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A Cationic Amino Acid Transporter NtCAT1 Promotes Leaf Senescence by the Accumulation of ABA in *Nicotiana tabacum*

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Abstract: Leaf senescence is a comprehensive process performed by integrating various internal and external signals, followed by nutrient reallocation, especially via organic nitrogen (e.g., amino acids) mobilization. Amino acid (AA) transporters play an important role in crop growth and development by participating in the process of organic nitrogen remobilization. However, the biological functions and downstream effectors of amino acid transporters involved in leaf senescence are still poorly understood. In the present study, we cloned and characterized a cationic amino acid transporter gene, *NtCAT1*, from tobacco (K326). We found that *NtCAT1* transcript levels were induced by age and abscisic acid (ABA). The NtCAT1 protein was highly localized in the plasma membrane. The overexpressing *NtCAT1* line (*OECAT1*) showed early leaf senescence, accompanied with increased reactive oxygen species (ROS) and ABA content. By contrast, the *NtCAT1* mutant (*ntcat1-36*) generated by the CRSPR/Cas9 system, showed a delayed-senescence phenotype with a decreased accumulation of ROS and ABA. Moreover, we discovered that the overexpression of *NtCAT1* could downregulate the expression of the target of rapamycin (TOR) kinase gene and upregulate the transcript levels of ABA-related genes during leaf senescence compared with wild type (WT), while the expression of these genes in *ntcat1-36* plants exhibited inverse trends. Furthermore, an analysis of the amino acid concentration demonstrated that *NtCAT1* transgenic plants displayed dramatic changes in the amino acid profile during leaf senescence. In summary, our results suggest that NtCAT1 could promote leaf senescence via the increased biosynthesis of ABA, and our study provides new insights into the molecular mechanism of leaf senescence.

Keywords: leaf senescence; ABA; amino acid transporter; tobacco

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1. Introduction

Leaf senescence, the last stage of leaf development, is a complicated developmental process needed for plant nutrient reallocation, involving the integration of various internal and external signals [1,2]. It is well known that leaf senescence is associated with source–sink transport, especially organic nitrogen (e.g., amino acids) remobilization, for grain filling and plant development [3–5]. Increasing evidence shows that the remobilization of organic N from senescing leaves to actively growing leaves, developing fruits, and seed is an essential process for plant fitness and productivity under challenging environmental conditions [2–4,6]. Despite its importance, the understanding of the molecular regulatory mechanisms underlying leaf senescence in tobacco remains limited.

During leaf senescence, as the main form of organic nitrogen (N), free amino acid remobilization requires a myriad of amino acid transport proteins to transfer them between source and sink organs [7–10]. Numerous studies have suggested that amino acid transporters play a fundamental role in the process of organic N recycling [8,11,12]. Moreover, an

increasing number of reports indicate that various amino acid transporters are involved in the regulation of plant growth and development and abiotic and biotic stress tolerance [12–16]. In Arabidopsis, overexpressing *AtCAT1* decreases the total biomass of soil-grown plants and enhances the resistance to pathogen attack (*P. syringae*) [12]. In rice, the altered expression of amino acid transporters OsAAP3, OsAAP4, and OsAAP5 influences rice development, including tillering [13], grain yield [13,14], and leaf senescence [15]. PtCAT11 has been found to facilitate amino acid transport during leaf senescence in poplar [17]. However, the mechanisms by which amino acid transporters influence leaf senescence remain to be further elucidated, and little is known on the biological roles of amino acid transporters in plants.

It has long been known that ABA is a crucial phytohormone in the regulation of stress tolerance and various developmental processes of plants [18,19]. Accumulating studies have reported that ABA is involved in promoting natural leaf senescence [20–22]. Several genes that promote ABA-induced leaf senescence have been characterized. Accumulating investigations have found that ABA can induce a myriad of senescence-associated gene (SAG) expression, such as SGR [23], NYC1 [24] and SAG12 [25]. Recent years have witnessed tremendous progress in understanding the molecular mechanisms by which ABA homeostasis in plants is fine-tuned by the balance between ABA biosynthesis and degradation [2]. Many reports have shown that the ABA biosynthesis and degradation pathways in plants involve several key genes, including 9′-cis-epoxycarotenoid dioxygenases genes (*NCEDs*) [26]; zeaxanthin epoxidase (*ZEP*) [27]; *ABSCISIC ALDEHYDEOXIDASE3* (*AAO3*) [20], as the last step in ABA biosynthesis; and ABA 8′-hydroxylase genes (*ABA8ox1*) [27], which are required for the oxidation of ABA. Several studies have indicated that the ABA signaling pathway consists of four modules: ABA receptors (*PYR/PYL/RCAR*), protein phosphatases (PP2C), protein kinases (SnRK2), and their downstream targets [28,29]. However, there is very little knowledge about the ABA-induced senescence of tobacco leaves.

Target of rapamycin (TOR) kinase, as an evolutionarily conserved crucial regulator, integrates internal signals (e.g., nutrients, energy, growth, and hormones) and environmental stimuli (e.g., abiotic stresses and biotic stresses) to improve organism growth in eukaryotes [30–33]. Emerging studies suggest that the TOR module plays vital functions in regulating nutrient metabolism and cell proliferation in plants [30,33,34]. TOR regulates the balance between plant growth and stress response by phosphorylating the PYL proteins. In the absence of stress, the phosphorylation of PYL inhibits ABA binding and its interaction with PP2C, resulting in blocked ABA signaling [34]. Recently, it is found that amino acid signals might control convergent ROP2-TOR signaling in Arabidopsis [35]. However, the regulatory network that connects its action to leaf senescence is largely unknown.

Previous studies have suggested that the altered expression of amino acid (AA) transporter genes influences plant developmental processes, such as plant height, tillering and leaf senescence, etc. [12,15]. However, the molecular frameworks by which AA transporters regulate leaf senescence remain obscure. In this study, we cloned and characterized a putative amino acid transporter gene *NtCAT1* from common tobacco (K326). The results showed that the overexpressing *NtCAT1* line displays early leaf senescence, while loss-of-function mutant *ntcat1* exhibits delayed leaf senescence. We also studied the mechanisms by which NtCAT1 regulates leaf senescence at physiological and molecular levels. Our data indicate that NtCAT1 promotes leaf senescence by enhancing the biosynthesis of ABA.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The tobacco cultivars K326 and Nicotiana benthamiana were used in the study. K326 was utilized for the generation of the transgenic plants, and Nicotiana benthamiana was used for the transient assays. The seeds of tobacco were surface-sterilized and plated on the 1/2 MS medium (2.2 g/L MS + 20 g/L sucrose + 1% agar, PH = 5.8). Then, the plates were placed in a light incubator (28 °C, 16/8 h light/dark cycle and 100 μmol photons m^{-2} s^{-1}) for 7 d. These mature materials were cultured in a greenhouse set at 26–30 °C and 300–400 μmol photons m^{-2} s^{-1} under 16 h light/8 h dark conditions.

2.2. RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted from K326 using TRIgent (Mei5bio, Beijing, China) following the manufacturer's instruction. The reverse transcription protocol was based on HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). An amount of 1 μg of total RNA was first treated to remove residual genomic DNA using 4×gDNA wiper mix in the kit, and then the first strand cDNA was synthesized with 5×HiScript III qRT SuperMix in the kit. qRT-PCR was performed on Thermal Cycler Dice® Real-Time System III (TaKaRa, Kusatsu, Japan). Reaction mixture and PCR protocol was set according to the instructions for 2X M5 HiPer SYBR Premix EsTaq (with Tli RNaseH) (Mei5bio, Beijing, China). The primers used for qRT-PCR are listed in Supplementary Table S2. The NtActin gene was used as an internal control gene, and the expression levels of genes were calculated with the $2 - \triangle\triangle$CT method.

2.3. Subcellular Localization

To clarify the subcellular localization of NtCAT1 protein, NtCAT1 gene CDS sequence was connected with linearized pSuper1300-GFP vector to construct pSuper1300-NtCAT1-GFP fusion expression vector, which was transformed into the agrobacterium tumefaciens strain GV3101. pSuper1300-NtCAT1-GFP and the plasma membrane marker mCherry-PM were injected into the leaves of Nicotiana benthamiana. To further explore the localization of NtCAT1 in K326, NtCAT1:GFP fusion expression vector was constructed, which was driven by the 2 kb upstream of NtCAT1 promoter (ProCAT1:NtCAT1-GFP). After dark treatment for 1 d and normal culture for 2 d, the fluorescence signals were observed by laser confocal microscope (LEICA TCS SP5II, Wetzlar, Germany).

2.4. Generation of Transgenic Lines

To construct NtCAT1-overexpressing vector, the CDS sequence of NtCAT1 was amplified from the cDNA of K326 and inserted into the linearized pCAMBIA2301-S2 vector with the ClonExpress II One Step Cloning Kit (Vazyme, China), following the manufacturer's instruction, to complete the construction of the overexpression vector. The pCAMBIA2301::35S::NtCAT1 plasmid was transformed into Agrobacterium tumefaciens EHA105. The knockout mutants were obtained by CRISPR-Cas9 technology. The target primer sgRNA was designed by E-CRISP (http://www.E-CRISP.org/E-CRISP/ 10 April 2018). After annealing, sgRNA was linked with AtU6-26sgRNA-SK vector, and NheI and SpeI digested the recombinant plasmid to obtain sgRNA cassette with a size of 642 bp. Finally, the sgRNA cassette was linked to pCAMBIA1300pYAO:Cas9 vector, and the recombinant plasmid was transferred into EHA105. These transgenic lines were obtained using the leaf disc method [36].

2.5. Leaf Senescence Experiments

Seeds of WT, OECAT1, and ntcat1-36 mutant plants were plated on 1/2 MS medium (2.2 g/L MS + 20 g/L sucrose + 1% agar) for 7 d and were obtained from different genotypes under the same growth conditions. Under similar conditions, the 7-day-old seedings were transferred to the soil to continue growing for phenotype observations. The 4th leaves of soil-grown WT plants were detached at different development ages, and these leaves at the same leaf position at 15 d, 45 d, 65 d, and 75 d were defined as young leaf (YL), mature leaf (ML), early senescence leaf (ES), and late senescence leaf (LS), respectively. These rosette leaves from different genotypes plants were utilized for senescence experiments or as samples of physiological measurements.

2.6. Assays for Antioxidant Enzymes Activity

For the enzyme assays, 1 g of fresh leaf sample was ground in 3 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 12,000× g for 20 min at 4 °C, and the resulting supernatants were used for assays for SOD, POD, and CAT activities. SOD, POD, and CAT

activities were measured as previously described [37]. All spectrophotometric values are measured via spectrophotometer (Persee TU-1810, Zhengzhou, China).

2.7. GUS Staining

The tissues were immersed in GUS staining solution at 37 °C for 24 h. They were then decolorized using 75% ethanol for 24 h and, finally, stored in 50% glycerin for imaging. GUS staining solution formula (200 mL) was composed of the following: X-Gluc 100 mg, 50 mmol/L K4[Fe(CN)6] 2 mL, 50 mmol/L K3[Fe(CN)6] 2 mL, 0.5 mol/L EDTA (PH = 8.0) 4 mL, methanol 5 mL, Triton X-100 0.2 mL, and 50 mmol/L phosphate buffer up to 200 mL [38].

2.8. Physiological and Biochemical Measurements

The total chlorophyll contents were measured as described previously [37]. In brief, the rosette leaves from each genotype were cut into sections, flash-frozen with liquid nitrogen, and then stored at −80 °C before used. Chlorophyll was extracted from the above samples with 95% ethanol, and then the absorbance was measured at 649 nm and 665 nm.

Measurement of MDA content: 1 g of fresh leaf sample was ground in 2 mL 10% (*w*/*v*) trichloroacetic acid. After the homogenates were centrifuged at 4000× *g* for 10 min, 2 mL of 0.6% trichloroacetic acid was added into the supernatant. The mixtures were reacted in a boiling water bath for 15 min, quickly cooled on ice, and centrifuged at 4000× *g* for 10 min. The supernatant was used to determine the content of MDA according to a previous study [39]. All spectrophotometric values are measured in spectrophotometer (Persee TU-1810, Beijing, China).

2.9. Measurement of ABA Content and Free Amino Acids

ABA content was measured by enzyme-linked immunosorbent assay [40]. Free amino acids were analyzed via a liquid chromatography–tandem mass spectrometry analysis performed by modifying a previously described method [41].

2.10. Statistical Analysis

Student's *t*-test was used to determine significance among treatments. All experiments were conducted in triplicate in this study. Asterisks indicate significant differences at $p < 0.05$.

3. Results

3.1. Screening and Identification of NtCAT1 in Nicotiana tabacum

According to the analysis of the RNA sequencing designed to screen senescence-associated genes, we identified an aging-induced gene from the common tobacco K326, and its coding with an amino acid transporter protein shared a good similarity (68.68%) with AtCAT1 in Arabidopsis. Thus, it was named NtCAT1 (Figure 1A). Based on the protein database of the National Center for Biotechnology Information (NCBI), multiple alignments of forty CAT1 proteins in different plant species were executed by DANMAN, and the phylogenetic tree was generated by the Neighbor-Joining (NJ) method, showing that NtCAT1, in tobacco, had high homology with SlCAT1, in tomatoes (Figure 1A). Three conserved domains were identified from these CAT1 protein sequences using an NCBI CD search named Motif 1–3, and the finding indicated that all forty CAT1 proteins had the conserved domains (cl29101) of the APC amino acid transporter protein family. Thus, the NtCAT1 protein was a member of the amino acid transporter protein family (Figure 1B,C).

3.2. NtCAT1 Is a Senescence-Associated Gene during Natural Leaf Aging

The above-mentioned RNA-seq analysis demonstrated that *NtCAT1* transcription was upregulated during leaf senescence. To further determine the expression profile of *NtCAT1* in tobacco leaves, we performed quantitative real-time PCR (qRT-PCR) to monitor the transcript level of *NtCAT1* in tobacco leaves at four different developmental stages, including young, mature, the early stage of senescence, and the late stage of senescence,

and in different areas of senescing leaves. The expression data showed that *NtCAT1* was expressed in all the leaves described above, with the highest level of expression in the late senescence leaves and the senescent area and the lowest level of transcription in young leaves (Figure 2A,B). These results suggest that *NtCAT1* is a senescence-associated gene and is upregulated during leaf senescence. The *NtCAT1* transcription pattern was further verified using transgenic plants expressing a GUS (β-glucuronidase) gene driven by the *NtCAT1* promoter containing a 2 kb genomic sequence upstream of the start codon (*NtCAT1pro:GUS*). The GUS staining analysis of 5-week-old leaves of *ProNtCAT1:GUS* exhibited that GUS activity gradually increased during the progression of leaf senescence, with higher GUS activity in yellow leaves (Figure 2C,D). This finding suggests that the transcript level of *NtCAT1* is induced during leaf aging.

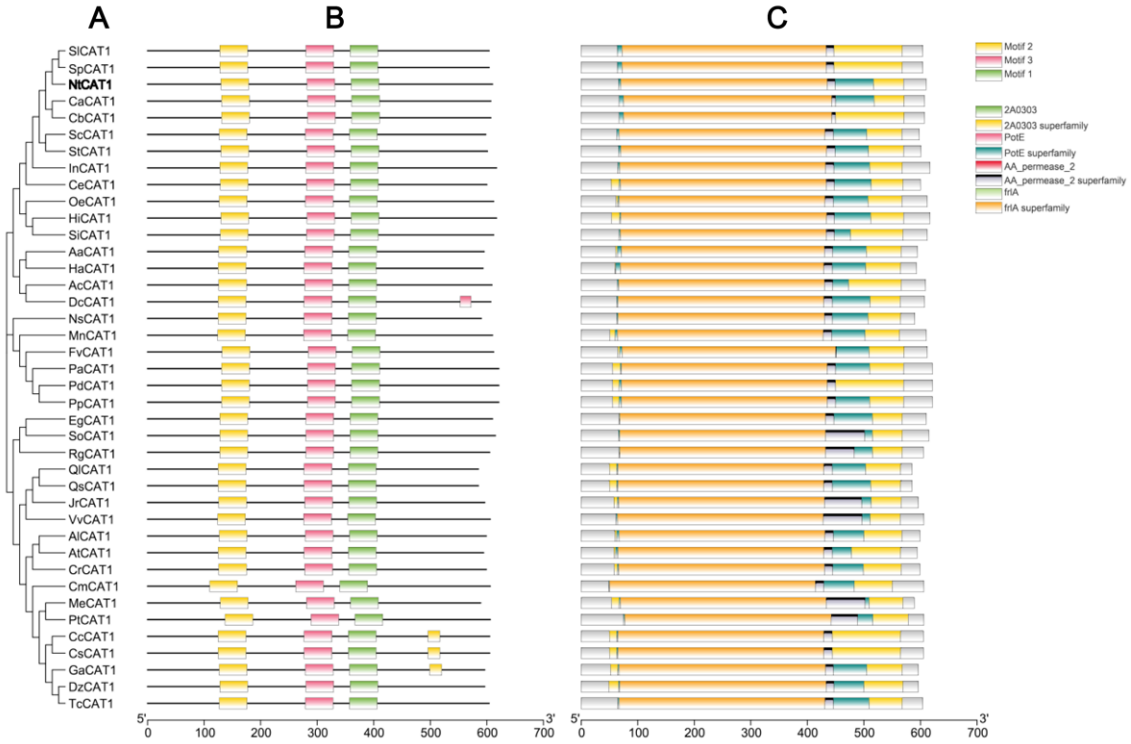

Figure 1. Phylogenetic analysis and characterization of NtCAT1. (**A**) Multiple sequence alignment of NtCAT1 and 39 other CAT1-like homologous proteins. The phylogenetic tree was constructed based on Neighbor-Joining (NJ) method using MEGA7.0. NtCAT1 was marked in bold. (**B**) The MEME motifs are shown as different-colored boxes on the protein sequence. (**C**) Analysis of CAT1 conserved domains using NCBI CD search. The NCBI accession numbers of these 40 CAT1 homologous proteins are listed in Supplementary Table S1.

3.3. Expression of NtCAT1 Is Induced by ABA Treatment

To detect the temporal and spatial expression pattern of *NtCAT1* in plants, we analyzed the transcript levels of *NtCAT1* in various organs (root, stem, leaf, flower, and fruit) of K326 using qRT-PCR. The analysis data showed that *NtCAT1* was expressed in all the organs of tobacco, with the highest level of expression in the root and the lowest level of transcription in the fruit (Figure 3A). We also determined the expression level of NtCAT1 in tobacco seedlings under senescence-related hormone (ABA, ethylene, and SA) treatment. Upon exposure to ABA treatment, the expression of *NtCAT1* started to increase gradually and peaked 6 h after exposure to ABA treatment, with a 2.5-fold increase compared to the control check (mock) plants, and then declined gradually (Figure 3B). In addition, when exposed to ACC and SA treatment, the expression level of *NtCAT1* showed no obvious changes (Figure 3C,D). The above results suggest that the transcript level of *NtCAT1* is upregulated under ABA treatment specifically.

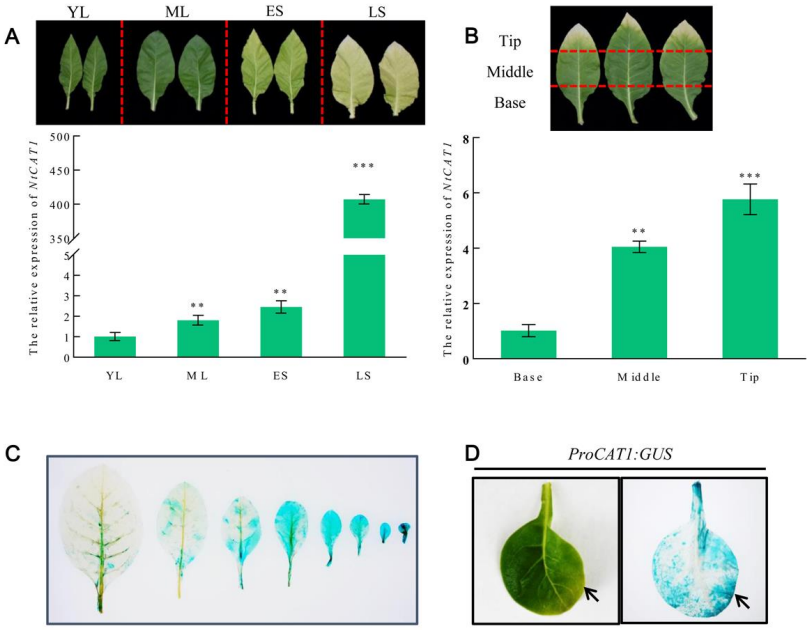

Figure 2. NtCAT1 expression in leaves is upregulated by senescence. (**A**,**B**) The expression levels of NtCAT1 increase in an age-dependent manner. YL, Young leaves; ML, mature leaves; ES, early senescence leaves; LS, late senescence leaves. (**C**,**D**) GUS staining of tobacco leaves of 5-week-old transgenic plant with GUS gene driven by the NtCAT1 promoter. The black arrows show that the GUS activity is higher in senescent leaf areas. The values are presented as means ± SD of three biological replicates. Asterisks indicate statistically significant differences level (Student's *t*-test; ** $p < 0.01$, *** $p < 0.001$) with corresponding controls.

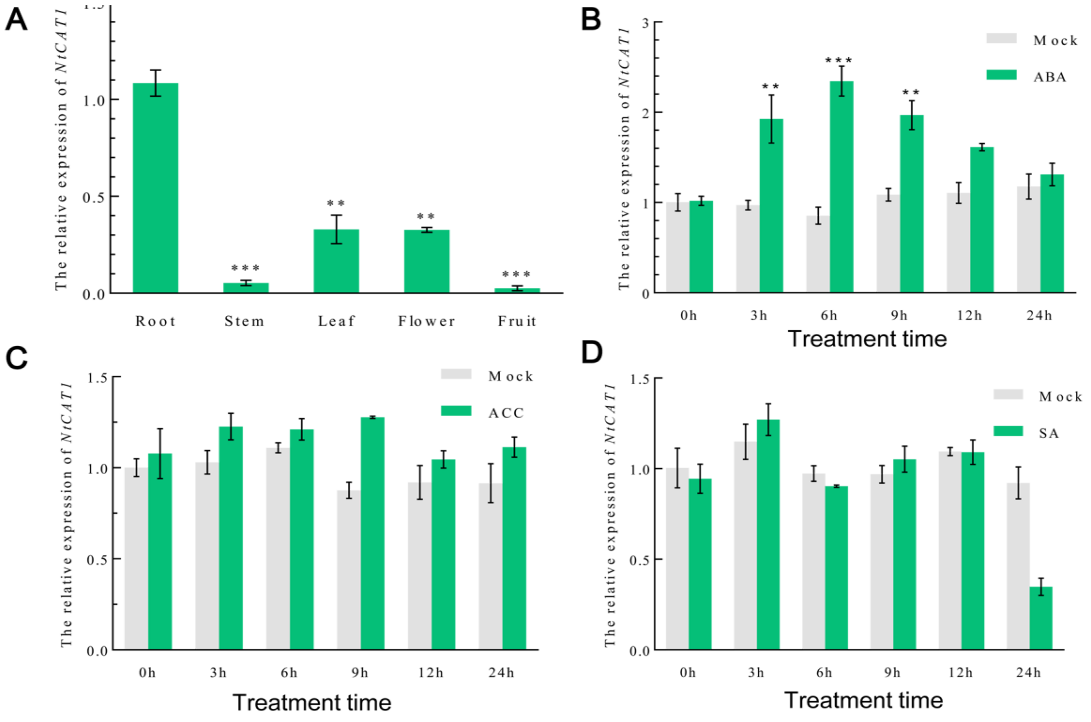

Figure 3. Expression pattern of NtCAT1. (**A**) The expression level of *NtCAT1* in different organs of tobacco K326. (**B**–**D**) qRT-PCR analysis of *NtCAT1* expression pattern in 10-day-old K326 seedlings under exogenous phytohormone treatment: ABA (100 μM), ACC (200 μM), SA (100 μM), and water as control (mock). The values are means ± SD of three biological replicates. Asterisks indicate statistically significant differences level (** $p < 0.01$ and *** $p < 0.001$) with corresponding controls.

3.4. NtCAT1 Protein Is Localized in the Plasma Membrane

To elucidate the subcellular localization of NtCAT1, a green fluorescent protein (GFP) subcellular experiment was performed. The CDS sequence of *NtCAT1*, fused to the N-terminus of the GFP, was transiently expressed in leaves of Nicotiana benthamiana. The fluorescence observation results showed that the GFP signal was intensively distributed in the plasma membrane, implying the localization of NtCAT1 at the plasma membrane (Figure 4A). The plasma membrane localization was confirmed with NtCAT1-GFP co-localizing with the plasma membrane marker protein CAN26-mCherry in epidermal cells of tobacco leaf. To further investigate the subcellular localization of NtCAT1 in tobacco plants, we generated transgenic plants (*ProNtCAT1:NtCAT1-GFP*) harboring the NtCAT1 CDS sequence driven by the *NtCAT1* native promoter. The fluorescence observation from the root of *ProNtCAT1:NtCAT1-GFP* transgenic lines showed that NtCAT1 is a plasma-membrane-localized protein (Figure 4B), in accordance with a previous subcellular localization of AtCAT1 [12]. These results suggest that NtCAT1 could play an important function in the intracellular and extracellular transmembrane transport of amino acids as an amino acid transporter.

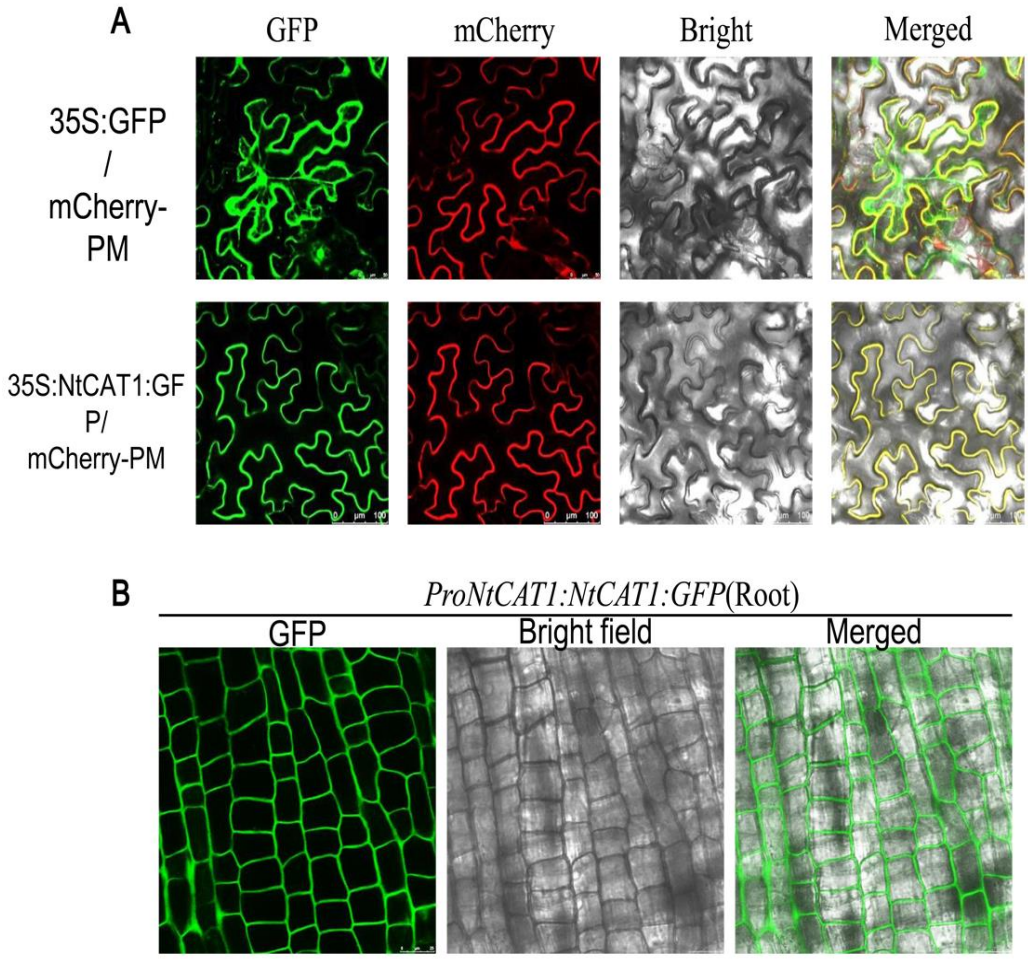

Figure 4. Plasma membrane localization of NtCAT1 protein. (**A**) Subcellular localization of NtCAT1 protein in tobacco leaf epidermal cells. Fluorescence from *35S:NtCAT1:GFP* transiently expressing in tobacco leaf epidermal cells. 35S:GFP transgenic tobacco leaf was used as a control. As plasma membrane marker, CAN26-mCherry constructs were transformed into tobacco epidermal cells. (**B**) Fluorescence observation of root epidermal cells of tobacco plant harboring the *NtCAT1:GFP* transgene driven by the *NtCAT1* promoter. The GFP and mCherry signals were captured under a Leica confocal microscope as green and red, respectively. (bar = 100 μm).

3.5. Transient Expression of NtCAT1 Accelerates Dark-Induced Leaf Senescence

Dark treatment is used frequently as an effective method to induce synchronous senescence [2]. To determine the potential functions of NtCAT1 in leaf senescence, the 35S:NtCAT1 construct was transiently expressed in tobacco (Nicotiana benthamiana) leaves. The expression of *NtCAT1* in transgenic leaves was upregulated approximately 25-fold relative to its expression in the control (Figure 5A). Then, these leaves were treated with darkness for various times. After a 4 d dark treatment, the leaves of *NtCAT1*-overexpressing lines displayed an accelerated yellowing phenotype compared with control leaves (Figure 5D). Consistently, the relative chlorophyll content in transgenic leaves after 4 d of darkness was only 0.5-fold compared with that in the control (Figure 5B). The results demonstrate that the total chlorophyll is metabolized at a higher rate in transgenic leaves than in the control (Figure 5B). In addition, the analysis of the ROS content found that the transgenic leaves had a higher level of H_2O_2 and O_2^- than the control after dark treatment (Figure 5C).

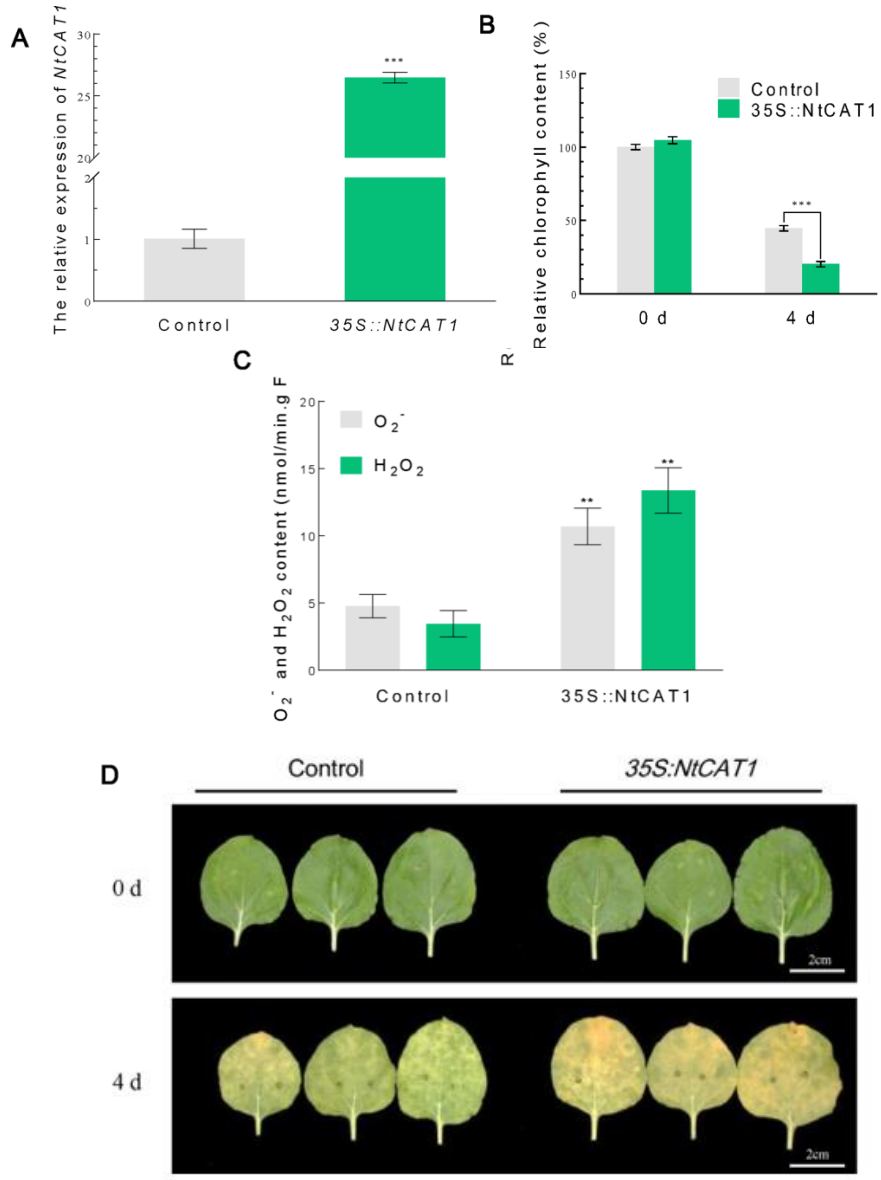

Figure 5. Transient transformation of *NtCAT1* in tobacco (*Nicotiana benthamiana*) under darkness. (**A**) The expression level of NtCAT1. (**B**) The relative chlorophyll content in *NtCAT1* transgenic leaves. (**C**) O_2-and H_2O_2 analysis of NtCAT1 transgenic leaves. (**D**) The phenotype of *NtCAT1* transgenic leaves after dark treatment. The data are means ± SD of three biological replicates. Asterisks indicate significant difference levels (Student's *t*-test; ** $p < 0.01$, and *** $p < 0.001$) with corresponding controls.

3.6. Overexpression of NtCAT1 Promotes Natural Leaf Senescence

To investigate the biological function of NtCAT1 in leaf senescence, we obtained *NtCAT1*-overexpressing lines (*OECAT1*) and the loss-of-function mutant of NtCAT1 (*ntcat1-36*) generated by the CRSPR-Cas9 system. We observed that *OECAT1* lines showed early leaf senescence (Figure 6A), while the *ntcat1-36* mutant exhibited weaker delayed leaf senescence compared with the wild type (WT). Accumulating reports demonstrate that the chlorophyll content and the photochemical efficiency of photosystemII (PSII) could reflect the progression of leaf senescence [42]. So, we determine the relative chlorophyll content and Fv/Fm ratio in leaves at different leaf positions of 6-week-old soil-grown plants. These results showed that the relative chlorophyll content of *OECAT1* exhibited lower values than that of WT, but a higher relative chlorophyll content was obtained in *ntcat1-36* plants. Consistently, the Fv/Fm ratio of OECAT1 was lower than that of WT, while NtCAT-36 had a higher Fv/Fm ratio than WT (Figure 6B,C). We also checked the expression level of *CYSTEINE PROTEINASE 1 (NtCP1, SENESCENCE-ASSOCIATED GENE 12/SAG12* homolog in tobacco) and the *RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN (NtRBCS*), two well-known senescence-induced genes [43]. Compared to WT, NtCP1 transcription was higher and NtRBCS expression was lower in the overexpressing lines of *OECAT1* (Figure 6D,E). In conclusion, these results demonstrate that NtCAT1 acts as a positive regulator in tobacco leaf senescence.

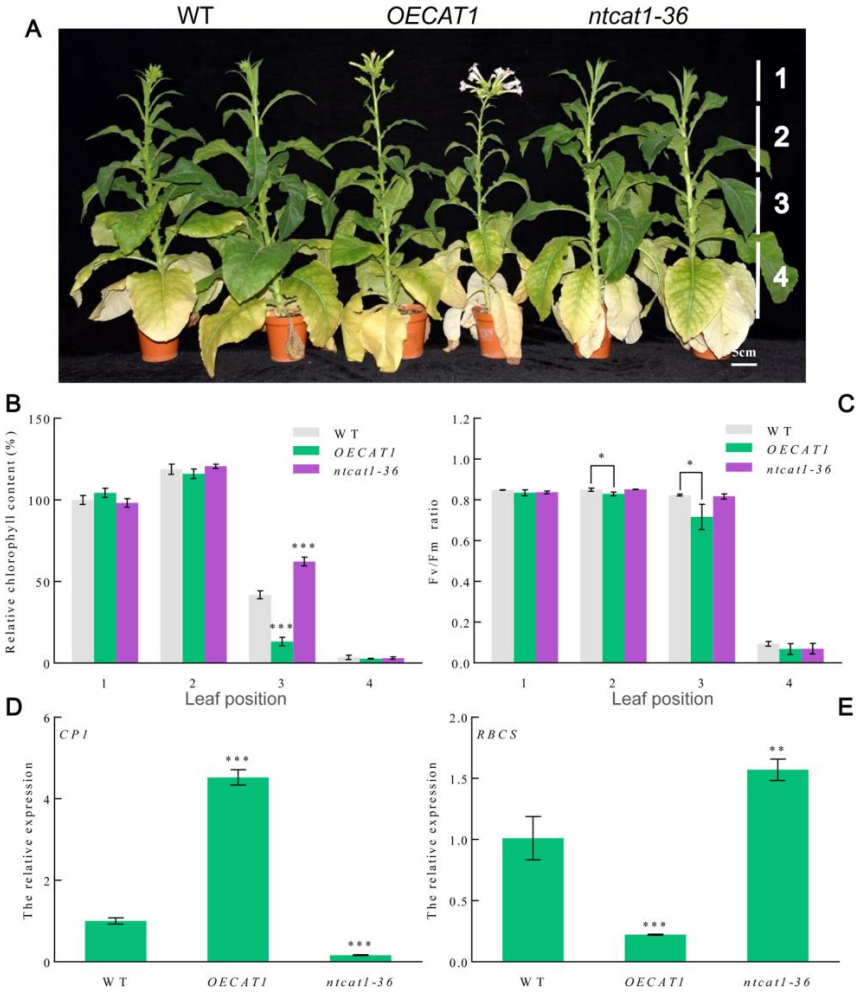

Figure 6. Overexpression of NtCAT1 promotes leaf senescence. (**A**) Phenotype of leaf senescence. (**B**) Relative chlorophyll content. (**C**) Analysis of Fv/Fm ratio. (**D**,**E**) Expression of CP1 and RBCS in tobacco. The data are means ± SD of three biological replicates. Asterisks indicate significant difference levels (Student's *t*-test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) with corresponding controls.

3.7. Overexpression of NtCAT1 Causes the Accumulation of Reactive Oxygen Species and ABA during Leaf Senescence

As we know, reactive oxygen species (ROS) and MDA accumulate gradually during leaf senescence. To further decipher the physiological function of NtCAT1 in leaf senescence, we detected the contents of ROS and MDA in 6-week-old leaves of transgenic plants and WT plants during leaf senescence. These data show that the contents of ROS and MDA in *OECAT1* was higher than that in WT, but *ntcat1-36* had a lower content (Figure 7A–C). Meanwhile, we discovered that *OECAT1* had a lower activity of SOD and POD compared to WT, while *ntcat1-36* showed a relative higher activity of SOD and POD during leaf senescence (Figure 7D), but there was no significant difference in CAT activity between the transgenic plants and the WT plants (data not shown). Moreover, the analysis of endogenous ABA content demonstrated that there was a higher level of ABA in *OECAT1* plants than in WT plants (Figure 7E). In conclusion, we confirm that NtCAT1 positively regulates leaf senescence at the physiological level.

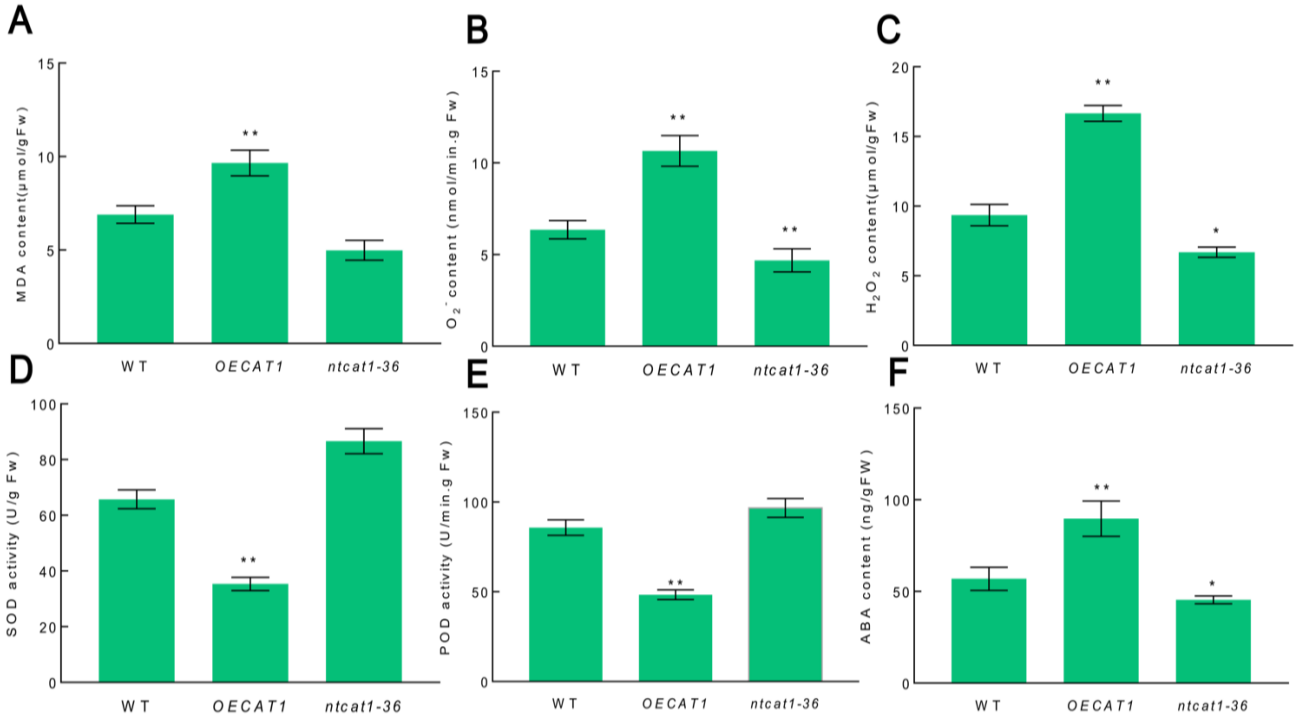

Figure 7. Overexpression of NtCAT1 causes accumulation of reactive oxygen species and ABA. (**A**) MDA content. (**B**) O_2^- content. (**C**) H_2O_2 content. (**D**,**E**) The activity of SOD and POD. (**F**) ABA content. The data are means ± SD of three biological replicates. Asterisks indicate significant difference levels (Student's *t*-test; * $p < 0.05$, ** $p < 0.01$) with corresponding controls.

3.8. Altered Expression of NtCAT1 Influences the Expression of ABA-Associated Genes and TOR Gene during Leaf Senescence

To reveal the molecular mechanisms of NtCAT1 regulating leaf senescence, we examined the expression level of various ABA-associated genes, such as *NtNCED1*, *NtNCED3-2*, *NtABA8ox-1*, *NtSnRK2.1*, *NtPYL4*, and *NtTOR*, by qRT-PCR. These results showed that the expression level of *NtNCED1*, *NtNCED3-2*, *NtSnRK2.1*, and *NtPYL4* was higher and the expression level of *NtABA8ox-1* was lower in *OECAT1* plants compared with the WT plants, while an inverse trend of the expression level of the above genes was observed in NtCAT1-36 (Figure 8A–D,F). Interestingly, we found that the transcript level of the target of rapamycin kinase gene (NtTOR and AtTOR homolog in tobacco) was obviously downregulated in *OECAT1* lines compared with the WT plants (Figure 8E). These data suggest that NtCAT1 could cause leaf senescence by an ABA-related network and the TOR complex.

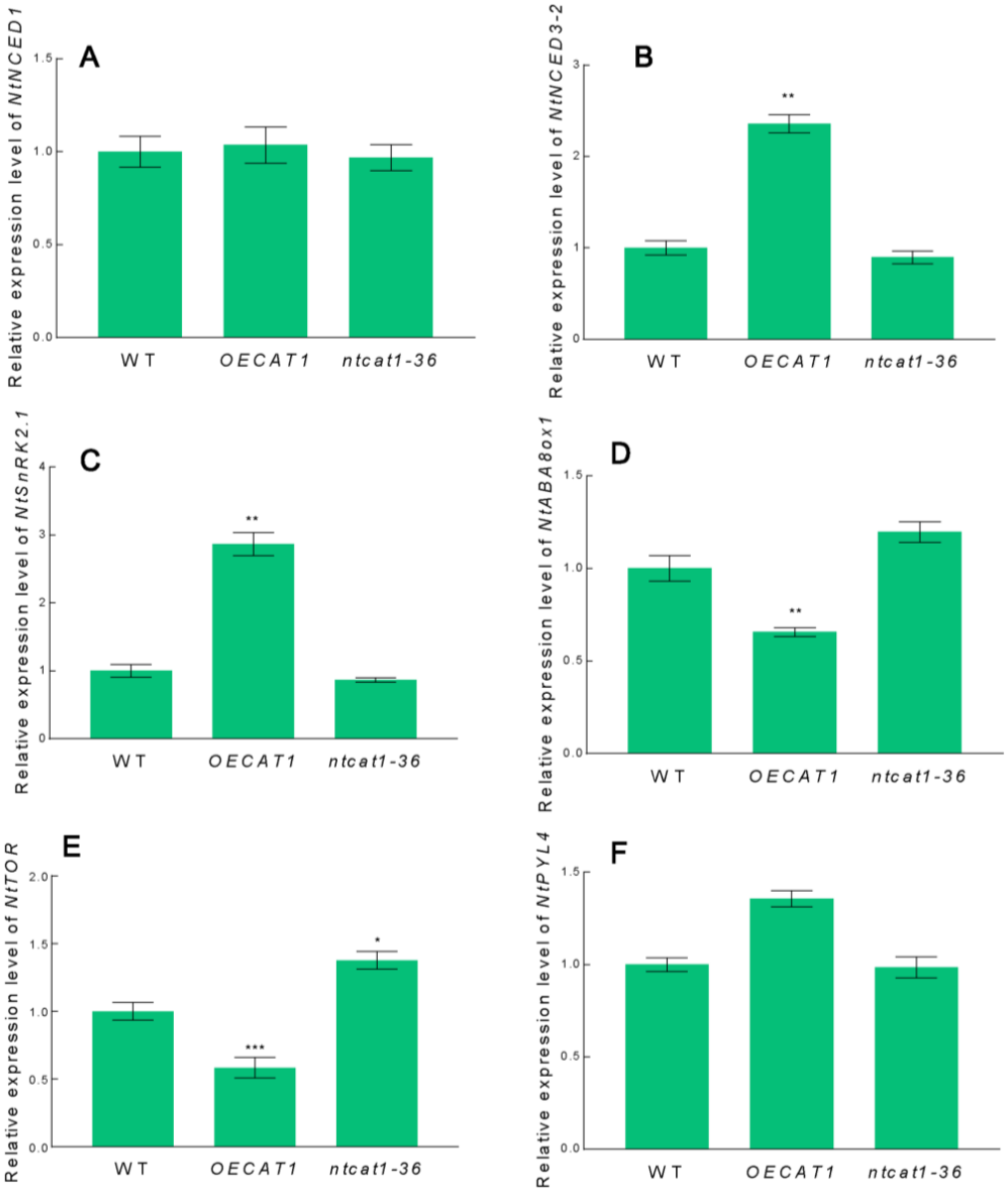

Figure 8. NtCAT1 influences the expression of several ABA key marker genes. (**A**) NtNCED1. (**B**) NtNCED3-2. (**C**) NtSnRK2.1. (**D**) NtABA8ox-1. (**E**) NtTOR. (**F**) NtPYL4. The data are means ± SD of three biological replicates. Asterisks indicate significant difference levels (Student's *t*-test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) with corresponding controls.

3.9. Overexpression of NtCAT1 Affects the Free Amino Acid Profile during Leaf Senescence

To test whether the overexpression of *NtCAT1* causes changes in free amino acid homeostasis, we examined the total amino acid content and the individual amino acid concentration in leaves of transgenic plants and WT plants. In the young leaf, there were no obvious changes in the total amino acid content of these plants. However, in senescent leaves, an obvious decrease in the total amino acid content was observed in *OECAT1*, while the total amino acid content in *ntcat1-36* was upregulated compared with in the WT plants (Figure 9A). We also measured the individual amino acid concentration in senescent leaves of transgenic lines and the WT plants. We observed that the amino acids Glu, Ser, Thr, and Arg were increased in *ntcat1-36* plants, while the content of the above amino acids was decreased in *OECAT1* relative to the WT plants (Figure 9B). These above results demonstrate that NtCAT1 influences amino acid homeostasis during leaf senescence.

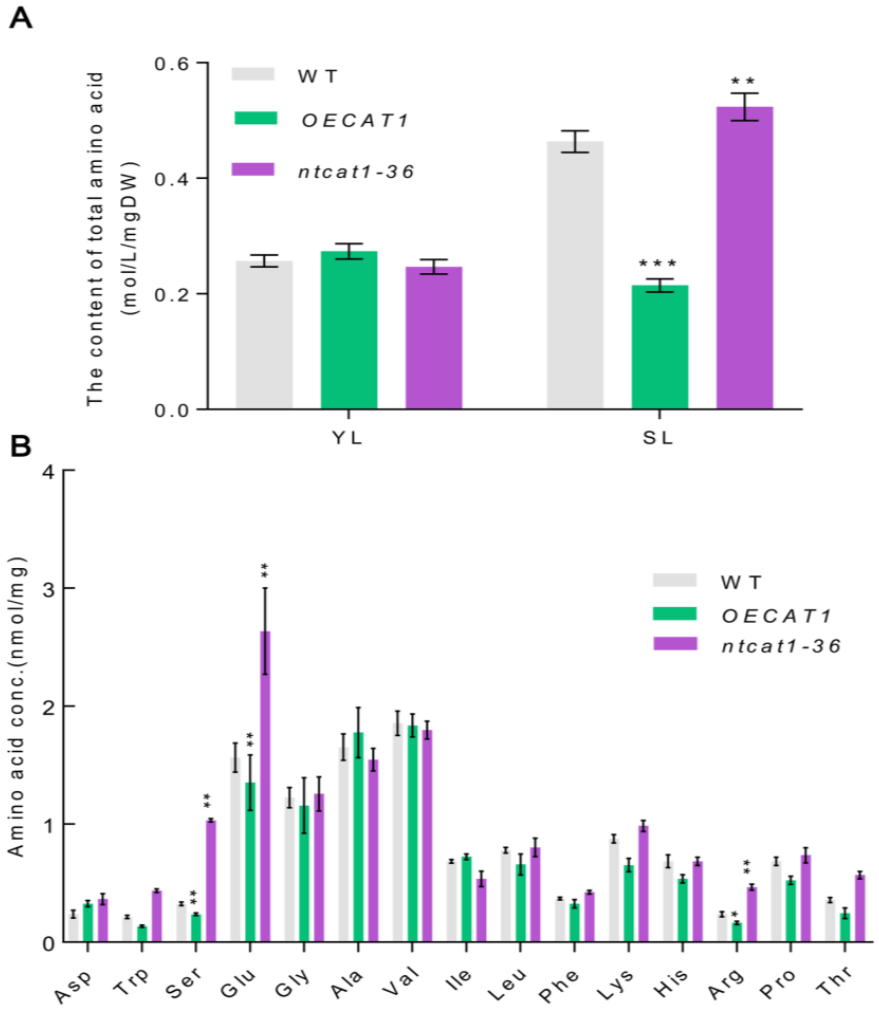

Figure 9. Analysis of free amino acid contents in leaves of OECAT1 and *ntcat11-36* plants. (**A**) Total free amino acid concentrations in 2-week-old young leaf (YL) and 6-week-old senescent leaf (SL) of transgenic plants. (**B**) Concentrations of selected individual amino acids in 6-week-old leaves of transgenic plants. The data are exhibited as means ± SD of three biological replicates. Asterisks indicate significant difference levels (Student's *t*-test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) with corresponding controls.

4. Discussion

Leaf senescence is a comprehensive response of plants to the age information of growth and development by integrating various internal and external signals. Senescence is closely related to agricultural production and crop economic benefits [44]. After the initiation of plant senescence, macromolecular substances in the senescent site degrade and produce small molecular substances such as oligopeptides and amino acids, which are then loaded into the phloem by various amino acid transporters for redistribution and utilization. Amino acid transporters play an important role in crop growth and development, seed yield, and fruit quality by participating in the process of organic nitrogen remobilization during senescence [4]. However, the molecular mechanisms involved in regulating amino acid transporters in organic N remobilization during leaf aging remains unclear. In this study, we identified an amino acid transporter NtCAT1 gene from common tobacco (K326), which had an upregulated expression in senescent leaves and was localized at the plasma membrane. The overexpression of *NtCAT1* exhibited an early leaf senescence phenotype with a higher level of ABA, whereas the loss-of-function mutant *NtCAT-36* showed delayed leaf senescence with a lower level of ABA compared with WT plants. Furthermore, we

observed that the amino acid concentration decreased in OECAT1, while the amino acid content was upregulated in NtCAT1-36. Taken together, our findings shed new insights into the connection between organic nitrogen remobilization and leaf senescence.

Amino acid transporters play vital roles in the redistribution of organic nitrogen, such as amino acids and oligopeptides produced by the degradation of chloroplasts, proteins, nucleic acids, and other biological macromolecules [3,22]. However, the molecular identity of transporters involved in the compartmentalization, storage, and remobilization of amino acids in plants is still unclear. So far, nine CAT members (AtCAT1-AtCAT9) have been identified in Arabidopsis [12], and CAT family members have also been studied in rice [45], but no CAT1 has been reported in tobacco. In this study, an amino acid transporter gene NtCAT1 with high homology (identity = 68.68%) to AtCAT1 (AT4G21120) was identified from common tobacco. Breeze et al. [46] found that many amino acid transporter genes and oligopeptide transporter genes were upregulated or downregulated in Arabidopsis senescent leaves. In Arabidopsis, the transcript level of AtCAT1 and AtCAT2 is significantly upregulated during leaf senescence [47]. In this paper, it was found that the expression of NtCAT1 was upregulated in an early senescence leaf, a late senescence leaf, and the yellowing area of a leaf, which suggested that NtCAT1 may be involved in the natural senescence process of leaves. NtCAT1-overexpressing tobacco exhibited an accelerated leaf senescence phenotype, while NtCAT1 knockout lines exhibited delayed leaf senescence, which demonstrated that NtCAT1, as a positive regulator, was involved in the senescence process of leaves. In addition, our study showed that the overexpression of NtCAT1 promoted the accumulation of ROS in senescent leaves. Palatnik et al. [48] found that the excessive accumulation of ROS during leaf senescence can lead to the degradation of chloroplast glutamine synthetase 2 (glutamine synthetase, GS2), but we still need to further verify that NtCAT1 directly or indirectly regulates ROS homostasis to accelerate leaf senescence.

In this work, we observed that the overexpression of *NtCAT1* enhanced ABA biosynthesis by regulating the expression of *NtNCED1, NtNCED3-2,* and *NtABA8ox1.* It is well known that ABA is a crucial phytohormone in regulating natural leaf senescence [2,20–22]. Many reports showed that the ABA biosynthesis and degradation pathways in plants involve several key genes, including 9′-cis-epoxycarotenoid dioxygenases genes (NCEDs) [26]; zeaxanthin epoxidase (ZEP) [27]; ABSCISIC ALDEHYDEOXIDASE3 (AAO3) [20], as the last step in ABA biosynthesis; and ABA 8′-hydroxylase genes (ABA8ox1) [27], which are required for the oxidation of ABA. It was reported that the overexpression of AtNAP promoted leaf senescence via directly regulating ABA biosynthesis [20]. In rice, OsNAC2 and OsNAP also upregulated the expression of ABA-biosynthesis-related genes, resulting in early leaf senescence [2]. However, the molecular mechanisms by which amino acid transporters influence ABA biosynthesis remain largely unknown.

The target of rapamycin (TOR) acts as a master regulator that coordinates cell growth with energy and nutrient availability [49]. Accumulating evidence has shown that TOR plays key functions in crosstalk with plant hormone signaling [50]. Belda-Palazón et al. [51] found that ABA inhibits TOR signaling by the nuclear export of SnRK1, a key kinase in ABA signaling. Wang et al. [34] showed that TOR could phosphorylate the ABA receptor PYL on a conserved serine residue, thereby inhibiting ABA signaling. These studies inspire us: NtCAT1 might affect the ABA pathway by regulating TOR signaling. Our results showed that the overexpression of NtCAT1 suppresses the expression of TOR, which verified our hypothesis. Amino acids (AAs) are key upstream signals for TOR activation in mammals, but how nitrogen signals regulate TOR signaling in plants is poorly understood [35]. A very recent study has shown that the TOR pathway is closely related to nitrogen metabolism in plants [52]. In Arabidopsis, glutamine can activate TOR signaling by activating the small GTPase Rho-related protein from plants 2 (ROP2) [35]. In addition, the excessive accumulation of branched-chain amino acids in plants can lead to the upregulation of TOR activity [35]. Therefore, during leaf senescence, NtCAT1 plays an amino acid transport function on the plasma membrane to transport organic nitrogen out of the cell, leading to

a decrease in free amino acid content, thereby inhibiting TOR signaling, and ultimately affecting the ABA pathway. In future research, the molecular mechanism by which NtCAT1 regulates TOR signaling during leaf senescence should be further investigated.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13071691/s1. Table S1: The GenBank accession numbers of NtCAT1 and its close homologues proteins as listed in Figure 1; Table S2: The qRT-PCR primer sequences used in this study.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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