Stay-Green Trait Assessment using the Leaf Incubation Method to Examine the Maintenance of Assimilation Rates under High Temperature Conditions during the Grain-Filling Period in Rice

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Abstract: Rice yield and quality decrease under high temperature conditions during the grain-filling period particularly in early-ripening cultivars that are directly subjected to midsummer heat. As the grain growth rate increases under high temperature conditions, the grain requires greater amounts of assimilates for the shortened grain-filling period and occasionally experiences a lack of assimilates. The stay-green trait that can maintain assimilation during the active grain-filling period is expected to mitigate the negative impact on grains due to the lack of assimilates. Our objectives were to evaluate the stay-green trait of rice maintaining assimilation rates under high temperature conditions during the active grain-filling period using the leaf incubation method. When whole leaves or leaf segments were floated on water at 20 – 40ºC under dark conditions, a leaf color reading (SPAD) showed steady genotypic differences at 25 – 35ºC. When flag leaf segments of early-ripening cultivars from the Japanese Rice Collection (JRC) were incubated at 35ºC under dark conditions, the SPAD values of incubated leaves well reflected those in the top three-leaves in standing plants during the active grain-filling period ($r = 0.774, p < 0.005$). Under ambient and elevated temperature conditions (+3 – 4ºC), the SPAD values and photosynthetic rates of genotypes that ranked higher for the trait by the leaf-incubation test, tended to be higher during the active grain-filling period. The results suggest that the leaf incubation method is suitable for the first-step selection of the stay-green trait of rice associated with the maintenance of assimilation rates under high temperature conditions during the grain-filling period.

Key words: High temperature, Leaf incubation, Photosynthesis, Rice, Stay-green.

Potential negative impacts of climate warming from the greenhouse effect are of concern for future crop production in high- and mid-latitude regions (Angus, 1990; Evans, 1996). During the past decade, the summer temperature has been rising in West to Middle Japan (JMA, 2014). Hot summer conditions seriously damage rice quality and production, particularly in early-ripening rice where most of the grain-filling period occurs during the midsummer heat (Terashima et al., 2001; Morita, 2004). The high temperature accelerates the rate of dry matter accumulation in the grain, but does not increase assimilation. As a result, the shortfall in the amount of assimilates supplied to grains during the active grain-filling period causes strong negative effects on grain quality and yield (Kobata and Uemuki, 2004; Kobata et al., 2004). Simple retardation of leaf senescence under high temperature conditions during the grain-filling period did not improve grain-filling in rice (Kim et al., 2011). Maintenance of assimilation during the active grain-filling period is expected to mitigate the reduction in grain yield and quality caused by high temperatures. The stay-green trait is expected to be one of the high temperature tolerance strategies to maintain assimilation during the active grain-filling period.

The stay-green trait reflects the ability to maintain high leaf chlorophyll contents (Spano et al., 2003). The stay-green trait that maintains chlorophyll contents and leaf assimilation is considered functional stay-green, whereas the maintenance of green color only is termed non-functional stay-green (Spano et al., 2003). The presence of the stay-green trait during the grain-filling period has contributed to an improvement in grain yield of sorghum (Borrell et al., 2000a, 2000b; Mahalakshmi and Bidinger, 2002), wheat (Christopher et al., 2008) and rice of Vietnam (Hoang and Kobata, 2009a, 2009b) under drought stress conditions. Sorghum genotypes that possess the stay-green trait maintain more photosynthetically active green leaves during post-flowering drought periods.
Rice cultivars that retained green leaves for a relatively longer period under drought conditions had a higher dry matter yield compared with other rice cultivars (Jearakongman et al., 1995; Hoang and Kobata, 2009a, 2009b). The maintenance capacity of photosynthesis in stay-green cultivars has been observed under normal conditions in wheat (Spano et al., 2003) and rice (Fu and Lee, 2008). In stay-green rice mutants, a mutation in the gene associated with the decomposition of chlorophyll, resulted in the maintenance of green color (Kusaba et al., 2007; Sato et al. 2007).

The stay-green trait can be evaluated by the change in leaf color of standing plants (Spano et al., 2003; Hoang and Kobata, 2009a, 2009b). However, observations of leaf color in standing plants require long periods of time and long-term maintenance of growth environmental conditions, e.g., temperature and soil water, are difficult when investigating genotypes with different growth durations. Therefore, the stay-green trait is frequently empirically evaluated using leaf incubation methods in which whole leaves (Spano et al., 2003; Kusaba et al., 2007; Hoang and Kobata, 2009a, 2009b) or leaf-sections (Kusaba et al., 2007; Sato et al., 2007) are floated on water. In local Vietnam rice cultivars in which a deeper green color was maintained on whole leaf incubation, the increase in plant dry matter accumulation was maintained in desiccated soils during the post-anthesis period (Hang and Kobata, 2009b). However, it is unknown whether the stay-green trait has been evaluated during the grain-filling period under high temperature conditions using incubation methods in rice. Our objective was to establish a simple and repeatable method for the first-step screening of the stay-green trait related to assimilation in the post-anthesis period of early-ripening rice under high temperature conditions in Japan.

Materials and Methods

1. Plant materials

Two wild and stay-green mutant genotypes, Koshihikari and nycl-1 (stay-green mutant of Koshihikari) and Nipponbare and sgr-2 (stay-green mutant of Nipponbare) (Kusaba et al., 2007) (Oryza sativa L.), were grown in paddy fields at the Nishikawatsu experimental field site (17 m above sea level at 133ºE, 35ºN) at Shimane University, Matsue, Japan, for an incubation test. For the test the whole leaf was used in 2010 and leaf segments in 2013. The soil at the Nishikawatsu site is silty clay loam (mud-rich sediments of alluvium). Three-week-old seedlings consisting of one plant per hill were transplanted into the paddy fields on 12 May, 2010 and 31 July, 2013 (Koshihikari and nycl-1) and 14 May, 2010 and 31 July, 2013 (Nipponbare and sgr-2). The seedlings were planted in rows spaced 0.30 m apart with a 0.15 m spacing between the seedlings. In 2010, fertilizer was applied at basal dressing rates of 40 kg N ha⁻¹ [as (NH₄)₂SO₄], 120 kg P ha⁻¹ (as CaHPO₄) and 40 kg K ha⁻¹ (as KCl), and an additional 40 kg N ha⁻¹ was applied on 16 July, 2010. In 2013, the same cultivars were grown in the paddy field using the same methods but with slow-release NPK fertilizer (100/100L 80%/20%, Komeso Ippatsu, MC Ferticom Co. Tokyo) applied at transplanting at concentrations of 100 kg ha⁻¹ of each element. Eighty percent of nitrogen in the fertilizer is released starting around 50 days after application and 20% is released just after application.

Variation of the stay-green trait was assessed by incubating leaf segments of the Japanese rice collection (JRC) (69 cultivars) that covers 95% of simple sequence repeats (SSR) in rice (Kojima et al., 2005; NIAS 2009). Six three-week-old seedlings for each cultivar, one plant per hill, were transplanted in rows spaced 0.30 m apart with a 0.15 m spacing between the seedlings on 12 May, 2009. Fertilizer was applied at basal dressing rates of 40 kg N ha⁻¹ [as (NH₄)₂SO₄], 120 kg P ha⁻¹ (as CaHPO₄) and 10 kg K ha⁻¹ (as KCl). An additional 10 kg N ha⁻¹ [as (NH₄)₂SO₄] was applied every two weeks until heading. The heading date was recorded. Twenty three cultivars that headed before 12 August were selected as early-ripening cultivars (Table 1). The cultivars with a heading date between 18 August and 10 September were excluded from the measurements as mid-late ripening cultivars. To observe leaf color during the mid-grain-filling period, seedlings of 13 cultivars randomly selected from the early-ripening JRC cultivars were transplanted into a pipe with a diameter of 0.06 m and a height of 0.17 m in compacted rice-seedling soil (Green soil, Green Epoch, Shimane, Japan) with N [0.1 g as (NH₄)₂SO₄], P (0.25 g as CaHPO₄), and K (0.1 g as KCl) fertilizer applied as a basal dressing at the time of transplanting. Plants were grown under flooded conditions in water pans under outdoor conditions.

The leaf color and photosynthetic rate were observed during the grain-filling period in Koshihikari, nycl-1, Nipponbare, sgr-2 and seven early-ripening JRC cultivars. Twenty seeds of Hiyadachiine, Akage, Hosogara, Hinode, Imanishiki, Karahoshi and Himenomochi cultivars that germinated from 17 to 19 April, 2011 and twenty seeds of Koshihikari, nycl-1, Nipponbare, sgr-2, Himenomochi, Hosogara and Hinode cultivars that germinated from 27 April to 4 May, 2012 were sown in 4 liter pots with compacted rice-seedling soil and only main culms were grown (Satake, 1972). Fertilizer was applied at basal dressing rates of 1 g N pot⁻¹ [as (NH₄)₂SO₄], 2.5 g P pot⁻¹ (as CaHPO₄) and 1 g K pot⁻¹ (as KCl). An additional 1 g N pot⁻¹ [as (NH₄)₂SO₄] was topdressed twenty days before heading and at full heading.

2. Temperature treatments

A chamber system was used to increase air temperature.
The chamber was constructed using steel frames that measured 0.6 by 1.5 m and 1.5 m tall. The frames were covered with high clear polyester sheets (Sixlight, Mitsubishi Plastics Agri Dream Co., Tokyo) (Kobata and Uemuki, 2004). Ambient air was introduced through a heat exchanger (VL-150KP, Mitsubishi co. Tokyo) at a rate of 0.04 m$^3$ s$^{-1}$ for 24 h and hence inside air was exchanged 1.8 times per min. The inside temperature was elevated using radiation and a fan heater with an electric temperature controller (SF-1013A, Sowa co. Saitama, Japan). The heater was switched on when the inside temperature dropped below 30ºC. Inside air was ventilated using a 0.08-m diameter fan attached to the upper part of the chamber when the air temperature exceeded 35ºC. A 0.10-m diameter fan was suspended near the height of the panicles to circulate inside air. Slit plastic curtains located on both sides were opened for measurements of leaf color and photosynthetic rate. In ambient plots, the top surface of the frame was covered with polymer sheets to simulate the reduced light intensity inside the temperature-controlled chamber. Temperature treatments were started at 10 July in 2011 and 12 July in 2012 until the end of September when all cultivars attained maturity. Three chambers were used as replications in a completely randomized design consisting of ambient and elevated temperature conditions. Inside and outside chamber temperatures were logged every 30 min using a data logger with a ventilated fan.

Table 1. SPAD values before incubation (at heading) (B) and after incubation for 7 days (A), and the ratio of after versus before incubation (A/B) of the SPAD values of leaf segments from early-ripening cultivars in the Japanese Rice Collection (JRC). A leaf segment approximately 0.05 m long was sectioned from the center of flag leaves, placed into plastic vials (Figure 1) and incubated under dark conditions at a temperature of 35ºC. * indicate cultivars used in the SPAD observations in the pot experiment (Figure 6).

| Japanese rice collection | Heading date | Day | A/B |
|--------------------------|--------------|-----|-----|
| Cultivar                 | NIAS ID      | 0   | 7   |     |
| Early heading type       |              |     |     |     |
| Iruma nishiki            | JRC 07*      | 28 July | 48.4 | 46.2 | 0.96 |
| Sekiyama                 | JRC 36       | 20 July | 47.6 | 42.5 | 0.89 |
| Fukoku                   | JRC 46       | 12 July | 44.2 | 40.5 | 0.91 |
| Hirayama                 | JRC 10       | 12 August | 45.6 | 40.4 | 0.89 |
| Hiyadaitou               | JRC 45       | 21 July | 46.6 | 40.3 | 0.87 |
| Morita wase              | JRC 30*      | 25 July | 40.6 | 38.2 | 0.94 |
| Hinoe                    | JRC 03*      | 28 July | 47.4 | 38.1 | 0.80 |
| Himenomochi              | JRC 50*      | 23 July | 39.9 | 36.3 | 0.91 |
| Shinshiru                | JRC 51*      | 28 July | 39.0 | 35.0 | 0.90 |
| Mansaku                  | JRC 22*      | 30 July | 47.0 | 33.4 | 0.71 |
| Rikutou rikun 2          | JRC 49       | 23 July | 42.8 | 32.6 | 0.76 |
| Okka modoshi             | JRC 08*      | 12 August | 40.5 | 31.5 | 0.78 |
| Hosogara                 | JRC 20       | 18 July | 46.5 | 31.2 | 0.67 |
| Yamada bake              | JRC 05*      | 12 August | 41.7 | 30.9 | 0.74 |
| Nagoya shiro             | JRC 38*      | 30 July | 40.1 | 26.7 | 0.66 |
| Akage                    | JRC 17       | 12 July | 43.1 | 22.2 | 0.51 |
| Kaneko b                 | JRC 06*      | 28 July | 43.1 | 20.1 | 0.47 |
| Karahoushi               | JRC 44*      | 28 July | 39.1 | 20.0 | 0.51 |
| Gaisen mochi             | JRC 01*      | 12 August | 24.3 | 16.6 | 0.68 |
| Hasokuhu                 | JRC 18       | 12 August | 23.8 | 16.5 | 0.69 |
| Bouzu mochi              | JRC 13*      | 30 July | 25.1 | 11.9 | 0.47 |
| Kahei                    | JRC 11       | 12 August | 20.9 | 11.6 | 0.56 |
| Oiran                    | JRC 12       | 12 August | 22.8 | 9.8  | 0.43 |
| Average                  |              |     |     |     |     |
| cv                       |              | 0.23 | 0.38 | 0.23 |
| LSD$_{95\%}$             |              | 8.9  | 21.2 | –   |

Data are the mean of 3 to 4 replicates. LSD was determined using the Tukey test ($p<0.05$).
3. Leaf incubation

(1) Whole-leaf incubation

Whole leaves were incubated at five temperatures under dark conditions for 60 days. Flag leaves after heading were harvested from the field, sealed in plastic bags and transported to the laboratory. Six leaves were floated on distilled water in plastic trays measuring 0.310 × 0.225 × 0.057 m and covered with a transparent cover. The trays were placed into a multiple-temperature-controlled incubator (TG-180-5L, Nippon Ika Co., Osaka, Japan) and temperatures of 20, 25, 30, 35 and 40°C were set. The temperature in each partition was measured and logged at 30-min intervals using a temperature logger. The observed temperatures were 21.4 ± 0.3°C (T20), 25.3 ± 0.9°C (T25), 30.1 ± 0.4°C (T30), 35.1 ± 0.3°C (T35) and 39.2 ± 0.3°C (T40) (average ± sd).

(2) Leaf-segment incubation

An alternative method that does not require large incubation spaces was tested. Sections approximately 0.05 m long were cut from the centers of the leaves and incubated in a watertight 0.06-L vial (New sample vial, NO. 7, Asone, Osaka, Japan). Incubation in a vial can save space in an incubator and many samples can be observed simultaneously. The vials had a 5-mm hole in the cap that was offset from the center position to facilitate the exchange of air (Fig. 1). Air exchange is important for leaf senescence processes because reduced \( \text{O}_2 \) levels inhibit ethylene biosynthesis, which is one of the dominant factors of senescence in detached plant organs (Zagory and Kader, 1988). The vials were laid on the floor and filled with distilled water up to the level of the hole. Four leaf tips per cultivar were floated on the water. The vials were incubated at 35°C in a single controlled incubator or at 20 – 40°C in a multiple-temperature-controlled incubator.

4. Measurements

(1) Leaf color and green leaf area

The leaf color was determined using a portable chlorophyll meter (SPAD-502, Minolta Co., Ltd., Tokyo). In the whole leaf incubation experiment, leaf color was determined at three positions along each leaf blade, whereas in the leaf segment incubation experiment, the leaf color of the central position of the leaf segment was assessed. The SPAD meter provides convenient and rapid estimates of the chlorophyll content (Inada, 1965), although the relationship between chlorophyll contents and SPAD values is exponential (Markwell et al., 1995). In addition, the relationship between gas exchange or chlorophyll fluorescence and SPAD values was weaker compared with the Rubisco content of rice leaves (Kumagai et al., 2009). In the whole leaf incubation, leaf color was maintained for long periods of time, but in the leaf segment incubation, loss of leaf color occurred rapidly.

The SPAD value was measured at the start of incubation and every 3 days for a period of 60 days for the whole leaf incubation and at 0 and 7 or 8 days for the leaf segment incubation.

The green leaf area (GLA) for whole-leaf incubation was determined as follows:

\[
\text{GLA} = \text{GLA}_0 \times (1 - \text{SLAR} / 100)
\]

where \( \text{GLA}_0 \) is GLA at heading measured using a leaf area meter (CI-203 area meter, CID, Inc., Vancouver, WA, USA) and \( \text{SLAR} \) is the percentage of senesced leaf area of each leaf. Senesced leaf area is defined as the area of a leaf that is occupied by yellow or white portions caused by degradation of the chlorophyll pigment. SLAR of each leaf was visually scored on a scale of 0 to 9 where leaves with an SLAR of 0 to 10%, were given a score of 0 and leaves with an SLAR of 90 to 100% were given a score of 9 (Hoang and Kobata, 2009a, 2009b).

The SPAD values and green leaf areas of 6 flag leaves from the whole-leaf incubation or 3 – 4 flag leaves from the leaf-section incubation were averaged. Using 13 early-ripening JRC cultivars grown in pots, the SPAD value of the top three leaves at the mid-grain-filling period was measured. Under ambient and elevated temperature conditions the SPAD values of flag leaves of Koshihikari, ryd-1, Nipponbare, sgr-2 and seven JRC early-ripening cultivars were monitored weekly during the post-anthesis period.
differences in the slope and intercept of the regression lines were tested using an analysis of covariance.

Results

1. Detection of the stay-green trait using the leaf incubation methods

Whole flag leaves of Koshihikari, nyc-1, Nipponbare, sgr-2 and 7 early-ripening JRC cultivars, the photosynthetic rate at 11:00 – 15:00 was measured at the center of a flag leaf every week after full heading using a portable photosynthesis measurement system with an LED radiation unit (LCcrop+Portable, ADC BioScientific Ltd. UK). The light intensity and temperature inside the chamber were set at 1300 μmol m⁻² s⁻¹ and 30°C, respectively. In these cultivars, the inflection point occurred at approximately 900 μmol m⁻² s⁻¹, beyond which the photosynthetic rate increased very slowly (Yabuta and Kawamitsu, unpublished) and the photosynthetic rate was approximately 95% of the rate measured at 1800 μmol m⁻² s⁻¹. Ambient air was introduced from a buffer chamber with a volume of 0.038 m⁻³ connected to an air inlet at a height of 4 m above the ground where the CO₂ (390 – 400 μmol m⁻³) and humidity (60 – 70%) of the inlet air were not controlled. Although leaf photosynthesis was measured at 30°C in ambient and elevated chambers, the photosynthetic rate of rice scarcely changes over a 30 – 35°C range (Nagai, and Makino, 2009), which approximates our experimental conditions.

5. Statistical Analysis

An ANOVA was used in conjunction with Tukey’s LSD to test for significant differences. The correlation coefficient (r) and probability (p) were calculated to evaluate the relationship between two parameters. The least-squares method was used to fit the regression lines and the coefficient of determination (R²) was calculated to assess the goodness of fit in the regression analysis. Significant differences in the slope and intercept of the regression lines were tested using an analysis of covariance.
incubation series, and these were compared with the SPAD values (Fig. 3). In Koshihikari and Nipponbare (bold lines), the slopes of the regression line at 40°C (T40) (bold dotted lines), determined using an analysis of covariance, were significantly \( p < 0.05 \) slower than at 20 – 35°C (T20 – T35) (solid). However, in nyc-1 and sgr-2 (narrow lines), the difference between T40 (dotted) and T20 – T35 (solid) was small, and was significant only in the intercept in sgr-2. In the T20 – T35 range, the slopes of the regression lines between the SPAD values and the cumulative temperatures for nyc-1 and sgr-2, were significantly \( p < 0.05 \) more gentle and had lower intercepts compared with their respective wild types, Koshihikari and Nipponbare. At T40, a significant difference \( (p < 0.05) \) was observed only between the slopes of sgr-2 and Nipponbare. Thus, a decrease in the SPAD value of whole leaves was temperature dependent over an incubation temperature range of 20 – 35°C, whereas the responses at 40°C were markedly different from those at 20 – 35°C.

The incubation method using a short time period and a small sample size, was examined by incubating the central parts of the flag, second and third leaves of nyc-1 and sgr-2 and each wild type, Koshihikari and Nipponbare, in vials at 35°C for 7 days under dark conditions (Fig. 1). Similar SPAD values were recorded for both the wild type and mutant genotypes at the start of the incubation period. However, after the leaf sections were incubated, the SPAD values of all nyc-1 and sgr-2 leaves were significantly higher compared with their respective wild types (Fig. 4). There was a significant correlation between the SPAD values of the leaves in the second or third positions and those of the flag leaves \( (y = 0.96x, R^2 = 0.989, p < 0.001) \). Therefore, trends in the SPAD values of the flag and two lower leaves would likely be similar to those after incubation.

The variation in green color maintenance was examined using flag leaf sections at heading were collected from 23 early-ripening (heading before mid-August) JRC cultivars and incubated at 35°C under dark conditions (Table 1). Prior to incubation, the average SPAD value of all the cultivars was 39.1 compared with 29.2 after a 7-day
incubation period. The SPAD values measured before incubation ranged from 20.9 in Kahei to 48.4 in Iruma nishiki and decreased to values ranging from 9.8 in Oiran to 46.2 in Iruma nishiki after incubation for 7 days (Table 1). The ratio of the SPAD values measured after versus before incubation (A / B), ranged from 0.43 in Oiran to 0.96 in Iruma nishiki. There were highly significant relationships between the SPAD values measured before and after incubation (\( r = 0.846, p < 0.001 \)) and between the SPAD values measured after incubation and the A / B value (\( r = 0.896, p < 0.001 \)). Furthermore, leaf segments from six early-ripening JRC cultivars that the SPAD values at heading was relatively higher-ranked (39 – 47) (Table 1) exposed to different temperatures (20, 25, 30, 35 and 40ºC) under dark conditions for eight days (Fig. 5). The differences in the SPAD values between cultivars after incubation (Table 1) were maintained at the temperature extremes. At 20ºC, however, the SPAD value of one cultivar was lower compared with the other temperatures, and at 40ºC, the SPAD values of most cultivars were higher than at 35ºC.

For the thirteen early-ripening JRC cultivars, there was a significant correlation between the average SPAD values of the three upper leaves in the potted plants measured at the mid-grain-filling period (19 ± 2 days after heading, the mean of observed cultivars ± se), and them of the flag leaf segments measured after incubation (Table 1) (\( r = 0.774, p < 0.005 \)) (Fig. 6a). Furthermore, there was a significant correlation between the SPAD value at the mid-grain-filling period / at the heading in the potted plants and the A / B of incubated flag leaf segments (Fig. 6b). The average temperatures in the potted plants were maintained within a similar range (29.6 ± 1.2ºC, mean and sd) for 20 days after heading. Therefore, early-ripening cultivars maintaining higher absolute and relative SPAD values in the incubation test were able to keep higher values at mid-grain-filling period.

2. Examination of stay-green trait under elevated temperature conditions

For all genotypes, the average daily temperature in the elevated temperature chamber was 3 – 4ºC higher than ambient conditions after heading (Fig. 7). This increase in temperature primarily depended on an increase in daytime temperature, and the maximum temperature at the midday occasionally reached around 40ºC.

From 0 to 30 days after heading for Koshikari, nry1-1, Nipponbare and sgr-2, the average temperature was maintained at 3.1 – 3.3ºC higher than ambient conditions.
The SPAD values were considerably higher in *nyc*1-1 and *sgr*-2 than in the wild types under both ambient and elevated conditions during the grain-filling period (Fig. 8). The photosynthetic rates measured within 7 days after heading, were higher in *nyc*1-1 and *sgr*-2 than in the wild types but then decreased to similar levels under ambient and elevated conditions within two weeks after heading (Fig. 8). Under elevated temperature conditions, the SPAD values were similar to those at ambient temperatures, but the photosynthetic rate in both genotypes was lower.

The A/B and SPAD values after incubation in seven early-ripening JRC cultivars used for the elevation temperature treatments widely varied (Table 1). The A/B and SPAD values in Iruma nishiki, Hiyadachitou, Hinode and Himenomochi were higher-ranked, those in Hosogara were intermediate-ranked; and those in Akage and Karahoushi lower-ranked. In Iruma nishiki, Hinode, Hosogara and Himenomochi, the SPAD values was maintained higher than in Akage and Karahoushi under ambient temperature conditions during the grain-filling period (Fig. 9a). There were large variations in the SPAD values in Hinode and Hosgara. Hosogara displayed a relatively high SPAD value after heading, although it was an intermediate-ranked cultivar from the incubation test (Table 1). Higher photosynthetic rates were maintained after heading in Iruma nishiki, Hiyadachitou, Hinode, Hosogara and Himenomochi compared with Akage and Karahoushi under ambient temperature conditions (Fig. 9b). In the temperature-controlled chamber, the average temperature was maintained at 3.1 – 3.6ºC higher than ambient conditions for 30 days after heading in all cultivars (Fig. 7). To clarify the effect of the elevated treatment on the SPAD values, the relationship in daily trends of SPAD
values during the grain-filling period between elevated and ambient temperature conditions was examined (Fig. 10a). The SPAD values of most of the cultivars grown under elevated temperature conditions distributed over the \( y = x \) line: hence the decrease in the SPAD values under elevated temperature conditions was smaller than under ambient conditions (Fig. 10a). In the relationship in daily trends between elevated and ambient conditions, the photosynthetic rates under elevated conditions were distributed below the \( y = x \) line; thus, a decrease in the photosynthetic rates under elevated conditions were greater than under ambient conditions (Fig. 10b).

At 20 – 21 days after heading, the average SPAD value and photosynthetic rate in these cultivars were calculated and compared (Table 2). In 2011, the average SPAD values in Himenomochi, Hinode, Hosogara, Iruma nishiki and Hiyadachitou were significantly higher than those in Karahoshi and Akage under both ambient and elevated temperature conditions (Table 2). The averages across ambient and elevated temperature conditions in Himenomochi, Hinode and Hosogara were significantly higher than those in Iruma nishiki and Hiyadachitou, and the averages in Karahoshi and Akage were significantly lowest. In 2012, the average SPAD value in Hinode was significantly lower than in other cultivars under ambient conditions, and that in Himenomochi was significantly higher than in Akage under elevated temperature conditions. The averages across ambient and elevated temperature conditions in Himenomochi and Hosogara were significantly higher than those in Hinode and Akage. In 2012, the average SPAD value in these four cultivars under both temperature conditions (20.6) was much lower than in 2011 (34.8). Hosogara showed a relatively high average SPAD value, although the SPAD value in the incubation test was intermediate (Table 1). The elevated temperature conditions significantly increased the average SPAD values shown by the ratio of elevated to ambient SPAD values (1.0 – 1.5) in both years (Table 2). Furthermore, there was a significant correlation in the average of the SPAD values, including data under ambient and elevated temperature conditions (Table 2), and the SPAD values measured after the incubation of leaf
Table 2. Average SPAD values and photosynthetic rates of early ripening JRC cultivars measured 20 – 21 days after heading under ambient and elevated temperature conditions in the post-anthesis period. The average temperature in elevated temperature conditions was by 3.2 – 4.2°C higher than the ambient. Temperature was elevated using a plastic covered chamber.

| Factor          | 2011 SPAD Value | 2012 SPAD Value | 2011 Photosynthesis Rate | 2012 Photosynthesis Rate |
|-----------------|-----------------|-----------------|--------------------------|--------------------------|
|                 | Ambient | Elevated | Average | Elevated / Ambient | Ambient | Elevated | Average | Elevated / Ambient | Ambient | Elevated | Average | Elevated / Ambient |
| Cultivar        |         |         |         |                  |         |         |         |                  |         |         |         |                  |
| Himenomochi     | 38.7 a   | 46.6 a   | 42.6 a  | 1.2              | 19.3 a   | 26.1 a   | 22.7 a  | 1.4              | 6.3 bc  | 7.6 a   | 7.0 b   | 1.2              |
| Hinode          | 40.5 a   | 39.6 ab  | 40.0 a  | 1.0              | 15.5 b   | 22.9 ab  | 19.2 b  | 1.5              | 12.5 a  | 11.1 a  | 11.8 a  | 0.9              |
| Hosogara        | 38.0 a   | 41.6 ab  | 39.8 a  | 1.1              | 19.9 a   | 22.8 ab  | 21.3 a  | 1.1              | 9.2 ab  | 6.9 a   | 8.0 b   | 0.8              |
| Iruma nishiki   | 32.5 a   | 34.9 b   | 33.7 b  | 1.1              | –        | –        | –        |                  | 9.2 ab  | 10.7 a  | 9.9 ab  | 1.2              |
| Hiyadachitou    | 31.6 a   | 31.3 b   | 31.4 b  | 1.0              | –        | –        | –        |                  | 8.9 ab  | 7.5 a   | 8.2 b   | 0.8              |
| Karahoushi      | 21.3 b   | 22.7 c   | 22.0 c  | 1.1              | –        | –        | –        |                  | –       | –       | –       | –                |
| Akage           | 15.2 b   | 18.2 c   | 16.7 c  | 1.2              | 18.6 a   | 20.0 b   | 19.3 b  | 1.1              | 2.6 c   | 1.9 b   | 2.3 c   | 0.8              |
| Average         | 31.1     | 33.6     | 32.3    | 1.1              | 18.3     | 22.9     | 20.6    | 1.3              | 8.1     | 7.6     | 7.9     | 0.9              |
| LSD0.05         | 9.0      | 8.6      | 5.7     | –                | 3.1      | 5.8      | 2.0     | –                | 4.5     | 5.2     | 3.1     | –                |

| Temperature     |         |         |         |                  |         |         |         |                  |         |         |         |                  |
|                 |         |         |         |                  |         |         |         |                  |         |         |         |                  |
| Cultivar        | ****    | ***     | ****    | ****             | ***     | ****    | ****    | ****             | ****    | ****    | ****    | ****             |
| Temperature     | *        | ****    | ns      | ****             | ns      | ****    | ns      | ****             | ns      | ****    | ns      | ****             |
| Cultivar × Temperature | ns | * | ns | **** |

Each data point is the average of three replicates. Within columns, means followed by the same letter are not significantly different at the 0.05 level of probability by Tukey test. *, ***, and **** indicate significant differences at 0.05, 0.005 and 0.0001 probability levels, respectively; ns is non-significant.
conditions in Hinode was the significantly highest, and those in Himenomochi, Hosogara, Iruma nishiki and Hiyadachitou were significantly higher than that in Akage. In 2012, the average photosynthetic rate both under ambient and elevated temperature conditions in Himenomochi, Hinode and Hosogara was significantly higher than in Akage (Table 2). The average of photosynthetic rates across ambient and elevated temperature conditions in Hinode was the significantly highest, and those in Himenomochi and Hosogara was significantly higher than in Akage (Table 2). Overall, the elevated temperature lowered the photosynthetic rate in both years (0.7 – 0.9), although the effect of temperature on photosynthesis was significant only in 2012 (Table 2). The interaction between cultivars and temperature was only significant in 2012. There was a stronger correlation between the photosynthesis values measured under ambient and elevated temperatures over the two years ($r = 0.843$, $p < 0.005$); therefore, photosynthetic rates under elevated temperatures strongly reflected photosynthesis under ambient conditions. There was a significant correlation between the average of photosynthetic rates during 20 – 21 days after heading and the relative value (the average / at heading), where data points in Himenomochi, Hinode, Hiyadachito and Iruma nishiki distributed in the higher range than in Akage (Fig. 11b); thus, the lower reduction of photosynthetic rates during the active-grain-filling period were approximately reflected in the higher rates.

**Discussion**

The senescence of excised rice leaves is promoted by ethylene production (Kao and Yang, 1983). However, extremely high temperatures (35 – 40°C) inhibit ACC synthase (EC 4.4.1.14), which produces a precursor of ethylene, and ethylene-forming enzyme activity declines in tomato pericarp tissue under high temperatures (Biggs et al., 1988). In our incubation test for Koshihikari and Nipponbare, the insensitivity of leaves to color loss at 40°C likely resulted from a reduction of ethylene production caused by the high temperature. Therefore, a temperature range of 20 – 35°C could be used to determine the ability of different genotypes to maintain leaf color. At 20°C, however, considerable decreases were observed in the SPAD values in some cultivars. Moreover, the incubation range of 25 – 35°C is similar to the temperatures that rice plants are subjected to in midsummer (Terashima et al., 2001; JMA, 2014).

In early ripening JRC cultivars, there was a significant correlation between the SPAD values measured after incubation of the leaf segments and the SPAD values measured in the upper three leaves of standing plants during mid-grain-filling period (Fig. 6). This suggests that the SPAD values measured after the incubation of flag leaf segments provide a good estimate of the SPAD values of the attached dominant leaves during the active grain-filling period, if the temperature range during the measurement of SPAD value
was relatively stable. Furthermore, there was a positive correlation between the SPAD values measured in these cultivars after and before incubation, and between the SPAD value measured after incubation and the A/B values (Table 1). Both the ability to maintain high levels of green color in the leaves after heading and high SPAD values at the time of heading (as a basis for assimilation capacity) (Takai et al., 2010) would be essential for high assimilation of the stay-green trait. However, the leaf incubation test cannot reflect the effect of leaf nutrient use by a sink (e.g., grain) on leaf color. Sinks, such as the grains of standing plants, may alter the relationship, because the leaf color maintenance in attached leaves is influenced by grain growth, which removes assimilate and nitrogen from leaves (Borrell et al., 2000b), in contrast to leaf segments. Therefore, the incubation method should be considered a procedure to exclude genotypes with a low rating for the stay-green trait and to select candidates with stay-green genotypes.

Elevated temperature significantly increased the SPAD values measured after heading (Table 2 and Fig. 10). Absorption of soil nitrogen by rice increases under high temperature conditions (Takahashi et al., 1976); thus, leaf nitrogen levels would also increase under elevated temperature conditions, resulting in the maintenance of higher SPAD values. Previous studies suggest that high temperatures do not accelerate leaf senescence in the early to mid-grain-filling period in rice (Kim et al., 2011). However, photosynthetic rates decreased under elevated temperature conditions. In rice, leaf photosynthesis increased from 22 to 32°C and then decreased to 42°C, where photosystem II (PSII) activity declined steadily in protoplasts, chloroplasts, and thylakoids of rice from 22 to 42°C (Al-Khatib and Paulsen, 1999). As a result, cultivar differences in the SPAD values and photosynthetic rates that are indistinguishable at ambient temperatures may be evident at elevated temperatures.

Although the stay-green mutants (nyc1-1 and sgr-2) maintained higher SPAD values for longer time periods after heading, the photosynthetic rates were not maintained during the active grain-filling period from 1 to 3 weeks after heading under ambient and elevated temperature conditions. Hence, the stay green trait in nyc1-1 and sgr-2 is considered non-functional in a broad sense (Spano et al., 2003; Kusaba et al., 2007). Hence, the incubation method can select candidates of stay-green genotypes of long-keeping green leaf color but not promise selections of functional stay-green.

The early-ripening JRC cultivars, Iruma nishiki, Hinode and Himenomochi, which exhibited higher SPAD values after incubation and A/B (Table 1 and Fig. 11), maintained higher SPAD values and photosynthetic rates during the grain-filling period in the pot experiments (Tables 1 and 2) and were, therefore, considered to be candidates of the functional stay-green cultivar, whereas Akage was considered non-stay green. The cultivar difference in SPAD values during the grain-filling period was clearly observed in 2011 when the SPAD values were much higher. Although the reason for the differences in SPAD values between two years was unknown, the higher temperature (1.8°C on the average) in 2012 may have accelerated soil reduction in pots and limited nitrogen absorption (Takai and Kamura, 1966), resulting in the lower SPAD values. The stay-green trait of rice is expressed under higher nitrogen absorption conditions (Hoang and Kobata 2009a, 2000b; Kobata and Hoang, 2010). Therefore, growth and cultivation conditions relating to nitrogen absorption should be carefully prepared to select for the stay-green trait.

**Conclusion**

Determination of the SPAD values of flag leaves using the leaf segment incubation method allows for convenient evaluation of the stay-green trait associated with assimilation capacity at elevated temperature conditions during the grain-filling period in rice, provided incubation and plant growth conditions are appropriately controlled. The results from this study suggest that this method is suitable for the first-step screening of the stay-green trait to mitigate the impact of high temperatures during the post-anthesis period on grain production in rice.

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