Exogenous ascorbic acid delayed leaf senescence of early flowering rice mutant FTL10

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Abstract

FTL10 is an early flowering mutant of *OsFTL10*-suppressed transgenic rice (*Oryza sativa* L.) with premature senescence phenotype. Early leaf senescence can cause negative effects on rice yield, therefore delaying leaf senescence and prolonging the leaf functional stage is one of the important approaches to increase the rice yield. It is well known that ascorbic acid (AsA) is involved in regulating plant growth. To explore the effect of AsA on leaf senescence of FTL10, we treated rice leaves with 0.28 mM AsA. Results showed that total antioxidant capacity was higher and reactive oxygen species were lower in the AsA-treated group. The expression of senescence-associated genes was higher in the control group. Exogenous AsA can stabilize chlorophyll and Rubisco protein contents, delay leaf senescence, and maintain net photosynthetic rate (P_N) of rice leaves. Our results suggest that exogenous application of AsA can delay leaf senescence, increase P_N, and then increase the rice yield.

Additional key words: nitroblue tetrazolium staining; nonenzymatic antioxidant; Rubisco large subunit; Rubisco small subunit; seed-setting rate; soluble protein.

Introduction

Rice (*Oryza sativa* L.) is one of the world most important crops and is the staple crop for approximately more than half the world's population (Iizumi and Ramankutty 2016). The yield is mainly provided by the photosynthesis of leaves after heading (Mae 1997). However, the grain yield of rice is often tightly linked to leaf senescence. Leaf senescence is considered the final stage of leaf development and is vital for plant fitness (Lim *et al.* 2007). It can be seen as an important phase in the plant life cycle (Nam 1997). Although senescence has great positive effects on plants, precocious senescence shortens the growth stage of crops and the functional period of leaves (Woo *et al.* 2013). Particularly in agricultural aspects, premature senescence results in poor grain quality, deterioration of the quality of vegetables, and reduction of crop yield (Jing and Nam 2012). It has been shown that premature senescence of flag leaves is negatively correlated with grain yield in rice (Zhang *et al.* 2007). The supply of assimilated carbon to grain at the grain-filling period can be enhanced by delaying leaf senescence, thereby enhancing the mass per grain (Jiang *et al.* 2007, Gregersen

et al. 2013). Leaf senescence can be triggered by many endogenous and external factors (Lee *et al.* 2015). These factors include increased accumulation of ROS, such as hydrogen peroxide, superoxide, and hydroxyl radical (Van Breusegem and Dat 2006).

ROS, when accumulated at high concentrations, can oxidize the cellular AsA and glutathione pool of the cell (Bienert *et al.* 2006, Mittler *et al.* 2004). Previous study showed that endogenous ROS burst resulted in programmed cell death of starchy endosperm at 12 d after flowering (Xu *et al.* 2010). In another study, a higher accumulation of ROS in the rice leaves during the senescence period led to reduced yield (Zeng *et al.* 2018). Excessive ROS would destroy the photosynthetic apparatus (Hideg and Schreiber 2007); to control the ROS content, plants have developed enzymatic and nonenzymatic mechanisms. AsA is included in the nonenzymatic mechanisms (Gest *et al.* 2013).

AsA belongs to water-soluble antioxidants and is one of the most important vitamins widely distributed in various plants (Guerreiro *et al.* 2010). Some previous studies have demonstrated that AsA plays important roles in the regulation of mitosis and cell expansion (Smirnoff and Wheeler 2000), in electron transport chain (Tóth *et al.*

2009), and particularly, in scavenging of ROS (Ren *et al.* 2015). AsA is an important antioxidant of the ROS scavenging mechanism; it helps to reduce peroxides (Potters *et al.* 2010). AsA is considered one of the most abundant nonenzymatic antioxidants, which can regulate the content of ROS in higher plants (Gest *et al.* 2013). Meanwhile, AsA is involved in physiological processes, such as senescence regulation and stress response (Mekki *et al.* 2015, Chiang *et al.* 2017). It was reported that decreasing AsA content accelerated the senescence rate of tomato plants (Zhang *et al.* 2013). Recently, we have reported that rice plants with more AsA had a greater yield (Yu *et al.* 2015). The rice plants with insufficient AsA were sensitive to environmental factors and accumulated more ROS in the leaves (Yu *et al.* 2017, Zhang *et al.* 2018). ROS burst out after flowering in the rice and accelerate the senescence of leaves (Xu *et al.* 2010). The *OsFTL10*-suppressed transgenic rice line FTL10 exhibited earlier flowering compared to wild type. This study aimed to explore the function of exogenous AsA in the process of senescence in rice line FTL10. The results suggest a close correlation between exogenous AsA and leaf senescence in FTL10. Exogenous AsA can effectively scavenge ROS, delay senescence, finally leading to the increased grain yield.

Materials and methods

Plant material: *Oryza sativa* L. cv. Zhonghua 11 (ZH-11), and an early flowering mutant FTL10 were used in this study (Fang *et al.* 2019). The pasteurized seeds were planted in an artificial climate incubator and grown under controlled conditions with an average temperature of 33°C light/28°C dark, air humidity of 80%, and a photoperiod of 14 h light/10 h dark [PAR of 100 μmol(photon) m^{-2} s^{-1}]. As the seedlings had four leaves they were taken out gently and then transferred to the field. Flag leaves of FTL10 were sampled at 10:30–11:00 h at 0, 10, 20, 30, and 40 d after heading (DAH), and all sampled leaves were stored at –80°C until further analysis.

Exogenous AsA: In the preliminary experiment, we set five exogenous AsA concentrations, 0.07, 0.14, 0.21, 0.28, and 0.35 mM, respectively. Preliminary experiment results showed that exogenous AsA of 0.28 mM could significantly delay the senescence of early flowering mutant FTL10. FTL10 rice plants grown in the field were sprayed with exogenous AsA (0.28 mM) solution at 12:00 h every day from 0 to 40 DAH until the leaves were thoroughly wet. Control groups (CK) were sprayed with an equal volume of distilled water.

Detection of H_2O_2 and $O_2^{\cdot-}$ in the tissue: H_2O_2 generation in leaves was detected by an endogenous peroxidase-dependent *in situ* histochemical staining procedure using 3,3'-diaminobenzidine (DAB). Leaves were placed in a solution containing 1 mg(DAB) mL^{-1} (pH 5.5) for 2 h at room temperature after a brief vacuum infiltration for three times (10 min each). Oxidation of DAB leads to its polymerization and deposition at the site of H_2O_2

generation. The leaves were boiled in ethanol (95%, v/v) for 10 min and then stored in 60% glycerol. H_2O_2 production is visualized as a reddish-brown coloration (Liu *et al.* 2007).

$O_2^{\cdot-}$ production was detected by nitroblue tetrazolium (NBT) staining. Leaves were placed in a solution containing NBT (1 mg mL^{-1}) plus NaN_3 (10 mM) solution in 10 mM potassium phosphate buffer (pH 7.8). After vacuum infiltration for three times (10 min each), the leaves were stained for 4 h at room temperature. The leaves were then boiled in ethanol (95%, v/v) for 10 min and stored in 60% glycerol. The NBT was visualized as a dark blue-colored formazan deposit (Liu *et al.* 2007).

RNA isolation and quantitative RT-PCR: Total RNA was extracted from flag leaves using an *Omega R6827* kit (*Omega*, GA, USA) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized with oligo(dT) primers using a M-MLV reverse transcriptase kit (*Takara*, Kyoto, Japan), and then the cDNAs were stored at –80°C until further analysis. Quantitative real-time PCR (q-PCR) was performed using an *Applied Biosystems* platform. Each 10 μL of PCR reaction contained 0.5 μL of cDNA, 3.5 μL of RNAse free water, 5.2 μL of SYBR® *Premix* EX Tap™ II (*Takara*, Kyoto, Japan), and 0.4 μL of each primer, which was synthesized by *Invitrogen*. Rice *OsUBQ* was used as an internal control. Normalization and fold changes were calculated using the method of Livak and Schmittgen (2001). The primers for real-time PCR were designed as follows: 5'-CCAGGACAAGATGATCTGCC-3' (forward) and 5'-AAGAAGCTGAAGCATCCAGC-3' (reverse) for *OsUBQ*; 5'-ACCCGCAGCAGCTTCTCGCCAA-3' (forward) and 5'-CAAGCACCAAAACCTCCTCCAT-3' (reverse) for *OsA12*; 5'-AGGGGTGGTACAACAAGC-TG-3' (forward) and 5'-GCTCCTTGCGGAAGATGT-AG-3' (reverse) for *OsSGR*; 5'-GCACGGAGGCGA-ACGA-3' (forward) and 5'-TTGAGCGGTAGCACC-CATT-3' (reverse) for *Osh36*; 5'-TATCTCAGATCATAC-CGGATG-3' (forward) and 5'-AAGTACTCCCTCAGC-GATGG-3' (reverse) for *OsFTL10*; 5'-CAAGAAGCC-GAACGGTTC-3' (forward) and 5'-GTTAGAGTGGAG-CAGCAT-3' (reverse) for *OsNAP*.

Detection of TAC of leaves: TAC was evaluated by measuring the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging rate according to Saha *et al.* (2008). In brief, rice flag leaves (0.2 g) were ground with 2 mL of 95% methanol in an ice bath, and the homogenate was centrifuged at 13,000 × *g* at 4°C for 10 min. The sample of 0.2 mL was added to 2.8 mL of 120 μM DPPH (dissolved in 95% methanol) for 5 min in the dark. The absorbance of mixture was measured at 517 nm (*UV-2450*, *Shimadzu*, Kyoto, Japan). Different concentrations of DPPH were used as a standard. The free radical-scavenging capacity of the mixture was calculated according to the absorbance of the mixture at 517 nm.

Measurement of Chl content: Fresh leaves (0.1 g) were extracted with 10 mL of 80% (v/v) acetone at 4°C for

24 h. The absorbance of the extracts was measured at 663 and 645 nm using a *UV-Vis 2450* spectrophotometer (*Shimadzu*, Tokyo, Japan). Chl *a* and Chl *b* were determined using the redetermined extinction coefficients and equations established by Lichtenthaler (1987): Chl *a* [mg L^{-1}] = 12.21A$_{663}$ − 2.81A$_{645}$ and Chl *b* [mg L^{-1}] = 20.13A$_{645}$ − 5.03A$_{663}$.

Rubisco protein determination was carried out according to Liu *et al.* (2000). Fresh leaves of 0.1 g were homogenized in 2 mL of grinding medium composed of 50 mM Tris-HCl (pH 7.8) buffer containing 5% (w/v) polyvinylpyrrolidone (PVP), 0.1% (v/v) β-mercaptoethanol, 0.1% (w/v) NaCl, 0.17% (w/v) phenylmethanesulfonyl fluoride (PMSF), and 2% (v/v) glycerol, and the homogenate was centrifuged at 13,000 × *g* for 10 min. Supernatant (50 μL) was added to an equal volume of sample buffer sample buffer (pH 7.6) composed of 10 mM Tris, 2% (v/v) 2-mercaptoethanol, 24% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), and 0.02% (w/v) bromophenol blue, and then incubated in boiling water for 5 min. The samples (10 μL) were loaded onto gels containing 12.5% (w/v) resolving polyacrylamide gel [12.1% acrylamide, 0.5 M Tris-HCl (pH 8.8), 0.1% SDS, 0.4% N,N'-methylene-bis-acrylamide, 0.08% ammonium persulphate and 0.08% N,N,N',N'-tetramethylethylene diamine (TEMED)] and 4% (w/v) stacking polyacrylamide gel [4% acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% N,N'-methylene-bis-acrylamide, 0.05% ammonium persulphate, and 0.08% TEMED]. Gel staining with *Coomassie Brilliant Blue R-250* (*Sigma*, USA) was followed by destaining overnight with gentle shaking to make sure that its background turned colorless. The molecular mass of the Rubisco large (R$_L$) and small subunit (R$_S$) is 55 and 15 kDa, respectively. The SDS-PAGE gel was scanned using the *GelDoc-It Imaging System* (*UVP*, Upland, CA, USA) and relative grey value of each band detected by using *TotalLab Quant* software (*TotalLab*, Newcastle upon Tyne, UK). On the other hand, the supernatant was collected and diluted for 50 times. Then 250 μL of diluted supernatant was added into the same volume of Bradford solution. After 5 min, the absorbance was read at a wavelength of 595 nm (*UV-Vis 2450* spectrophotometer, *Shimadzu*, Tokyo, Japan).

Measurement of P_N: The P_N of flag leaves was determined at 10:00–11:30 h using the *Li-6400* portable photosynthesis system (*LI-COR, Inc.*, USA) on sunny days. The PPFD was set at 800 μmol m^{-2} s^{-1}, the ratio of red and blue light of the irradiance in the leaf measurement chamber was set at 9:1, the corresponding mean temperature was 32 ± 2°C, and the mean relative air humidity was 45%. P_N was recorded when the values were relatively stable.

Yield and its component traits were also examined for FTL10 and ZH-11 plants, including mass of single spike, yield per plant, and seed set. Five plants in the middle of the inner two rows of each plot in the field were investigated to score the following traits for each plant: mass per panicle (mass in g of per panicle at 12–14% moisture), yield per plant (mass in g of grains per plant at 12–14% moisture)

and seed set (the number of filled grains per main panicle at maturity).

Statistical analysis: Values were expressed as means ± standard error (SE). Statistical significance was determined by *Student*'s *t*-test using *SPSS Statistics 19.0* (*IBM*, NY, USA). Means were considered to be significantly different at the level *p*<0.05. Data were processed by using the *SigmaPlot* software (version *10.0*, *SYSTAT Software Inc.*, Richmond, CA, USA).

Results

The early flowering of FTL10: On the 50 d after transplanting, FTL10 has begun heading into reproductive growth and ZH-11 was still in the vegetative growth stage. FTL10 showed the phenotype of early flowering compared to ZH-11. Leaf color was the most direct manifestation of rice senescence. Flag leaves of FTL10 were significantly more yellow than those of ZH-11 at 80 d after transplanting, and it indicated that leaf senescence of FTL10 was faster than that of ZH-11 (Fig. 1*D*). The *OsA12*, *OsSGR*, and *Osh36* were all senescence-related genes in rice, and their
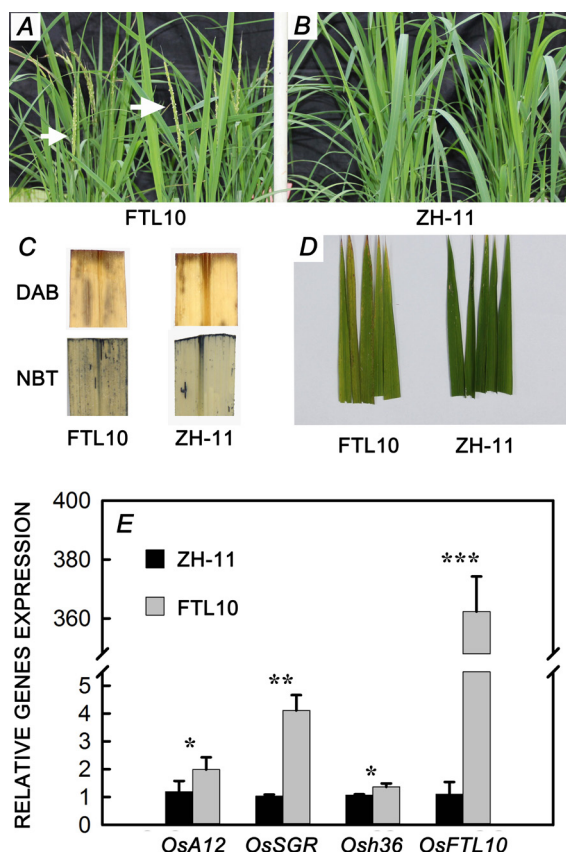

Fig. 1. The growth phenotypes of FTL10 (*A*) and ZH-11 rice (*B*), accumulation of H$_2$O$_2$ and O$_2$$^{•-}$ in the flag leaves visualized by 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively (*C*), senescence of rice leaves (*D*), and relative genes expression (*E*). Data are presented as the means ± SE (*n* = 5). Asterisks indicate significant differences (two-sided Student's *t*-test, * – *p*<0.05, ** – *p*<0.01, *** – *p*<0.001).

expression was positively correlated with the senescence process of rice (Fig. 1E). At 80 d after transplanting, the expression levels of *OsA12*, *OsSGR*, and *Osh36* genes in FTL10 were significantly 2 times, 4 times, and 1.2 times, respectively, higher than those in ZH-11. Expression of *OsFTL10*, an early flowering gene, was 360 times higher in FTL10 than that of ZH-11. The location of H_2O_2 and $O_2^{\cdot-}$ in flag leaves of rice 80 d after transplantation showed that the accumulation of H_2O_2 and $O_2^{\cdot-}$ in leaves of FTL10 was higher than that of ZH-11 (Fig. 1C). The results suggested that early flowering resulted in early senescence.

Senescence of leaves: On the 40 DAH, there was a significant yellowing of the leaves without AsA treatment. AsA delayed the senescence of FTL10 leaves. Exogenous AsA could reduce the expression of senescence-related and early flowering genes (Fig. 2C). The expression levels of *OsFTL10*, *OsNAP*, and *OsSGR* genes decreased by 27.2, 5.8, and 33.1%, respectively. The TAC of rice leaves increased gradually during senescence, and it was higher in the leaves after the AsA treatment than that of CK at all stages, suggesting that exogenous application of AsA could improve the TAC of leaves (Fig. 2A). ROS localization in rice leaves showed that the accumulation of H_2O_2 and $O_2^{\cdot-}$ was higher in the leaves without AsA treatment, suggesting that the addition of exogenous AsA reduced the accumulation of H_2O_2 and $O_2^{\cdot-}$ in leaves of FTL10 ((Fig. 2B).

Photosynthetic capacity: The Chl contents in leaves decreased gradually with heading time (Fig. 3A). It was higher in the leaves with AsA treatment compared with CK at all stages. On the 40 DAH, the content of Chl was 20.8% higher than that in CK. Degradation of soluble proteins is one of the basic characteristics of leaf senescence. The content of soluble protein decreased slowly and remained higher between 0 and 20 DAH, and then decreased fast. The application of exogenous antioxidant AsA effectively slowed down its degradation rate, so that the soluble protein content in the leaves with AsA treatment was higher than that of control group by 53.6% at 40 DAH (Fig. 3C). At the same time, the P_N of leaves also gradually decreased. Exogenous application of AsA slowed down the decline of P_N in leaves (Fig. 3D). Rubisco, as the main enzyme of photosynthesis, was higher in the leaves with AsA treatment, and the R_L and R_S were 14.3 and 35.4%, respectively, higher than that of CK at 40 DAH (Fig. 3B).

Yield and grain phenotypes: The yield characteristics of FTL10 after 40 d of heading are shown in Fig. 4. Under natural aging conditions, the seed-setting rate of FTL10 was lower. The seed-setting rate could be significantly increased and the vacancy rate decreased by the application of exogenous AsA. Statistical analysis showed that the mass of single spike, yield per plant, and seed-setting rate of FTL10 in the control group were 88.5, 91.3, and 91.4% of that in the AsA treatment group, respectively.

Discussion

Leaf senescence is a normal physiological phenomenon in rice, which has a very positive effect on rice itself, and is also necessary for the transport of organic matter from source to sink (Lim *et al.* 2007). However, premature senescence is unfavorable for the functional period of rice. In agricultural production, premature senescence of leaves shortens the functional period of leaves and thus reduces the rice yield. The FTL10 is an early flowering line by overexpressing the *OsFTL10* gene (Fang *et al.* 2019). The expression level of *OsFTL10* gene in FTL10 was significantly higher than that in the wild type. The results showed that the FTL10 was heading and flowering, while the wild type was still
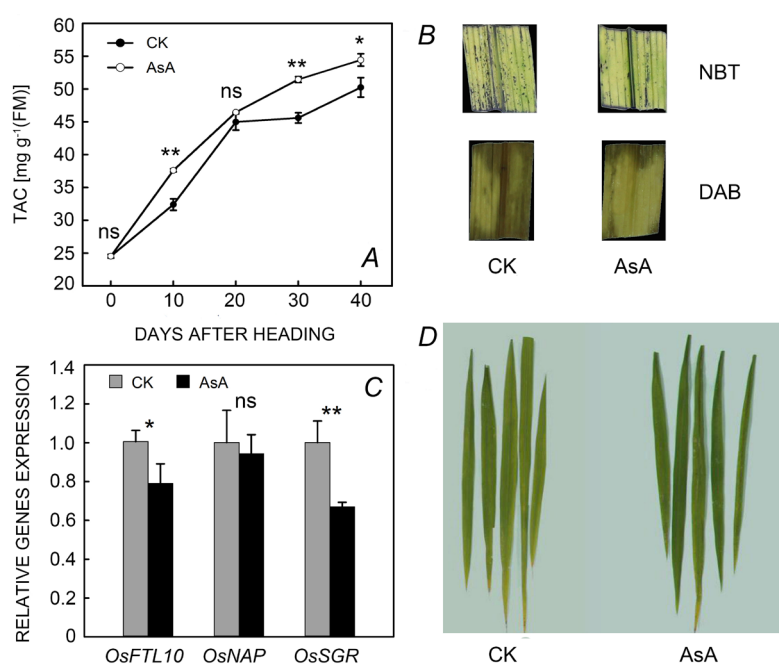

Fig. 2. Effect of ascorbic acid (AsA) treatment on total antioxidant capacity (TAC) of FTL10 rice leaves (*A*), accumulation of $O_2^{\cdot-}$ and H_2O_2 in the flag leaves visualized by nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) staining, respectively (*B*), relative genes expression (*C*), and phenotypes of rice leaves (*D*). Data are presented as the means ± SE (*n* = 5). Asterisks indicate significant differences (two-sided Student's *t*-test, * – $p<0.05$, ** – $p<0.01$, ns – not significant).
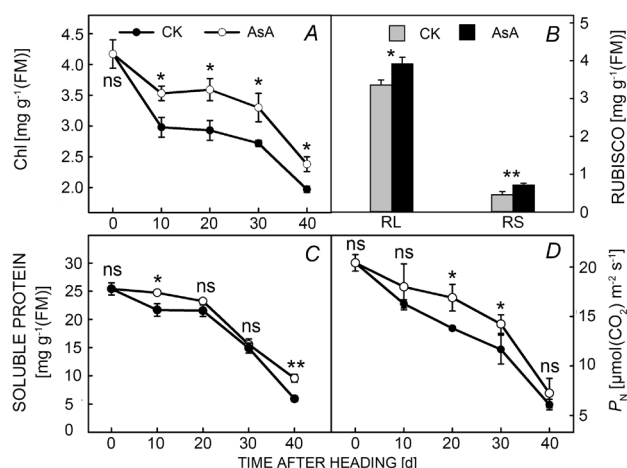
Fig. 3. Effect of ascorbic acid (AsA) treatment on chlorophyll (Chl) content (*A*), Rubisco large (R$_L$) and small subunit (R$_S$) contents 40 d after heading (*B*), soluble protein content (*C*), and net photosynthetic rate (*P$_N$*) (*D*) of FTL10 rice leaves. Data are presented as the means ± SE (*n* = 5). Asterisks indicate significant differences (two-sided *Student*'s *t*-test, * – *p*<0.05, ** – *p*<0.01, ns – not significant).
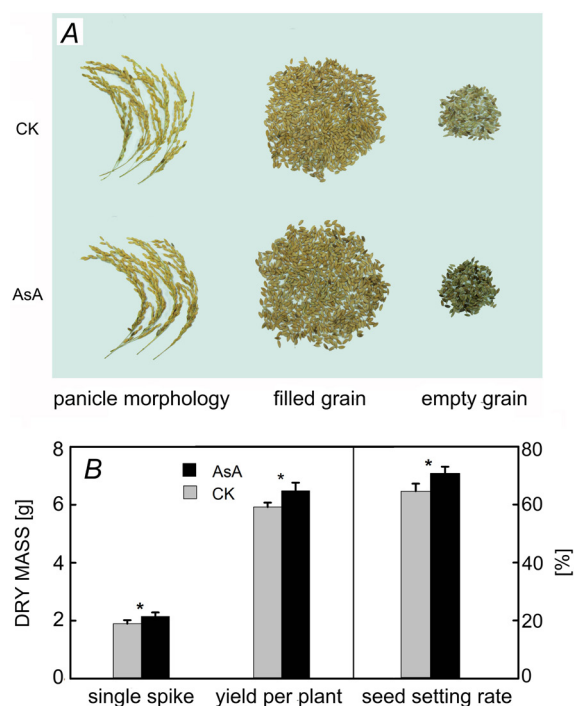

Fig. 4. Grain phenotypes (*A*) and statistical results of mass of single spike, yield per plant, and seed-setting rate (*B*) of FTL10 rice treated with ascorbic acid (AsA). Data are presented as the means ± SE (*n* = 5). Asterisks indicate significant differences (two-sided *Student*'s *t*-test, * – *p*<0.05).

in the vegetative growth stage at 50 d after transplanting. The rice leaf phenotype showed that FTL10 was more senescent and it proved that early flowering rice shows the phenotype of premature senescence (Fig. 1). The balance between light energy capture and utilization was broken

and it led to the formation of a large amount of ROS during the leaf senescence stage (Li *et al.* 2009). It has been reported that the response of leaves to senescence signals is regulated by ROS (Van Breusegem and Dat 2006). At 80 d after transplanting, the accumulation of ROS in FTL10 was higher than that in the wild type and the expression of senescence-related genes was also higher (Fig. 1). This was consistent with previous observations in other rice cultivars (Gregersen *et al.* 2013).

AsA plays a very important role in plants metabolism and developmental growth (Alhagdow *et al.* 2007, Li *et al.* 2016), mediating the interaction of plants with the environment and oxidative stress (Gallie 2013). As one of the main antioxidants in plants, it can effectively scavenge ROS. In *Arabidopsis*, AsA deficiency leads to increased accumulation of ROS (Müller-Moulé *et al.* 2003). Our previous studies showed that the accumulation of ROS in grains was reduced by increasing the content of endogenous AsA (Yu *et al.* 2017). In this study, the application of exogenous AsA increased the TAC of leaves and reduced the accumulation of ROS (Fig. 2*A*,*B*). Low tissue AsA content is linked to premature senescence (Barth *et al.* 2004) and programmed cell death in plants (Pavet *et al.* 2005, De Pinto *et al.* 2012). At the same time, ROS can be used as signal substances in plants to cause plant senescence (Van Breusegem and Dat 2006). Previous studies have proved that AsA could regulate plant leaf senescence by controlling the content of ROS (Gallie 2013). On the 40 d after AsA treatment, the expression of *OsFTL10*, *OsNAP*, and *OsSGR* genes decreased significantly compared with CK (Fig. 2*C*). The *OsSGR* gene is involved in the disaggregation of Chl and the *OsNAP* gene is linked to the onset of leaf senescence (Liang *et al.* 2014). The phenotypes of leaves on the 40 d after AsA treatment showed that the leaves of the control group were more yellow (Fig. 2*D*). These results indicated that exogenous AsA can effectively scavenge ROS and then slow down leaf senescence.

Degradation of photosynthetic pigments and proteins is an important indicator of plant leaf senescence (Liang *et al.* 2014, Yang *et al.* 2016). With the senescence of rice leaves, ROS gradually accumulate. The Chl and soluble protein in leaves are gradually destroyed, resulting in a gradual decline of *P$_N$* in leaves (Fig. 3). Previous studies have found that different exogenous hormones can promote or slow down the degradation of Chl (Buchanan-Wollaston *et al.* 2005). In this study, the content of Chl was stabilized by applying exogenous AsA. Soluble protein, as a biological macromolecule, can be increased by increasing the expression of endogenous antioxidants. In the AsA-treated group, the soluble protein content was higher and Rubisco content was also higher. As the two most important substances of photosynthesis, Chl and Rubisco are closely related to *P$_N$*, the degradation of them leads to the decline of *P$_N$*. Our previous studies showed that the *P$_N$* of super high-yield rice was relatively high, and the *P$_N$* decreased slowly at the late growth stage (Ou *et al.* 2003). Therefore, maintaining the stability of photosynthetic apparatus during the rice senescence is the basis of yield. The *P$_N$* of the leaves treated with AsA was higher than that of the

control group, and the yield was higher in the treatment group. There was a significant difference between the single pike mass, yield per plant, and seed-setting rate. In summary, our results suggest that exogenous AsA may play a role in delaying the senescence of rice leaves. It can effectively remove active oxygen, slow down the speed of leaf senescence, prolong the functional period of leaves, and increase the rice yield.

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