candidate genes obtained from the comparison between genes in the locus of Stg3 and SAGs set. Zm00001d042215 and Zm00001d042241 were the common genes identified. (B) Candidate genes obtained from the comparison between genes in the locus of Stg7 and SAGs set. Zm00001d022017 was the common gene obtained. (C) Genes in SAGs set or DEGs in the reported leaf senescence QTL which are involved in autophagy and the main auxin biosynthesis pathways. Boxes
Fine-tuning interaction between Stg3 and Stg7 on leaf senescence

To further validate the function of the stacking effect of ZmATG18b and ZmGH3.8, we analysed the expression patterns of these two genes in the NILs and Si-287 at seven DBA and 30 DAA (Fig. 7A, B). Compared with seven DBA, ZmATG18b showed a down-regulation (negative fold-change) at 30 DAA in all the NILs, but it was up-regulated (positive fold-change) in Si-287. For ZmGH3.8, it was up-regulated (positive fold-change) in all the NILs and Si-287, while its expression in NIL-Stg3 Si-144/Stg7 Si-144 and NIL-Stg3 Stg7 Si-144 were significantly higher ($P<0.01$) than that in NIL-Stg3 Si-144 and Si-287 at 30 DAA. Combining the leaf senescence phenotype of these lines, we found that down-regulation of ZmATG18b and up-regulation of ZmGH3.8 was a favourable combination in delaying leaf senescence. It should be noted that the expression of ZmATG18b was the lowest at 30 DAA compared with other three lines (NIL-Stg3 Si-144, NIL-Stg7 Si-144 and Si-287), while that of ZmGH3.8 was relatively moderate (higher than that in NIL-Stg3 Si-144 and Si-287, and lower than that in NIL-Stg7 Si-144) in NIL-Stg3 Si-144/Stg7 Si-144 at 30 DAA, which suggests that a subtle balance between the expression of these two genes is needed in NIL-Stg3 Si-144/Stg7 Si-144, leading to the delay of leaf senescence.

Considering the combination of Stg3 Si-144/Stg7 Si-144 and Stg3 Stg7 Si-287 type lines exhibited significant differences with different colours represent expression of each gene. The red triangles represent SAGs identified in this study, and the red asterisks represent DEGs in the reported leaf senescence QTL in maize. YUC, Yuca; ATG, Autophagy; PI3K, Phosphatidylinositol 3-kinase. (D, E) Schematic representation of the structure and sequence variations of ZmATG18b (D) and ZmGH3.8 (E). The white boxes represent UTR regions, and the green boxes represent exons. The upstream region of the 5′UTR indicates the promoter region. The vertical black lines indicate variations between Si-287 and Si-144. The pentagons represent variation sites in the major cis-elements, different colours indicate different cis-elements. Variations of the marked sites between Si-287 and Si-144 were displayed with yellow and green background, respectively. Stg3 Stg7 type and Stg7 Stg7 indicate alleles of ZmATG18b and ZmGH3.8 in Si-287, respectively. Stg3 Stg7 type and Stg7 Stg7 indicate alleles of ZmATG18b and ZmGH3.8 in Si-144, respectively.
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in senescence phenotypes and yield character, as well as differences in the expression patterns of \textit{ZmATG18b} and \textit{ZmGH3.8} (Fig. 7C), we inferred that the interaction between autophagy and auxin pathways might be the cause of these performances. However, the underlying mechanism remains to be ascertained.

### Discussion

**Identification of candidate genes involved in leaf senescence by integrated analysis of QTL mapping and transcriptome profiling**

Although previous QTL mapping studies identified multiple loci associated with leaf senescence across the 10 chromosomes of maize (Zheng et al., 2009; Almeida et al., 2014; Belicuas et al., 2014), the position of \textit{Stg3} and \textit{Stg7} identified in this study are not in those reported regions, suggesting that they are two novel loci for maize leaf senescence. In sorghum, four classical loci regulating leaf senescence were identified (Xu et al., 2000). Interestingly, the collinearity loci of \textit{Stg3} and \textit{Stg7} identified in this study were close to \textit{Stg1} and \textit{Stg3} in sorghum, respectively, indicating that mechanistic similarities might exist between maize and sorghum (Supplementary Fig. S5). Moreover, the yield data of improved hybrids and Jidan27 further confirmed the tolerance function of these two loci to drought stress. Under drought stress, ear weight losses of \textit{NIIL-Stg3Si-144/Stg7Si-144} × \textit{Si-144} was significantly lower than that of Jidan27 (Fig. 6E). These results were consistent with previous reports that timely delaying of leaf senescence can facilitate yield homeostasis under stress conditions (Thomas and Ougham, 2014; Zhang et al., 2019). Therefore, we speculate that these two QTL identified in this study not only play important roles in delaying leaf senescence, but also have potential effects on maize yield stability under drought stress.

QTL mapping and transcriptomic analysis are important approaches for detecting the essential pathways or genes controlling quantitative traits of interest. Through integrated analysis of these two methods, two candidate genes \textit{ZmATG18b} and \textit{ZmGH3.8} were identified, and they were included in the 1655 common genes identified in the \textit{SAGs} set in a previous study (Sekhon et al., 2019) and ours (Fig. 4C), indicating their
potential importance in the regulation of leaf senescence. It has been reported that \textit{ZmATG18b} plays an essential role in plant development and stress responses (Klionsky and Ohsumi, 1999; Li et al., 2015), and that \textit{ZmGH3.8} is involved in rice morphogenesis and response to stress tolerance (Du et al., 2012).

The potential function of the candidate genes in the regulation of leaf senescence in maize

Expression pattern analysis revealed that the expression of \textit{ZmATG18b} was higher in Si-287 than in NIL-\textit{Stg}\textsuperscript{3\text{Si}}-144 at 30 DAA (Fig. 7A), suggesting that this gene might be a positive regulator of maize leaf senescence. It has been reported that autophagy can be activated during leaf aging, and that \textit{ATG18} is involved in the formation of autophagosome in Arabidopsis (Wang and Schippers, 2019). Some studies have also shown that the increase of autophagic activity can promote leaf senescence and cell death (Avila-Ospina et al., 2014), suggesting the possibility of \textit{ZmATG18b} in controlling maize leaf senescence. However, the underlying mechanism is still unclear.

Meanwhile, the expression of \textit{ZmGH3.8} in NIL-\textit{Stg}\textsuperscript{7\text{Si}}-144 was 20.4-fold higher than that in Si-287 at 30 DAA (Fig. 7B), suggesting its negative role in the regulation of maize leaf senescence. As \textit{GH3} is involved in IAA (indole-3-acetic acid) conjugation (Staswick et al., 2005), we speculated that changes in IAA concentration might be a main factor regulating leaf senescence. The role of auxin in leaf senescence is complex (Quirino et al., 1999; van der Graaff et al., 2006; Crane et al., 2019), and the potential mechanism is still much less understood. Higher concentrations of free IAA were detected in the senescent line than in the non-senescent line (our ongoing work); further studies on auxin conjugation are needed to better understand the mechanisms.

Natural allelic variations of these two genes significantly contributed to leaf senescence phenotype and yield trait in maize, and better performance in both inbred lines with the optimal allelic combination and the improved stacking NILs than in Si-287/ Jidan27 indicated the underlying interaction between \textit{Stg3} and \textit{Stg7}. However, the relevant mechanisms need to be studied further. Generally, reactive oxygen species (ROS) are a crucial component in plant responses to external stimuli (Gorlach et al., 2015; Signorelli et al., 2019). The accumulation of ROS can be influenced by auxin concentration and autophagic activity (Du et al., 2012; Signorelli et al., 2019). Further studies on the role of ROS in the connection between autophagy and auxin pathways in controlling leaf senescence are necessary.

In this study, four combinations were found for the natural allelic variations of \textit{ZmATG18b} and \textit{ZmGH3.8}. Lines with \textit{Stg3}\textsuperscript{3\text{Si}}-144/\textit{Stg7}\textsuperscript{7\text{Si}}-144 type showed higher green leaves ratio and increased ear weight compared with those of \textit{Stg3}\textsuperscript{3\text{Si}}-287/\textit{Stg7}\textsuperscript{7\text{Si}}-287 type. We questioned whether these combinations had been effectively used in maize breeding, so the elite inbred lines which have been widely used in hybrid maize breeding were examined. Amongst them, Qi-319 belongs to \textit{Stg3}\textsuperscript{3\text{Si}}-144/\textit{Stg7}\textsuperscript{7\text{Si}}-144 type, Zheng58, Huangzao4 and Ye478 are lines carrying \textit{Stg3}\textsuperscript{3\text{Si}}-144/\textit{Stg7}\textsuperscript{7\text{Si}}-287 type, and Chang7-2 is the line with \textit{Stg3}\textsuperscript{3\text{Si}}-287/\textit{Stg7}\textsuperscript{7\text{Si}}-144 type (Supplementary Table S1). As mentioned above, lines with \textit{Stg3}\textsuperscript{3\text{Si}}-144/\textit{Stg7}\textsuperscript{7\text{Si}}-287 and \textit{Stg3}\textsuperscript{3\text{Si}}-287/\textit{Stg7}\textsuperscript{7\text{Si}}-144 type displayed no significant differences in leaf senescence and yield traits compared with those in Si-287, hence improvement of lines with these two types into \textit{Stg3}\textsuperscript{3\text{Si}}-144/\textit{Stg7}\textsuperscript{7\text{Si}}-144 type would help the breeders to increase leaf stay-green rate and yield in maize.

Taken together, we identified two novel loci, \textit{Stg3} and \textit{Stg7}, controlling maize leaf senescence. Further analysis revealed that autophagy and auxin pathways might play crucial roles in leaf senescence at the transcriptional level. Natural allelic variations showed that the alleles of \textit{Stg3}\textsuperscript{3\text{Si}}-144/\textit{Stg7}\textsuperscript{7\text{Si}}-144 type, which could delay leaf senescence, is a favourable combination for increasing maize yield. In addition, the improved hybrids of NIL-\textit{Stg3}\textsuperscript{3\text{Si}}-144/\textit{Stg7}\textsuperscript{7\text{Si}}-144 × Si-287 exhibited lower yield loss under drought stress. The underlying mechanisms of \textit{ZmATG18b} and \textit{ZmGH3.8} in leaf senescence awaits further investigation.

Supplementary data

The following supplementary data are available at JXB online.

Fig. S1. Gene expression stability of the candidate reference genes calculated by NormFinder (A) and geNorm (B).

Fig. S2. Genetic composition of NIL-\textit{Stg}\textsuperscript{3\text{Si}}-144 and NIL-\textit{Stg}\textsuperscript{7\text{Si}}-144 on the chromosomes in maize.

Fig. S3. Senescence phenotypes of Si-287 and NILs.

Fig. S4. Candidate gene selection between Zm00001d042215 and Zm00001d042241 in \textit{Stg3}.

Fig. S5. Collinearity of the two candidate loci \textit{Stg3} and \textit{Stg7} in maize and sorghum.

Table S1. Detailed information of maize inbred lines used in this study.

Table S2. Primers used for RT–qPCR analysis.

Table S3. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist.

Table S4. Molecular markers used for the identification of the candidate loci.

Table S5. Statistical analysis of filtered and mapped data of all samples in QTL-seq.

Table S6. Primers used for the amplification of candidate genes.

Table S7. QTL detected for leaf senescence in F\textsubscript{2} population.

Table S8. Detailed information of NILs (NIL-\textit{Stg3Si}-144, NIL-\textit{Stg7Si}-144 and NIL-\textit{Stg3Si}-144/\textit{Stg7Si}-144) used in this study.

Table S9. Statistical analysis of RNA sequencing data of all samples.

Table S10. List of genes in the candidate region of \textit{Stg3}.

Table S11. List of genes in the candidate region of \textit{Stg7}.
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Conflict of interest

The authors declare no conflict of interest.

Author contributions

HCJ conceived and designed the experiments; XF and HQH performed the experiments; LLI, ZGL, and FS provided support for field phenotyping; XYW provided support for bioinformatics analysis; DYH provided support for materials; XF analysed the data and wrote the paper; HCJ and HQH revised the manuscript.

Data availability

The transcriptome raw data of Si-287 and Si-144 reported in this paper have been deposited in the Genome Sequence Archive (Wang et al., 2017) in National Genomics Data Center, China National Center for Bioinformation, Chinese Academy of Sciences, under accession number CRA003926 that are publicly accessible at https://bigd.big.ac.cn/gsa. All other supporting data of this study are available within the paper and its supplementary data published online.

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