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Characterization of nitrogen metabolism and photosynthesis in a stay-green rice cultivar

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Abstract: A field experiment was carried out in the years 2008–2011 in China to assess the nitrogen metabolism enzyme activities and photosynthetic characteristics in stable-yielding stay-green rice (*Oryza sativa* L.) cv. Shennong196. The results showed that higher levels of nitrogen content, nitrate reductase activity, and glutamine synthetase activity occurred in leaves of cv. Shennong196 compared with cv. Toyonishiki (control). Leaf color of cv. Shennong196 was positively correlated with nitrogen levels and nitrogen metabolism enzyme activities ($P < 0.05$). Superoxide dismutase activity and malondialdehyde contents were 18.53 unit/g fresh weight and 3.32 nmol/g, respectively, which were lower in flag leaves of cv. Shennong196 than cv. Toyonishiki. Cv. Shennong196 had a higher level of net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate in flag leaves of diurnal variation of photosynthesis at the ripening stage. The high net photosynthetic rate in cv. Shennong196 was positively correlated with the stomatal density of flag leaves ($P < 0.01$). Considering the yield-increasing potential and to prevent premature senescence of crop, these traits of cv. Shennong196 are useful for improved rice cultivar.

Keywords: macronutrient; carotenoid; leaf senescence; stay-green plant; plant density; antioxidant enzyme

Stay-green is a trait to maintain leaf greenness for a longer time (Thomas and Howarth 2000). Antonietta et al. (2014) reported that the leaves of stay-green cultivars of maize (*Zea mays* L.) delay senescence and remain green at maturity under different plant densities (6–10 plants/m^2). The stay-green gene has also been found by Rong et al. (2013). The overexpression of the stay-green rice-like (SGRL) gene can reduce the levels of chlorophyll and chlorophyll-binding proteins while accelerating their degradation in plant leaves. This indicates that the SGRL protein is associated with the degradation of chlorophyll. Moreover, a close association has been demonstrated between leaf color and nitrogen (N) content in plants (Zheng et al. 2008). Although the stay-green phenotype is similar in different species and genotypes, the physiological properties may be different.

The senescence of leaves has a major impact on rice production (Yang et al. 2004). Leaf senescence is an extremely complex and highly organized process (Procházková and Wilhelmová 2007). Cultivar japonica rice of North China has encountered a series of the problems such as rice premature senility, low seed setting percentage, and the lower grain plumpness in the late growing season, all of which are closely related to stay-green. Therefore, it is important to enhance leaf stay-green and resisting to premature senescence in development germplasm resources

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of rice. The objectives of this study were to analyze nitrogen metabolism enzyme activities and photosynthetic characteristics in the stay-green cv. SN196 to improve premature crop cultivar in the future.

MATERIAL AND METHODS

Plant materials and growth conditions. Cv. Shennong196 (SN196): a mutant material with the super green color of the leaf, which was obtained by our laboratory in the practice of super rice breeding. After years of homozygous inbreeding, the field traits of cv. SN196 reached consistency. Cv. Toyonishiki (control): a traditional Japanese Japonica cultivar. All plants were grown in experimental fields at Shenyang Agriculture University (41°50'N, 123°24'E) from 2008 to 2011, during the growing seasons April–October. The experimental field was flat, and soil was sandy loam. In the 0–20 cm soil layer, the soil organic matter, and total N, P and K were 29.8, 1.11–1.28, 2.80, and 34.0 mg/g, respectively. The area of each cultivar plot was 8.1 m^2 with three replications.

Determination of chlorophyll and nitrogen contents, nitrogen metabolism enzyme activities, and antioxidant enzyme activity in rice leaves. The uppermost fully expanded leaves were measured at the days after transplanting 13, 60, 79, 94 and 108 days. Chlorophyl *a* and *b* contents were determined by extraction using a mixture of ethanol and acetone (Wang et al. 2012). The content of carotenoid (Caro content) was determined using the method of Huang et al. (2013). N content (%) was measured with the Kjeldahl method (Foss Kjeltec2300 Auto Kjeldahl Analysis Equipment, Höganäs, Sweden). Nitrate reductase (NR) activity was determined by colorimetry using sulfonamide. Glutamine synthetase (GS) activity was assessed using the method of Duke and Ham (1976).

Additionally, the top-three leaves were taken at the ripening stage, and fresh samples were used to measure malondialdehyde (MDA) content and superoxide dismutase (SOD) activity. MDA content was determined spectrophotometrically as described by Lichtenthaler (1987). The SOD activity was measured according to Giannopolitis and Ries (1977).

Determination of diurnal variation in photosynthetic parameters and stomatal density. The diurnal variations in flag leaf net photosynthetic rate (P_n); stomatal conductance (g_s), intercellular carbon dioxide concentration (C_i) and transpiration rate (T_r) were measured on the main stem with an LI-6400 photosynthetic analyzer (LI-COR Biosciences, Lincoln, USA). The measurement was conducted from 07:30 to 16:30 on sunny days, interval time was one hour. The flow rate of air in the leaf chamber was adjusted to 1000 μmol/s, and the CO_2 concentration was a natural state. The irradiance was kept at a natural level on the measured leaves (cv. SN196 = 5 cm^2, cv. Toyonishiki = 4 cm^2), and the chamber maintained at 28°C.

Stomatal density (S_d) was measured at the maximum leaf width, circa 0.5 cm^2 of abaxial surface of the fully expanded leaf, using a desktop scanning electron microscope (TM1000, Hitachi High-Technologies Corp., Tokyo, Japan).

Statistical analyses. The treatment effects and significant differences were analyzed with one-way ANOVA using SPSS Statistics 13.0 (IBM SPSS, Somers, USA). Significant differences between means (0.05 or 0.01 level) were determined using Duncan's multiple range methods. All data were analyzed as means ± standard deviation.

RESULTS

Physiological characteristics. In the ripening stage (108 days after transplanting), the two cultivars rice showed the largest difference in carotenoid content. The carotenoid content of cv. SN196 was 0.43 mg/g FW (fresh weight), which was 2.8 times that of cv. Toyonishiki (0.15 mg/g FW). The reduction rate of carotenoid content of cv. SN196 was only 40.8%, in the whole growth season, while cv. Toyonishiki was 71.8%. The mean chlorophyll *a* (Chl *a*) content in cv. SN196 (2.85 mg/g FW) was significantly high than in cv. Toyonishiki (1.85 mg/g FW) in the growing season. As for chlorophyll *b* (Chl *b*), the cv. SN196 had higher ($P < 0.05$) content than cv. Toyonishiki at physiological maturity. On average, the Chl *a* and *b* contents of cv. SN196 were 35.1% and 44.6%, which were higher than those of cv. Toyonishiki, respectively.

The N content was significantly higher ($P < 0.05$) in leaves of cv. SN196 (15.2%), compared to cv. Toyonishiki during the growing season (except the 13 days after transplanting). The leaves gradually senesced after the grain-filling stage and the average N content reduction in cv. SN196 was 27.2%, which was slower compared with cv. Toyonishiki (33.1%). This indicates that cv. SN196 has a large source (leaf) than cv. Toyonishiki and has the potential to increase production. The observation of stomatal density in leaves of the two rice cultivars, the result showed that cv. SN196 had the highest number of stomatal density (856 stomata/mm^2) at the heading stage

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Table 1. Nitrogen (N) content; nitrate reductase (NR) activity; glutamine synthetase (GS) activity and stomatal density (S_d) in rice leaves at different growth stages in 2009

Item	Cultivar (line)	Stage				
		tillering	jointing	heading	grain filling	ripening
N (%)	SN196	3.80 ± 0.20^a	2.98 ± 0.01^a	2.37 ± 0.03^a	1.47 ± 0.02^a	1.07 ± 0.02^a
	Toyonishiki	4.15 ± 0.36^a	2.60 ± 0.01^b	2.18 ± 0.02^b	1.24 ± 0.02^b	0.83 ± 0.01^b
S_d (/mm^2)	SN196	542 ± 8^a	680 ± 11^a	856 ± 8^a	767 ± 3^a	732 ± 7^a
	Toyonishiki	428 ± 3^b	723 ± 11^a	693 ± 24^b	711 ± 5^b	723 ± 6^a
NR (μg/g FW/h)	SN196	1.11 ± 0.04^a	1.31 ± 0.15^a	2.08 ± 0.14^a	6.26 ± 0.04^a	4.31 ± 0.16^a
	Toyonishiki	0.97 ± 0.02^a	1.10 ± 0.13^a	2.15 ± 0.13^a	5.18 ± 0.06^b	3.17 ± 0.06^b
GS (A/mg protein/h)	SN196	–	20.66 ± 0.46^b	14.58 ± 0.43^a	19.63 ± 1.75^a	59.40 ± 4.85^a
	Toyonishiki	–	50.51 ± 4.58^a	14.80 ± 1.37^a	18.23 ± 0.70^a	37.12 ± 0.37^b

Within each factor, data are the mean ± standard deviation of three replications. Lowercase letters represent significant at P < 0.05. FW – fresh weight

(79 days after transplanting), which was significantly higher than that of cv. Toyonishiki (Table 1).

After the grain-filling (94 days after transplanting), the NR activity in cv. SN196 leaves had significantly stronger by 20.8% and 36.0% compared with cv. Toyonishiki (Table 1). The two cultivars showed the largest difference in glutamine synthetase activity in leaves at the ripening stage (108 days after transplanting), glutamine synthetase activity in cv. SN196 was 22.28 A/mg protein/h, which was higher than that in cv. Toyonishiki (Table 1, P < 0.01).

The SOD activity in the flag leaf of cv. SN196 was lower 18.53 unit/g FW than cv. Toyonishiki (Figure 1a). In contrast, the SOD activity in the third leaf of cv. SN196 (647.40 unit/g FW) was higher than that of cv. Toyonishiki (618.91 unit/g FW). The malondialdehyde content in the 2nd and 3rd leaves of cv. SN196 were significantly lower than cv. Toyonishiki, by 15.6% and 8.1%, respectively (Figure 1b). The result indicates that a lower level of membrane lipid peroxidation in the functional leaves of cv. SN196 compared with cv. Toyonishiki.

Diurnal variation in photosynthetic characteristics. The maximum P_n appeared at 13:30 in both cultivars and the average P_n of one day in cv. SN196 was 7.81 μmol/m^2/s, which was higher 9.6% compared with cv. Toyonishiki (Figure 2a). The average g_s of a day in cv. SN196 was 0.0845 mol/m^2/s, which was higher than cv. Toyonishiki (Figure 2b). The results indicated that cv. SN196 has higher g_s, which can ensure the exchange of H_2O and CO_2 even at midday with high light intensity and transpiration.

With regard to diurnal variation, the C_i of cv. SN196 was higher than cv. Toyonishiki and the average C_i was higher (7.8%) than cv. Toyonishiki (Figure 3). Cv. SN196 maintained higher transpiration rate

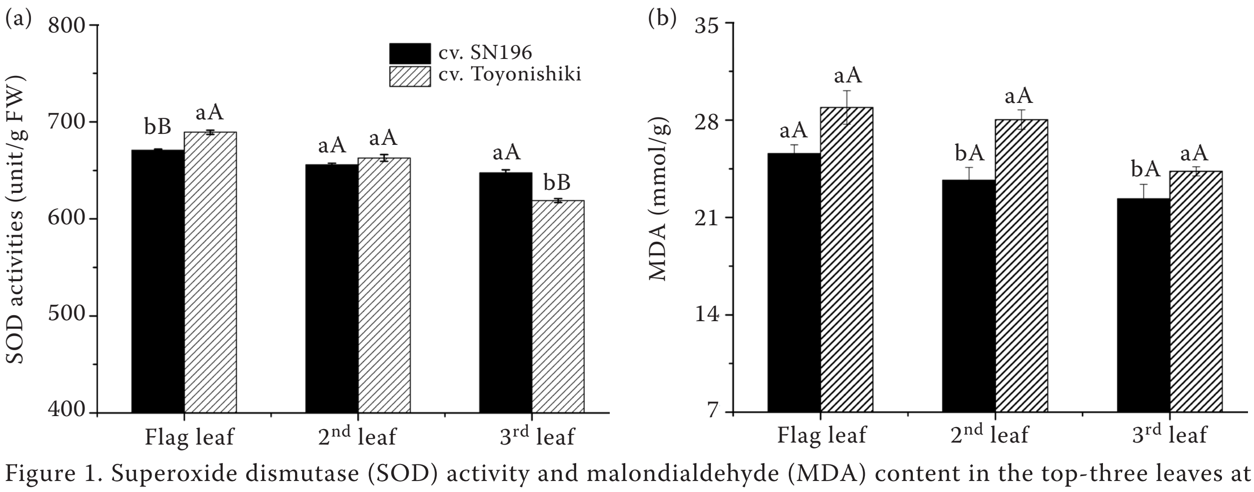

Figure 1. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in the top-three leaves at ripening stage in 2010. FW – fresh weight. Lowercase letters represent significant at P < 0.05, capital letters represent significant at P < 0.01

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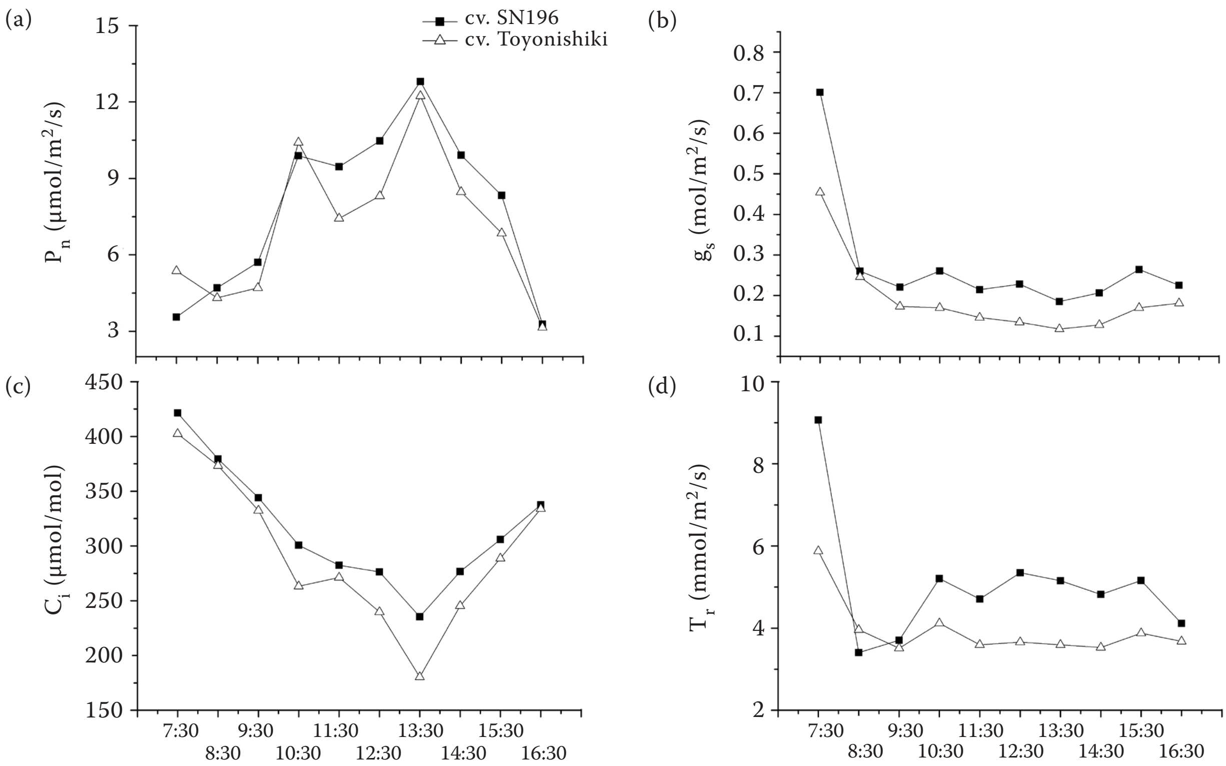

Figure 2. Diurnal variation curve of photosynthetic parameters in 2011. (a) Photosynthetic rate (P_n); (b) stomatal conductance (g_s); (c) intercellular CO_2 concentration (C_i) and (d) transpiration rate (T_r)

throughout the day compared with cv. Toyonishiki (Figure 2d).

DISCUSSION

The activity of numerous antioxidant enzymes is gradually decreased during plant senescence (Procházková et al. 2001). Thus, higher antioxidant capacity is conducive to delay leaf senescence. MDA, a product of membrane lipid peroxidation (Wang et al. 2014), is often used as an indicator of plant senescence; whereas, SOD is one of the production with enzymatic defense systems when the level of radical metabolism is destroyed, and the membrane lipids are peroxidized in plant cells (Wang et al. 2014). In the current study, the results showed that the MDA content was lower in the top-three leaves of cv. SN196, and the SOD activity was lower in both the flag leaf and the second leaf from of cv. SN196 compared with the control (cv. Toyonishiki). However, our results are inconsistency with Tian et al. (2012) that the increment in MDA of the stay-green wheat mutant *tasg1* is smaller with wide-type during the late senescence; this phenomenon is associated with the high activity of antioxidant enzymes (e.g., SOD, guaiacol peroxidase, and catalase). In the present study, the SOD activity did not increase in leaves of cv. SN196. It is possible that the radical metabolism level was not destroyed and the membrane lipids were not peroxidized in the flag/second leaves, which preventing the SOD defense system. The SOD activity in the 3rd leaf of cv. SN196 was higher than cv. Toyonishiki, which delayed leaf senescence, indicating higher antioxidant enzyme activity in the senescent leaves of cv. SN196.

NR is a rate-limiting enzyme (Tian et al. 2009), whose activity directly affects the N uptake by plants. Glutamine synthetase is a key enzyme of ammonia assimilation, plays an essential role in the transformation from inorganic N into organic N (Xu and Zhou 2004). Additionally, glutamine synthetase, in the translocation and re-utilization of N during leaf senescence, is important to understand the physiological mechanism in stay-green plants (Gu et al. 2013). In our experiment, the NR and glutamine synthetase activities were higher (by 36.0% and 60.0%, respectively) in leaves of cv. SN196 compared with cv. Toyonishiki at the ripening stage. The glutamine synthetase activity was possibly triggered in cv. SN196 when the leaves entered the senescence stage,

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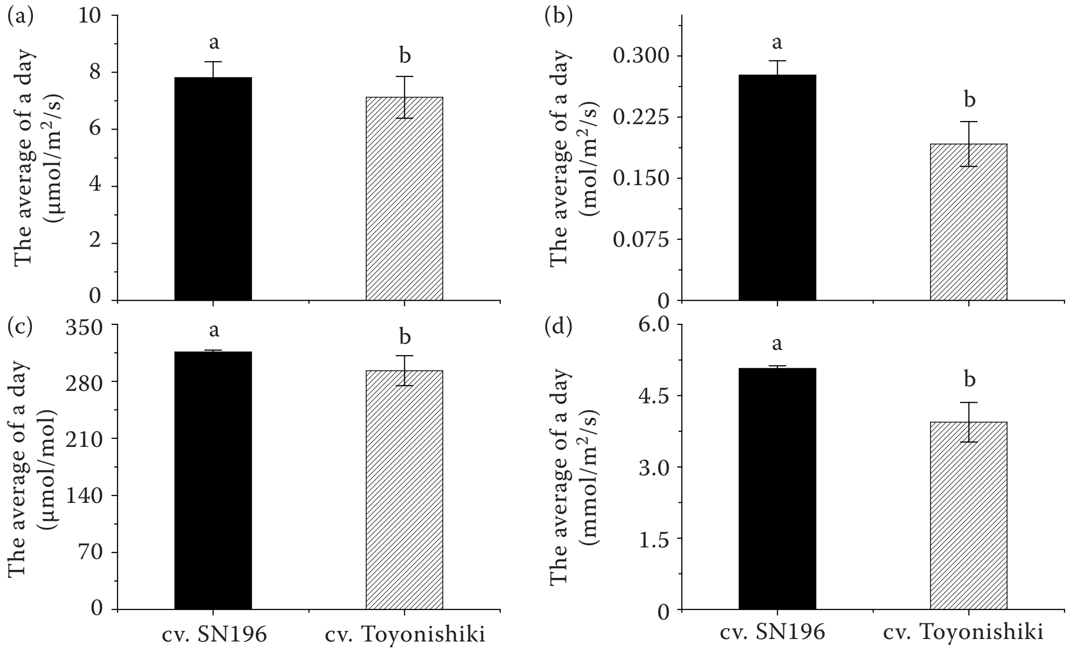

Figure 3. The average of photosynthetic parameters in one day. (a) Photosynthetic rate; (b) stomatal conductance; (c) intercellular CO_2 concentration and (d) transpiration rate. Superscript lowercase letters indicate significant at $P < 0.05$

ensuring N translocation and re-utilization in rice, delaying the rate of leaf yellowing. It means that the strong ability of N metabolism and re-utilization may be another characteristic of the stay-green cv. SN196. These results were confirmed in another experiment (Van Oosterom et al. 2010). The impact of different N supply levels in sorghum cultivars leaf senescence, Van Oosterom et al. (2010) found that the rate of leaf senescence was significantly reduced in AQL39/RQL36 (short cultivar) and A35/RQL36 (stay-green cultivar) with higher N levels around 77 days after senescence. This further demonstrates that the supply and metabolism level of N directly affect the degree of leaf senescence in plants.

Photosynthesis in cv. SN196. Photosynthesis, one of the important physiological processes in plant growth and development, refers to the absorption, transfer, and conversion of light energy on the thylakoid membranes in the chloroplast (Han et al. 2014). G_s, which indicates stomatal aperture, affects photosynthesis, respiration, and transpiration in plants (Dong et al. 1993). In our study, we found that the average P_n, g_s, and T_r of a day were significantly higher in flag leaves of the stay-green cv. SN196 compared with cv. Toyonishiki (Figure 3). However, the difference in P_n between cv. SN196 and cv. Toyonishiki was not significantly correlated with the chlorophyll content in flag leaves (Table 2), which indicates that chlorophyll content is not a major factor affecting P_n. Our result is inconsistent with the finding in a yellow-leaf mutant of rice reported by Wu et al. (2007) who believed that changes in the chlorophyll content of rice leaves can directly affect the P_n. Our study showed that the high chlorophyll content in flag leaves of cv. SN196 was directly related to its high carotenoid content (Table 2), which plays a role in protecting chlorophyll. A previous study has shown that the stay-green wheat mutant *tasg1* maintain high photosynthesis after anthesis is associated with the delay of chlorophyll degradation process (Tian et al. 2012).

Chl *b*, which can absorb and transmit light energy, plays a major role in the stability of light-harvesting complex II (Horn and Paulsen 2004). In the present study, the Chl *b* content in flag leaves of the stay-green rice cv. SN196 was 7.7 times that of the control (Figure 4d). Similarly, Tian et al. (2012) found that the Chl *b* content in leaves of the stay-green wheat mutant *tasg1* was higher than wild-type (control) around 30 days after anthesis in a natural state. A higher Chl *b* content is possibly conducive to protect the structure and function of chloroplast grana. On the other hand, the Chl *b* content in flag leaves of cv. SN196 was 5-fold higher than that of the control (Figure 4c), which indicated higher stability of cv. SN196 in the chloroplast ultrastructure. This result also indirectly suggests that stay-green plants

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Table 2. Correlation between chlorophyll content and photosynthetic characteristics, nitrogen (N) metabolism enzymes and stomatal density

Item	*Chl*	Caro	P_n	g_s	C_i	T_r	N	S_d	NR	GS
Chl	1	0.0001***	0.1565	0.0241*	0.1723	0.0524	0.0494*	0.2473	0.0328*	0.0079**
Caro		1	0.0535	0.0036**	0.0571	0.0112*	0.0112*	0.0938	0.0065**	0.0006***
P_n			1	0.0031**	0.0001***	0.0007***	0.0002***	0.0001***	0.0004***	0.0026**
g_s				1	0.0007***	0.0001***	0.0001***	0.0026**	0.0001***	0.0001***
C_i					1	0.0001***	0.0001***	0.0001***	0.0002***	0.0032**
T_r						1	0.0001***	0.0004***	0.0001***	0.0002***
N							1	0.0003***	0.0001***	0.0002***
S_d								1	0.0008***	0.0071**
NR									1	0.0001***
GS										1

Chl – chlorophyl; Caro – carotenoid; P_n – photosynthetic rate; g_s – stomatal conductance; C_i – intercellular carbon dioxide concentration; T_r – transpiration rate; S_d – stomatal density; NR – nitrate reductase; GS – glutamine synthetase; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

are characterized by improved stability of chloroplast membranes and chloroplast protein complexes in leaves (Kusaba et al. 2007).

In conclusion, stay-green cv. SN196 contained higher levels of Chl *a* and *b*, and N contents as well as NR and glutamine synthetase activities, but lower levels of

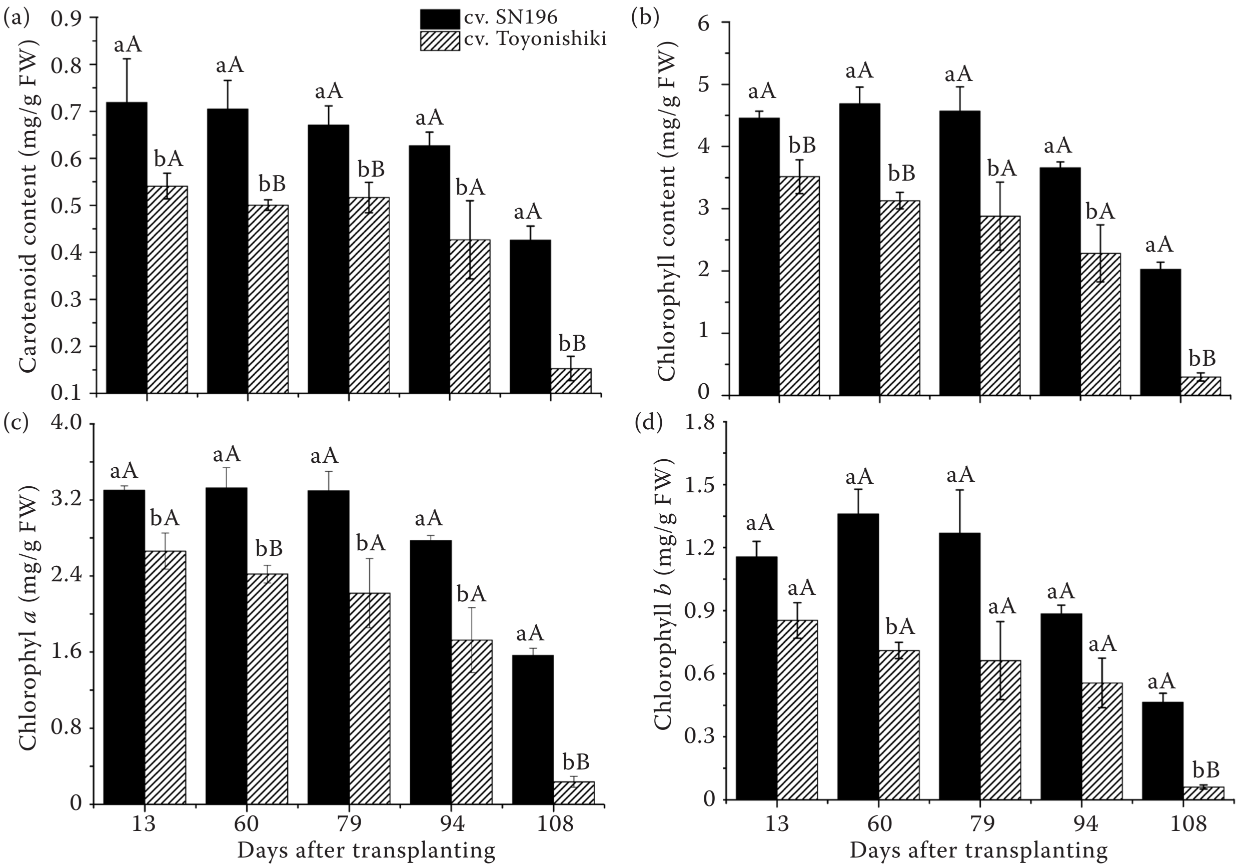

Figure 4. Change on (a) carotenoid content; (b) chlorophyll content; (c) chlorophyl *a* and (d) chlorophyl *b* of rice leaves in different growth stages of 2008. Lowercase letters represent significant at $P < 0.05$, capital letters represent significant at $P < 0.01$. FW – fresh weight

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MDA content and SOD activity in leaves. Cv. SN196 has stay-green leaves possibly by maintaining chloroplast ultrastructure in the late growing season, which contributes to improve P_n in the field condition. These data provide new insights into the physiological mechanism of stay-green rice and could facilitate the solution of cv. SN196 in agricultural practices in the fields.

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