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BRIEF COMMUNICATION

Photosynthetic characterization at different senescence stages in an early senescence mutant of rice *Oryza sativa* L.

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Abstract

An early senescence (*es*) mutant of rice *Oryza sativa* L. with progressing death of most of leaves before heading stage was identified in the field in Hainan province. After tillering stage, the brown striations were found in the base of green leaves randomly, and then expanded to whole leaves. No fungi, bacteria, and viruses were detected in the brown striations suggesting that it was a genetic mutant. The ultrastructure of leaf cells at the site of brown striations showed breakdown of chloroplast thylakoid membrane structures and other organelles, and condensation of the cytoplasm at severe senescence stage. The photosynthetic activity and chlorophyll (Chl) contents decreased irreversibly along with leaf senescence process.

Additional key words: photosynthesis; pigments; senescence; ultrastructure.

Senescence in plants can refer to the aging of various tissues and organs as the whole plant matures and the process of whole plant death that sometimes occurs after fertilization (Greenberg 1996). Besides, abiotic and biotic stresses, such as drought, heat, fungi, and exogenous H_2O_2, also can induce plant senescence, *e.g.* irreversible deathward phenomena combined with hypofunction, environmental stability degradation, and structural component degeneracy, *etc*. (Doorn and Woltering 2004). Morphological changes of senescence cell include cell crinkle, increase of membrane permeability and friability, infolding of nuclear membrane, mitochondrion attenuation, accumulation of lipofuscin or abnormal material in cell, which results in final apoptosis or necrosis. During rice senescence after ripening, the significant changes were the breakdown of Chl and Chl-protein complex (Tang *et al*. 2005). The degradation of thylakoid membrane usually caused the increase of reactive oxygen species (ROS) because the light energy could not be converted into photochemical energy. Plant senescence induced by H_2O_2 confirmed the association between senescence and free radical theory (Beckman and Ames 1998). Superoxide dismutase (SOD; EC 1.15.1.1) constitutes the first line of defense against ROS (Alscher *et al*. 2002, Apel and Hirt 2004).

In this study, an early senescence (*es*) rice mutant was identified from rice *O. sativa* L. Japonica cv. Nipponbare in Danzhou City of Hainan province. Its photosynthetic abilities and structural changes of leaves at different aging states were assayed and discussed here.

The early senescence mutant was identified in the T-DNA insertional lines obtained from rice *O. sativa* L. cv. Nipponbare (Japonica). Genetic analysis showed that it was not caused by T-DNA insertion because bar gene could not be detected in this mutant. Wild-type rice cv. Nipponbare and *es* mutant seedlings, which germinated

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Abbreviations: Chl – chlorophyll; *es* – early senescence; F_0 – minimal fluorescence level in dark-adapted leaves; F_0' – minimal fluorescence level in light-adapted leaves; F_m – maximal fluorescence level in dark-adapted leaves; F_m' – maximal fluorescence level in light-adapted leaves; F_v – variable fluorescence level in dark-adapted leaves; F_v' – variable fluorescence level in light-adapted leaves; F_v/F_m – maximal efficiency of PSII photochemistry; F_v'/F_m' – efficiency of excitation energy capture by open PSII reaction centers; MDA – malondialdehyde; NBT – nitroblue tetrazolium; NPQ – nonphotochemical quenching; POD – peroxidase; PPFD – photosynthetic photon flux density; PSII – photosystem II; q_P – photochemical quenching coefficient; SOD – superoxide dismutase; β-Car – β-carotene; Φ_{PSII} – actual PSII efficiency.
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on April 22, 2009, were used. Seedlings with 7 leaves were planted in red clay soils of experimental farm in Baodaoxincun, Danzhou (19°51′51N; 109°55′63E) on May 20, 2009. In growing season, the average temperature in June, July, and August were 30.3°C, 30.8°C, and 29.5°C, and precipitations were 77.7 mm, 175.6 mm, and 182.2 mm, respectively, with around 97.5% humidity. In August 2009, plants with different senescence degrees before heading stage were used for following assays.

The electron microscopic analysis was done according to Wang *et al.* (2006). The intact leaves were thoroughly rinsed with tap water and distilled water, and extra water on leaf surface was sucked by filter paper. The samples (1 mm^3) were fixed in 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h, and washed with the phosphate buffer for 1 h. Then the samples were fixed with 1% (w/v) OsO_4 (pH 7.2) for 1 h, and rinsed with the phosphate buffer for 3 × 10 min. After washing, the samples were stepwise dehydrated in gradient acetone and ethanol, and then embedded in Spurr's resin for 3 d. Dry sections (1–2 μm) were cut with a diamond knife using an ultramicrotome, and mounted on copper grids. Electron microscopic observation was made at 100 kV on a *JEM1230* transmission electron microscope (*JEOL*, Tokyo, Japan).

Rice fungi and pathogenic bacteria were measured based on the method of Notteghem and Silue (1992). 1 mm of fresh leaf tissue with brown scars was cultivated in 2% potato dextrose agar media (for fungi measurement) and 4% lysogeny broth media (for bacteria measurement) for 1 day at 37°C, and 6 replicates were made. The culture media were carefully checked under the low-power microscope *Leica DM3000B* (*Leica Microsystems GmbH*, Wetzlar, Germany). For virus detection, the leaves were extracted according to the methods of Kimura (1976) and checked by *GE Ultrospec™ 2100 pro UV/Visible Spectrophotometer* (USA) according to Lin *et al*. (1989).

Chl was extracted with 80% acetone from 0.1-g samples. The extract was measured spectrophotometrically at 475, 645, and 663 nm with *GE Ultrospec™ 2100 pro UV/Visible Spectrophotometer* **(**USA**)**. Specific Chl contents were determined according to the method of Lichtenthaler (1987).

Modulated Chl fluorescence measurements were made in attached leaves in the field at midday with a *PAM-2000* portable fluorometer (*Walz*, Effeltrich, Germany) connected to a computer with data acquisition software *DA-2000* (*Heinz*, Walz). The experimental protocol described by Demmig-Adams *et al*. (1996) was essentially followed. The minimal fluorescence level (F_0) in dark-adapted state was measured by the measuring modulated light, which was sufficiently low (<0.1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. To determine the minimal fluorescence level during illumination (F_0'), a black cloth was rapidly placed around the leaf and the leaf-clip holder in the presence of far-red light (7 μmol m^{-2} s^{-1}) in order to oxidize the PSII centers fully. Upon darkening of the leaf, fluorescence dropped to the F_0' level and immediately rose again within several seconds. The maximal fluorescence level in the dark-adapted state (F_m) and the maximal fluorescence level during natural illumination (F_m') were measured by a 0.8-s saturating pulse at 8,000 μmol m^{-2} s^{-1}. F_m was measured after 30 min of dark adaptation. F_m' and F_s was measured when photosynthetic photon flux densities (PPFDs) were approximately 200 and 1,400 μmol m^{-2} s^{-1}, respectively. Other parameters were calculated based on measured parameters above.

0.5–1.0 g of fresh leaves was homogenized with 2 ml of 80% methanol, containing 1 mmol L^{-1} 2,6-di-tert-butyl-4-methylphenol on ice. After 4 h of fully extraction at 4°C, the supernatant obtained by a centrifugation at 1,000 × *g*, 4°C for 15 min was passed through C-18 solid phase extract column. Samples were air dried with nitrogen, and resuspended by 1.5 ml of phosphate buffered saline buffer with 1:1000 (v/v) Tween-20, and 1:1000 (w/v) glutin, pH 7.5. ABA determination was performed with ELISA method with ABA antigen, antibody, and ABA standard. Secondary antibodies were goat anti-rabbit IgG coupling with horseradish peroxidase, the optical density values were measured by *DNA EXPERT* (*Tecan*, Austria) at 490 nm.

SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Beyer and Fridovich (1987) with some modifications. For the total SOD assay, a 5 ml of the reaction mixture contained 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3, 13 mM methionine, 0.025% (w/v) Triton X-100, 75 μm NBT, 2 μm riboflavin and an appropriate aliquot of enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 350 μmol m^{-2} s^{-1}. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. Peroxidase (POD; EC 1.11.1.7) activity was determined spectrophotometrically by the method of Van Loon and Geelen (1971). Proline was determined following Bates *et al*. (1973). Malondialdehyde (MDA) was assayed by the thiobarbituric acid method as described by Aust *et al*. (1985).

All of the measurements were performed 6 times, and the means and standard deviations (SD) were calculated.

The *es* mutant did not show any aging symptom at early growing stages. However, after tillering stage, the leaves at plant base began to grow old. Its senescence started from the emergence of brown striation. This striation occured randomly at the base of green leaves, and then expanded to whole leaves, until leaves withered and died before the stage of inflorescence emergence. Even so, the canopy of three leaves around rice ear did not die until the ear growing up and seed harvest. The examination of brown scars did not show any fungi,

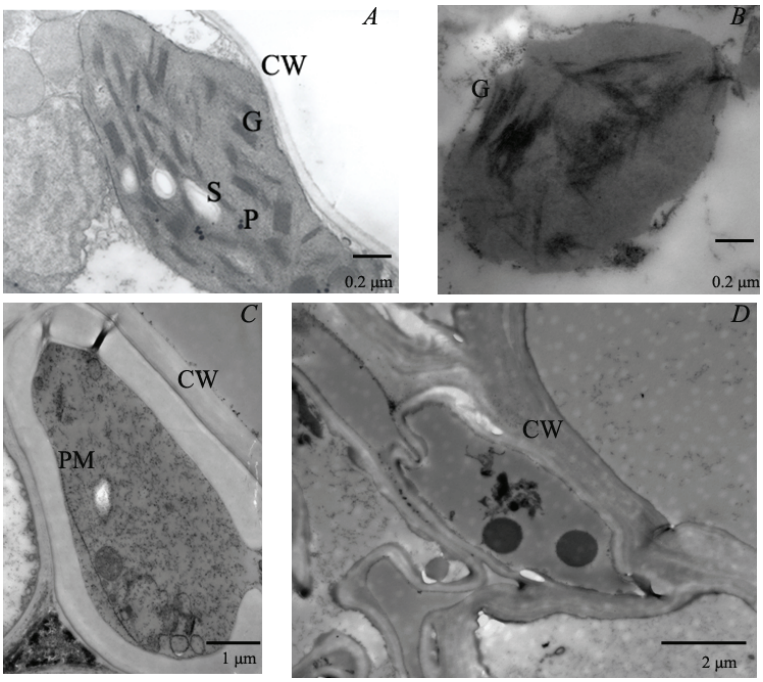

Fig. 1. Ultrastructure of *es* mutant. *A*: normal chloroplast of green leaves; *B*: breakdown chloroplast of semisenescent leaf; *C*: breakdown cell of semisenescent leaves, the plasmalemma is separated from the cell wall; *D*: cell of senescent leaf. CW – cell wall; G – grana; P – plastoglobulus; PM – plasmalemma; S – starch grain.

Table 1. PSII photochemistry of *es* mutant at different leaf stages (means and SD are shown, $n = 6$). ^{A}, ^{B}, and ^{C} means significant difference, *ANOVA, P*<0.01. nd – not detected.

	Leaf type Items	Green leaves	Semisenescent leaves	Senescent leaves
Stress indices	Proline [μmol g^{-1}(FM)]	0.36 ± 0.02^{B}	0.82 ± 0.24^{A}	nd
	SOD [U mg^{-1}(protein)]	50.1 ± 5.05^{A}	19.5 ± 3.04^{B}	nd
	MDA [mol g^{-1}(FM)]	1.05 ± 0.12^{A}	1.15 ± 0.22^{A}	nd
	POD [μg mg^{-1}(FM) min^{-1}]	5.3 ± 0.42^{B}	14.1 ± 2.08^{A}	nd
	ABA [μg g^{-1}(FM)]	7.4 ± 0.57^{B}	22.5 ± 0.73^{A}	nd
Chl contents	Chl *a* [mg g^{-1}(FM)]	2.88 ± 0.12^{A}	0.93 ± 0.09^{B}	0.56 ± 0.12^{C}
	Chl *b* [mg g^{-1}(FM)]	0.91 ± 0.07^{A}	0.35 ± 0.06^{B}	0.22 ± 0.12^{C}
	β-Car [mg g^{-1}(FM)]	0.54 ± 0.04^{B}	0.61 ± 0.02^{A}	0.43 ± 0.12^{C}
	Chl(*a*+*b*) [mg g^{-1}(FM)]	3.79 ± 0.10^{A}	1.28 ± 0.11^{B}	0.78 ± 0.12^{C}
	Chl *a*/*b*	3.17 ± 0.02^{A}	2.63 ± 0.01^{B}	2.51 ± 0.12^{B}
Chl fluorescence parameters	F_v/F_m	0.83 ± 0.02^{A}	0.77 ± 0.01^{AB}	0.70 ± 0.02^{B}
	ETR [μmol(electron) m^{-2} s^{-1}]	21.5 ± 0.82^{A}	20.1 ± 0.64^{A}	14.0 ± 0.02^{B}
	q_P	0.80 ± 0.05^{A}	0.76 ± 0.04^{A}	0.74 ± 0.02^{A}
	NPQ	0.63 ± 0.02^{B}	0.61 ± 0.05^{B}	0.81 ± 0.01^{A}
	Φ_{PSII}	0.51 ± 0.02^{A}	0.48 ± 0.01^{A}	0.33 ± 0.02^{B}

bacterial pathogen, or virus, which suggested that *es* mutant was not caused by pathogen or virus infection.

At the early state of brown scars accompanying with the initiation of DNA degradation (indicated by DNA electrophoresis according to the description of Labarca and Paigen 1980), the cytoplasm became less dense and then shrank, and the plasma membrane was separated from the cell wall (Fig. 1*C*). Chloroplasts remained intact, but they were swollen and their internal membranes, such as lamella were broken (Fig. 1*B*). The cell wall remained undamaged even though the cytoplasm was broken down and plants were senescent (Fig. 1*D*).

As shown in Table 1, the Chl *a* and Chl *b* contents of semisenescent and whole-senescent leaves were decreased by 32.2%, 38.8%, 19.4%, and 24.5% (*ANOVA, P*<0.01) compared with those of green leaves, respectively. The β-carotene (β-Car) contents of semisenescent and whole-senescent leaves were increased and decreased by 111.7% and 79.4% (*ANOVA, P*<0.01) compared with those of green leaves, respectively. The straight degradation of Chl *a* and Chl *b* is an indicator of plant death, which was associated with increase of chlorophyllase activity in Chl catabolites pathway. The faster degradation of Chl *a* than Chl *b* resulted in the decrease of

Chl *a*/*b* ratio. The increase of β-Car in semisenescent leaves suggested that plant could compensate for the loss of Chl by enhancing β-Car content, which can quench reactive oxygen at the early stage of a damage. The maximal efficiency of PSII photochemistry (F_v/F_m) of semisenescent and senescent leaves decreased by 7.4% and 15.8% comparing to green leaves. The electron transport rate (ETR), photochemical quenching (q_P), and the actual photochemical efficiency of PSII (Φ_{PSII}) of semisenescent leaves and senescent leaves were also decreased compared to those of green leaves, respectively. However, the nonphotochemical quenching (NPQ), which reflects the process competing with PSII photochemistry for absorbed excitation energy (Campbell *et al*. 1998), decreased by 4.7% in semisenescent leaves and increased by 29.1% (*ANOVA, P*<0.01) in senescent leaves. These indicated that after the photosystem structures breakdown, the light energy absorbed by chloroplast could not transfer to PSI but dissipated as heat through NPQ.

In semisenescent leaves, SOD activity decreased by 60.9%, while the proline content, MDA content, POD enzyme activity, and ABA content increased by 116.4%, 275.8%, 9.1%, 166.7%, and 202.4%, respectively (*ANOVA, P*<0.01). No values were detected in senescent leaves. These results indicated that the senescence caused defense enzyme up-regulated to cope with the damage of lipid membrane and protein in plant cell and increase of stress indicators, ABA.

Senescence is the final phase of plant vegetative and reproductive development, preceding the widespread death of cells and organs. In the present study, relatively high contents of POD and MDA indicated that the mutation was accompanied by oxidative stress. Morphological results and photosynthetic results indicated that mutant experienced an irreversible death process. The great reduction of Chl *a* and Chl *b* contents is the symbol of death of functioning chloroplasts in preventing plant cell death (Thomas *et al*. 2003). Our data also show that the Chl *a* and Chl *b* contents both in semisenescent and senescent leaves were lower than in green leaves. Besides, a straight decrease of F_v/F_m in *es* mutant suggests a potential damaging effect of light on its PSII, which can be observed by chloroplast breakdown. This is same with the observation in normal senescence that photosynthetic CO_2 assimilation rate, carboxylase activity of Rubisco, Chl, and carotenoids contents, and the Chl *a*/*b* ratio decreased significantly (Tang *et al*. 2005). The order for the stabilities of Chl–protein complexes during leaf senescence (LHCII > CP43 > CP47 > LHCI > PSI core) means that the light absorption was first reduced.

Membrane integrity and cellular compartmentalization are maintained until late stages of the senescence process, suggesting that there is little or no leakage of cellular contents (Fig. 1*C*,*D*). Previous ultrastructural studies with leaf mesophyll cells indicated that nuclei of cells undergoing tobacco mosaic virus-induced PCD maintained their structural integrity even at late stages of cell death, and they assumed a granular appearance (Goodman and Novacky 1994). MDA, one of the products of lipid peroxidation, appears to be produced in relatively constant proportion to the extent of lipid peroxidation and is thus a convenient indicator of the rate of lipid peroxidation. The correlation between ROS and ABA was found in stress research. In this study, the great increase of MDA content, POD enzyme activity and ABA contents were found in semisenescent leaves, but the activity of SOD enzyme was reduced remarkably, which means that the first line to defense the ROS has been damaged in the mutant. Superfluous content of ABA during the leaf development could accelerate premature senescence of the leaf.

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