Overexpression of salt-induced protein (salT) delays leaf senescence in rice

Keming Zhu1, Huimin Tao1, Shuo Xu1, Kaixia Li1, Sundus Zafar1, Wei Cao1 and Yanhua Yang1
1Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, People’s Republic of China.

Abstract

Senescence, a highly programmed process, largely determines yield and quality of crops. However, knowledge about the onset and progression of leaf senescence in crop plants is still limited. Here, we report that salt-induced protein (salT), a new gene, may be involved in leaf senescence. Overexpressing salT could prolong the duration of leaves with higher concentrations of chlorophyll compared with the wild type. Moreover, overexpression of salT could delay the senescence of rice leaves though the inhibition of senescence associated genes (SAGs). Overall, the characterization of salT suggested that it is a new gene affecting the leaf senescence induced by natural and dark conditions.

Keywords: Leaf senescence, salt-induced protein (salT), rice, stay-green.

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Introduction

Senescence is the final phase of plant development, in which the plant goes through a series of programmed cell death (PCD) processes (Cao et al., 2003; Cao, 2003 #1). This process combines chlorophyll degradation with carotenoid retention or anthocyanin accumulation (Park et al., 2007) so the leaves of plant generally change from green to yellow during senescence. It has been noted for long that leaf senescence is a major determinant of yield for many crops (Richards, 2000; Long et al., 2006; Yang and Zhang 2006). Delaying leaf senescence has usually been considered to be associated with the retention of the high photosynthetic capacity and yield increment (Thomas and Howarth, 2000; Masclaux-Daubresse and Chardon, 2011; Wu et al., 2016).

Rice (Oryza sativa L.) is one of the most important crops in the world and provides nearly half of the calories consumed by humankind (Zuo and Li, 2014). The leaf is the most important source organ for rice, with 60-80% of the nutrients required for grain filling after heading provided by leaf photosynthesis (Lu et al., 1988). Early-senescence of leaves will seriously reduce rice yield, disrupt the filling dynamics, and reduce grain quality (Zhao et al., 2014). The production can be increased by 2% with delaying rice senescence by one day (Liang et al., 2014). Therefore, finding an economic and effective method to delay leaf senescence in rice production can greatly improve the yield of rice.

During leaf senescence, the expression of senescence associated genes (SAGs) is up-regulated, which is a hallmark of leaf senescence (Kajimura et al., 2010). More than a thousand SAGs have been isolated, including transcription factors, signal transduction components, proteases, metabolic enzymes and various transporters of nutrients (Watanabe and Imaseki, 1982; John et al., 1997; Lee et al., 2001; Buchanan-Wollaston et al., 2003; Gregersen and Holm, 2007, Liu et al., 2008; Hayashi et al., 2015; Wu et al., 2016). Some important genes have been cloned and functionally characterized. STAY-GREEN (SGR) is an important member of the metabolic pathway of chlorophyll degradation, which encodes a chloroplast transit peptide and regulates chlorophyll degradation by inducing light-harvesting proteins of photosystem II (LHCPII) disassembly through direct interaction (Jiang et al., 2007; Hörtensteiner, 2009). RED CHLOROPHYLL CATABOLITE REDUCTASE 1 (RCCR1) encodes a red chlorophyll catabolite reductase, plays a key role in the chlorophyll degradation pathway, and strongly participates in senescence (Tang et al., 2011). NON-YELLOW COLORING 1 (NYC1) encodes the chlorophyll b oxidation-reduction enzyme, and chlorophyll b cannot be degraded in the nyc1 mutant because of abnormal binding of light-harvesting chlorophyll and carotene (Kusaba et al., 2007). NON-YELLOW COLORING 3 (NYC3) encodes a plastid-targeted α/β folding proteolytic enzyme that affects chloroplast structure, and the nyc3 mutant exhibits a senescence phenotype (Morita et al., 2009). OsNAP encodes a transcription factor with a crucial role in regulating the senescence process, as demonstrated by the delayed senescence phenotype of Osnap mutants (Liang et al., 2014). In general, the onset and progression of leaf senescence is influenced by a number of endogenous and external factors. The mechanism of
the onset of leaf senescence, especially the mechanism of delayed senescence in rice, is still largely unknown.

Here, we investigated the role of the salt-induced protein (salT) gene in rice. In our previous studies, we found OsISP specifically expressed in indica rice and the protein was identified as a salt-induced protein (salT) (Zhu et al., 2014). However, salT function is still not clear in rice. Here, we found that overexpression of salT in rice could delay the senescence of leaf by inhibiting the expression of SAGs and CDGs.

Material and Methods

Plant materials and growth conditions

All rice lines used in the study were derived from the japonica cultivar Nipponbare. Rice plants were cultivated in the experiment field at Jiangsu University, Zhenjiang, Jiangsu, during the natural growing season except where specifically indicated. For hydroponic culture, rice seedlings grown in a constant-temperature incubator with light/dark of 16/8 h and 30-28 °C, with approximately 200 μmol photons/m²/s photon-density and 70% humidity. For dark incubation, the third fully expanded leaves of each genotype were excised and incubated under continuous darkness at 28 °C. All experiments were carried out using the same location of the leaves. The samples were collected with a sharp scalpel to minimize the impact of wounding, immediately frozen in liquid nitrogen, and stored at -72 °C until needed for RNA isolation and chlorophyll extraction. All experiments were repeated three times independently.

Vector construction and plant transformation

Total RNA from rice leaf tissues (Zhonghua11) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After removal of genomic DNA contamination by DNase I (TaKaRa, Dalian, China), 200 ng of poly(A)+ mRNA was converted into cDNA by MMLV Reverse Transcriptase (Vazyme, Nanjin, China). The cDNA template was subsequently used for PCR analysis. For salT (Os01g0348900), a full-length cDNA was obtained using the primers 5’-ATGACGCTGGTGAAGATTGG-3’ and 5’-TCAAGGGTGGACGTAGATGC-3’, and inserted into the Sal I site of pCAMBIA1300-Actin1-ocs, creating an overexpression vector, salT-OE. The inserted sequences were confirmed by restriction enzyme digestion and sequencing. To construct a vector for the constitutive expression of salT, a 438 bp full-length salT cDNA was PCR amplified from its cDNA clone with the primers 5’-AAGTGCACATGCGGTAGAAGATGGG-3’ and 5’-AAACTCGACGTCAAGGGTGGACGTAGATGC-3’, and inserted into the Sal I/Pst I site of pCAMBIA1300-Actin1-ocs, creating an overexpression vector, salT-OE. The inserted sequences were confirmed by restriction enzyme digestion and sequencing. To construct the salT RNAi vector, part of the salT cDNA was PCR amplified from its cDNA clone with the primers 5’-AAAGATCCATGCGGTAGTGGTGAAGATGGG-3’ and 5’-AAGTGCACATGCGGTAGTGGGATTCAGAAATCTCCTT-3’ and inserted into the BamHI/Sal I and Xho I/Bgl II sites of the pUCCRNAi vector. Then, the pUCCRNAi vector was cut with Pst I and inserted into the Pst I site of pCAMBIA1300-Actin1-ocs, creating a salT RNAi vector, salT-RNAi. The inserted sequences were confirmed by restriction enzyme digestion and sequencing. The two binary plasmids were introduced into Agrobacterium tumefaciens EHA105 by electroporation and transformed into rice according to a published method (Hiei et al., 1994; Jeon et al., 2000).

DNA, RNA extraction, and qRT-PCR

Genomic DNA was extracted from rice leaves using the CTAB method and total RNA was extracted using the TRIzol reagent (Invitrogen). RNA was reversely transcribed from 3 μg of total RNA with the M-MLV reverse transcriptase (Vazyme) according to the manufacturer’s instructions. The qRT-PCR assays were carried out in a total volume of 20 μL, each containing 2 μL of cDNA (200 ng), 10 μL of SYBR Green Master Mix (Vazyme), 0.4 μL of 50ROX Reference Dye I, 0.4 μL of primers (10 μM) and 7.2 μL of H2O. Cycling conditions included a hot start (5 min at 95 °C), followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s, and finished with 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. Amplification was performed using an ABI7300 PCR thermocycler (Applied Biosystems, USA). Rice Actin1 was chosen as a control to normalize all data. For analysis, a threshold was set for the change in fluorescence at a point in the linear PCR amplification phase. Melt curve analysis was performed to ensure a single product species. All experiments were done in duplicate for both target gene and internal control and were repeated three times independently. For the CT calculation to be valid, the amplification efficiencies of the target and reference must be approximately equal. The relative expression levels of genes were calculated using the 2-ΔΔCT method (He et al., 2016; Tan et al., 2015). The statistical significance was analyzed by Student’s t-test. The primer sequences that were used are listed in Table S1. PCR products were separated by electrophoresis on 1.0% (w/v) agarose gels.

Chlorophyll measurement

Fresh rice leaves (20 mg) were chilled with liquid nitrogen and ground to a powder. Chlorophyll was extracted with 80% acetone until the residue turned to white color, and diluted to a final volume of 25 mL. Chlorophyll was determined by measuring absorbance at 663 and 645 nm using a W14546 spectrophotometer (Xin Mao Instrument, Shanghai, China). The chlorophyll content was calculated according to the formula (Ca = 12.7 OD663 - 2.69 OD645, Cb = 22.9 OD645 - 4.68 OD663, Ct = 8.02 OD663 + 20.21 OD645). Three seedlings of each sample were used in the chlorophyll assay. Statistical significance was analyzed by Student’s t-test. Values are reported as mean ± standard deviation of 10 measurements.
Results

Overexpressing salT delayed the senescence of rice leaves

The OsISP protein was a protein marker for indica rice varieties, and was identified as salt-induced protein (salT), however the function of salT is still not clear in rice (Zhu et al., 2014). To further investigate its function, we constructed salT-overexpression (salT-OE) and salT-RNAi vectors, and generated transgenic plants by Agrobacterium mediated transformation (Hiei et al., 1994; Jeon et al., 2000). A total of eight positive salT-OE transgenic plants and 16 positive salT-RNAi transgenic plants were obtained. All eight salT-OE transgenic plants (T₀) maintained a stay-green phenotype in the mature stage (Figure 1A,B), but the phenotype of salT-RNAi transgenic rice was similar to the wild type (data not shown). Thus, we used the salT-OE transgenic rice lines for our study.

Total DNA was extracted from T₀ transgenic rice plant and wild type leaves. In all T₀ transgenic rice plants a 220 bp fragment could be identified with hygromycin (hpt) gene-specific primers, which was similar to the positive control. However, no PCR products were detected in the wild type plants (Figure 2A). Moreover, we tested hygromycin resistance in excised leaves with hygromycin detection solution. All excised leaves from salT-OE transgenic plants stayed green and showed resistance to hygromycin while the leaves from untransformed control plants turned brown and died, indicating no resistance to hygromycin (Figure 2B). Furthermore, we extracted RNA from a salT-OE transgenic plant, and the qRT-PCR results showed that the salT gene expression in salT-OE transgenic plant was about two times higher than that in wild type (Figure 2C). These results indicate that we successfully constructed salT-OE transgenic plants.

Phenotypic analysis of salT-OE transgenic plants was carried out. As shown in Figure 1, at the mature stage of rice, the wild type leaves turned yellow (Figure 1C and D) while the leaves of salT-OE rice had a stay-green phenotype (Figure 1A,B). We also quantified the chlorophyll content of flag leaves from the first week to the eighth week of the filling stage. During this period, the chlorophyll content of the leaves decreased gradually, but in salT-OE rice it decreased much slower than in wild type (Figure 1E). These results showed that overexpression of the salT gene in rice could delay the senescence of leaves.

salT inhibits the expression of senescence associated genes

The expression of SAGs are up-regulated during leaf senescence (Kajimura et al., 2010). Thus, we used quantita-

![Figure 1 - Phenotype of wild-type and T₀ transgenic plants carrying salT overexpression constructs (salT-OE) in the mature stage. (A) Phenotypes of the T₀ salT-OE plants in the mature stage. (B) Flag leaf of the salT-OE plants keeps green in the mature stage. (C) Phenotypes of the wild type in the mature stage. (D) Flag leaf of the wild type turned yellow in the mature stage. (E) Chlorophyll contents of leaves of age-matched wild-type and salT-OE plants.](#)

![Figure 2 - Analysis of the salT-OE transgenic plants. (A) PCR analysis of hsg gene in the T₀ salT-OE transgenic plants. M: DL2000 plus DNA maker; -: untransformed wild-type plants; 1-8: T₀ salT-OE transgenic plants; P: pCAMBIA1300-Actin1-ocs plasmid. (B) Leaf assay of hygromycin resistance in transgenic rice plants. 1-8: T₀ salT-OE transgenic plants; 9-12: untransformed wild type plants. (C) Transcript levels analysis of salT overexpression fold. WT: wild type; salT-OE: salT-OE transgenic plant. *p < 0.05, **p < 0.01, ***p < 0.001. Student’s t-test was used to generate p-value.](#)
tive real-time PCR to detect two SAGs, OsNAP and OsCATB, in the flag leaves from wild type and salT-OE rice during the filling stage. As shown in Figure 3A, during the first week of the filling stage, the two genes were barely expressed in flag leaves. In the eighth week of the filling stage, the expression level of these two genes increased rapidly in the wild type, but not in the salT-OE plants. This result indicated that salT could inhibit leaf senescence by inhibiting the expression of SAGs.

Chlorophyll plays a central role in photosynthesis, and its degradation is an important phenomenon in leaf senescence (Morita et al., 2009). Therefore, we also quantified the expression levels of four chlorophyll degradation-related genes (CDGs), OsSGR, OsNYC1, OsNYC3, and OsRCCR1, by real-time PCR during the filling stage. Our results showed that the expression of the four genes had almost no difference between wild type and salT-OE plants in the first week of the filling stage. At the eighth week of the filling stage, the expression level of these genes increased sharply in wild type. In salT-OE plants, the expression levels of the four genes also increased, but they were lower than in wild type (Figure 3B). This result suggests that overexpression of salT could keep leaves green through inhibiting chlorophyll degradation.

Responses of salT to the dark treatment

Dark treatment is the simplest and most efficient method to induce leaf senescence. While the detached leaves of Nipponbare (wild type) turned yellow 3 days after dark incubation (DAD), salT-OE retained the greenness at 5 DAD (Figure 4A). The chlorophyll content of rice seedling was examined during dark incubation. Initial chlorophyll contents were similar between wild type and salT-OE, but a prominent decrease was observed at 7 DAD in wild type (Figure 4B). The qRT-PCR results showed that two CDGs, OsSGR, and OsRCCR, had lower expression in salT-OE than in wild type plants after 7 DAD (Figure 4C). These observations indicate that salT-OE plants retain more chlorophyll than the wild type during senescence through inhibiting the expression of CDGs.

Discussion

Leaf senescence, the final stage of leaf development, occurs autonomously in an age-dependent manner and leads to the death of a cell or an organ. The aging process that leads to senescence and limitation of life is a common biological phenomenon in most organisms (Woo et al., 2001). Up to now, it is clear that leaf senescence is con-

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Figure 3 - Overexpression of salT inhibits the expression of senescence genes. (A) Relative transcript levels of senescence-associated genes in wild type and salT-OE plants during the filling stage. (B) Relative transcript levels of chlorophyll degradation-related genes (OsRCCR1, OsSGR, OsNYC1 and OsNYC3) of age-matched wild type leaves and salT-OE plants. Overexpression of salT gene delays dark-induced leaf senescence. Transcript levels are expressed relative to rice Actin1 in each sample, and values are reported as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001. Student’s t-test was used to generate p-value.
trolled by genes, and many leaf senescence genes in Arabidopsis, rice, maize, and barley have been cloned (Schippers, 2015; Raines et al., 2016; Sakuraba et al., 2016; Deng et al., 2017). However, there are few studies on delayed leaf senescence, especially in rice (Ren et al., 2007; Hörtensteiner, 2009; Morita et al., 2009). In this study, we found that salT could slow down the degradation of chlorophyll by reducing the expression of SAGs and CDGs, which could delay the senescence of leaves (Figures 1 and 2).

During leaf senescence, the most striking phenotypic change is the yellowing of the leaf caused by the preferential breakdown of chlorophyll and chloroplasts (Hilditch et al., 1989; Woo et al., 2001). The change in leaf color and chlorophyll content is integrally related to leaf senescence and is widely used in the quantification of senescence. CDRGs (OsSGR, OsNYC1, OsNYC3, and OsRCCR1) play important roles in regulating chlorophyll degradation, and the transcription levels of these genes are up-regulated during natural and dark-induced leaf senescence (Jiang et al., 2007; Park et al., 2007; Sato et al., 2009; Tang et al., 2011; Rong et al., 2013; Liu et al., 2016). The sgr mutant maintains greenness during rice leaf senescence, while over-expressing SGR in rice produces yellowish-brown leaves (Park et al., 2007). The SGR gene encodes a chloroplast protein and is essential for the initiation of chlorophyll breakdown in plants (Park et al., 2007; Hörtensteiner, 2009; Liu et al., 2016).

The rice leaf nyc1 mutant also stays green during senescence, because chlorophyll degradation is impaired in the nyc1 mutant (Kusaba et al., 2007), and NYC1 is suggested to play essential roles in the regulation of LHCII and thylakoid membrane degradation during senescence (Kusaba et al., 2007). OsNYC3 encodes a plastid-targeted α/β hydrolase-fold family protein with an esterase/lipase motif, which affects chloroplast structure (Morita et al., 2009). OsRCCR1 encodes a red chlorophyll catabolite reductase, which plays a key role in the chlorophyll degradation pathway. The transcription level of OsRCCR1 is much lower in young leaves, but is about 20-fold higher in senescent leaves (Tang et al., 2011). These evidences suggest that OsSGR, OsNYC1, OsNYC3, and OsRCCR1 play important roles in regulating chlorophyll degradation during leaf senescence.

In this study, we found that the chlorophyll degradation rate of salT-OE plant leaves was lower than that of wild type during the mature period of rice (Figure 1E). Our results showed that the transcription levels of OsSGR, OsNYC1, OsNYC3, and OsRCCR1 were down-regulated during rice leaf senescence (Figure 3B). In addition, disadvantageous environmental factors, such as darkness, can also trigger senescence during leaf development (Zhang and Zhou, 2013). The results in Figure 4A showed that darkness can induce senescence in wild type, while overexpression of salT could delay leaf senescence in detached leaves. Moreover, the chlorophyll degradation rate of salT-OE plant leaves is lower than that of wild type during dark incubation (Figure 4B). The expression of OsSGR and OsRCCR1 was also inhibited with darkness treatment (Figure 4C). Our results showed that the leaves of salT-OE plants can stay green, because overexpression of the salT gene could inhibit the expression of CDGs (OsSGR, OsNYC1, OsNYC3, and OsRCCR1), which inhibits chlorophyll degradation during senescence in rice.

Figure 4 - Changes in the chlorophyll content of wild type leaves and of salT-OE lines during dark-induced senescence. (A) Changes in leaf color in detached leaves stored in darkness. (B) Chlorophyll contents and (C) relative transcript levels of chlorophyll degradation-related genes (OsSGR and OsRCCR1) of wild-type and salT-OE plants cultivated in darkness. Transcript levels are expressed relative to rice Actin1 in each sample, and values are reported as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001. Student’s t-test was used to generate p-value.
Previous studies showed that some genes, such as SAGs, are up-regulated in senescent leaves. For example, the expression of AtNAP increases with aging; overexpression of AtNAP in wild type triggers precocious senescence and significantly blocks the function of the transcription factor that delays senescence (Guo and Gan, 2006). OsCATB is significantly induced by ABA (Abscisic Acid) (Agrawal et al., 2001; Ye et al., 2011). As a matter of fact, the up-regulation of SAGs is one of the hallmarks of leaf senescence (Kajimura et al., 2010). The results in Figure 3A show that the expression of SAGs (OsNAP and OsCATB) was not different from the wild type before leaf senescence, but was down-regulated during rice leaf senescence. This result indicates that the overexpression of salT could inhibit the expression of SAG gene and inhibit leaf senescence. In general, overexpression of the salT gene could delay leaf senescence in rice by inhibiting the expression of SAGs and CDGs. Thus, salT is a new anti-aging factor gene, but its mechanism is still unclear and needs to be further explored.

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Conflict of Interest

The authors declare no conflict of interests.

Author Contributions

KZ and HT performed most of the experiments and wrote the manuscript; SX helped with the Realtime PCR analysis of genes; KL and WC helped with the transcriptome sequencing analysis; SZ and YY edited the article. All the authors discussed the results and contributed to the manuscript.

References


Supplementary material

The following online material is available for this article:
Table S1 - Primers used in this study.

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