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High mesophyll conductance in the high-yielding rice cultivar Takanari quantified with the combined gas exchange and chlorophyll fluorescence measurements under free-air CO₂ enrichment

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ABSTRACT

An effective strategy for increasing crop production is increasing the rate of photosynthesis. In this study, we conducted gas exchange and chlorophyll fluorescence measurements for a high-yielding rice cultivar, Takanari, to identify the leaf physiological properties that contribute to high capacity for photosynthesis of the uppermost leaves before (panicle initiation stage) and after heading (grain-filling stage) in the Tsukuba free-air CO₂ enrichment (FACE) facility. The higher photosynthesis rate of Takanari compared with that of the commonly cultivated cultivar, Koshihikari, was mainly attributed to the greater stomatal conductance for CO₂ (g_{sc}) at the panicle initiation stage and to the greater mesophyll conductance (g_m) at the grain-filling stage in both current and elevated atmospheric CO₂ concentrations [CO₂]. Takanari had a higher level of leaf nitrogen content (N_l) compared with Koshihikari at the grain-filling stage, which led to greater g_m and maximum carboxylation rate ($V_{c,max}$), but N_l alone did not explain the variations of g_m within the variety. A clear correlation was found between $V_{c,max}$ and N_l . Calculating $V_{c,max}$ taking g_m into consideration removed the artifact of $V_{c,max25}$ in relation to N_l that was observed when g_m was assumed to be infinite. Our results emphasize the need to separate the roles of $V_{c,max}$ and g_m to accurately understand the ecophysiological processes that control leaf photosynthesis in Takanari.

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Introduction

In the face of the world's growing population, global crop production needs to be increased. Increasing the

rate of photosynthesis is one strategy to increase crop production (Hubbart, Peng, Horton, Chen & Murchie, 2007; Long, Zhu, Naidu & Ort, 2006; Mann, 1999;

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Murchie, Pinto & Horton, 2009; Ort et al., 2015). Investigations have been conducted to understand the ecophysiological traits of available crop cultivars that support high rates of photosynthesis in order to maximize the use of available genetic resources.

High mesophyll conductance (g_m) supports a high rate of leaf photosynthesis. However, due to the difficulty of accurately quantifying g_m , the effect of mesophyll conductance is often neglected in terrestrial ecosystem models that investigate crop photosynthesis at a canopy scale (Ikawa et al., 2018). Researchers often use maximum carboxylation rate ($V_{c,max}$) based on intercellular CO_2 concentration (C_i) instead of on actual CO_2 concentration at the carboxylation site (C_c), assuming that g_m is infinite and, therefore, C_c is equal to C_i . Such an assumption leads to an underestimation of $V_{c,max}$ (apparent $V_{c,max}$) and overestimation of C_c . However, a growing number of studies have suggested that the impact of g_m on photosynthesis is not trivial, highlighting the need to disentangle the roles of $V_{c,max}$ and g_m in photosynthesis (Adachi et al., 2013; Knauer et al., 2019; Lauteri, Haworth, Serraj, Monteverti & Centritto, 2014). Furthermore, Sun et al. (2014) demonstrated that photosynthesis models based on the apparent $V_{c,max}$ assuming infinite g_m are accurate only within the limited conditions in which the $A-C_i$ curve was observed.

Takanari (*Oryza sativa* L. cv. Takanari) is known for its high grain yield (Imbe et al., 2004). The Tsukuba rice free-air CO_2 enrichment (FACE) experiment revealed that both high sink (i.e. large panicles) and source (i.e. high carbon supply) capacities of Takanari (Chen et al., 2014; Ikawa et al., 2018; Nakano et al., 2017) contribute to the greater grain yield with less loss in the quality in elevated atmospheric CO_2 concentration [CO_2] compared to a commonly grown cultivar, Koshihikari (Hasegawa et al.,

2013, 2019; Zhang et al., 2013, 2015). Based on a canopy scale model, Ikawa et al. (2018) attributed Takanari's high canopy photosynthesis to greater stomatal conductance (g_{sc} for CO_2) and a better nitrogen allocation compared to Koshihikari. However, their model did not explicitly consider the effect of g_m . Chen et al. (2014) reported that the high leaf photosynthesis of Takanari was supported mainly by high g_m in later growth stage. However, the method to quantify g_m used in Chen et al. (2014) depends on the curve-fitting method, which is not necessarily recommendable if any alternative method is available (Pons et al., 2009).

Recent advancements in gas exchange measurement systems enable a wide range of users to quantify g_m more reliably and easily. Using a newly-released photosynthesis system (LI-6800, LICOR, USA), this study aims to redefine the physiological traits of Takanari and determine why Takanari has a high rate of leaf photosynthesis. We set three hypotheses: (1) Takanari has greater $V_{c,max}$, g_m , and g_{sc} than Koshihikari both in current and elevated [CO_2]; (2) the difference in g_m between [CO_2] treatment and variety is greater than that of either $V_{c,max}$ or g_{sc} with respect to their effects on leaf photosynthesis; and (3) Takanari's high $V_{c,max}$ and g_m are explained by high leaf nitrogen contents. The rationale for the first two hypotheses is based on the results of the study by Chen et al. (2014). The third hypothesis is based on the fact that the uppermost leaves of Takanari have a high nitrogen content (Taylaran, Adachi, Ookawa, Usuda & Hirasawa, 2011), and also on recent studies reporting that the $V_{c,max}$ and g_m of rice leaves are correlated to leaf nitrogen content (Cai et al., 2018).

To test these hypotheses, the objectives of this study are: (1) to calculate the $V_{c,max}$, g_m , and g_{sc} using combined gas exchange and chlorophyll

Table 1. Measurement date, time, block, measurement order, and meteorological conditions during the combined gas exchange and fluorescence measurements in 2017. The order of the measurements was randomized for each variety (KH: Koshihikari and TN: Takanari) and for CO_2 treatment [A- CO_2 : a control plot with current (CO_2 ; $\sim 390 \mu mol mol^{-1}$) and E- CO_2 : elevated (CO_2 ; $\sim 590 \mu mol mol^{-1}$)]. Air temperature (T_a) and relative humidity (Rh) were measured at 2 m above the ground and wind speed (U) was measured at 2.5 m above the ground. PPFD (photosynthetic photon flux density) is the value on the horizontal plane.

Date	Time	Block	Measurement order	T_a ($^{\circ}C$)	Rh (%)	U (m/s)	PPFD ($\mu mol m^{-2} s^{-1}$)
<i>Panicle initiation stage</i>							
7/11	9:31–12:07	1	KH (A- CO_2) \rightarrow TN (A- CO_2) \rightarrow KH (E- CO_2) \rightarrow TN (E- CO_2)	30.0	64	3.1	1638
7/13	9:20–11:50	2	TN (A- CO_2) \rightarrow KH (A- CO_2) \rightarrow TN (E- CO_2) \rightarrow KH (E- CO_2)	30.8	69	3.3	1610
7/14	9:04–11:20	3	KH (E- CO_2) \rightarrow TN (E- CO_2) \rightarrow KH (A- CO_2) \rightarrow TN (A- CO_2)	30.4	66	2.3	1562
7/15	9:33–13:25	4	TN (E- CO_2) \rightarrow KH (E- CO_2) \rightarrow TN (A- CO_2) \rightarrow KH (A- CO_2)	30.9	64	1.0	1551
<i>Grain-filling stage</i>							
8/13	8:06–10:20	1	KH (A- CO_2) \rightarrow TN (A- CO_2) \rightarrow KH (E- CO_2) \rightarrow TN (E- CO_2)	27.0	80	0.8	896
8/18	8:12–10:43	2	TN (A- CO_2) \rightarrow KH (A- CO_2) \rightarrow TN (E- CO_2) \rightarrow KH (E- CO_2)	27.1	82	0.6	1051
8/20	7:56–10:30	3	KH (E- CO_2) \rightarrow TN (E- CO_2) \rightarrow KH (A- CO_2) \rightarrow TN (A- CO_2)	25.6	83	0.3	700
8/22	8:22–10:28	4	TN (E- CO_2) \rightarrow KH (E- CO_2) \rightarrow TN (A- CO_2) \rightarrow KH (A- CO_2)	28.6	77	0.8	956
8/24	7:55–10:27	1	KH (A- CO_2) \rightarrow TN (A- CO_2) \rightarrow KH (E- CO_2) \rightarrow TN (E- CO_2)	29.0	71	0.6	938
8/25	7:46–10:07	2	TN (A- CO_2) \rightarrow KH (A- CO_2) \rightarrow TN (E- CO_2) \rightarrow KH (E- CO_2)	30.1	72	0.5	744

fluorescence measurements for Koshihikari and Takanari both in current and elevated $[\text{CO}_2]$; (2) to conduct a sensitivity analysis of each parameter using the Farquhar, von Caemmerer, and Berry leaf photosynthesis model (Farquhar, von Caemmerer & Berry, 1980); and (3) to measure leaf nitrogen content and compare it with $V_{c,\text{max}}$ and g_m .

Materials and methods

Site descriptions

Leaf-level gas exchange measurements were conducted in the Tsukuba FACE experimental facility in Tsukubamirai, Ibaraki prefecture, Japan ($35^\circ 58' \text{ N}$, $139^\circ 60' \text{ E}$, 10 m.a.s.l.). The experimental facility includes four FACE rings (hereafter referred to as E- CO_2 plots), with a diameter of 17 m and four control blocks (A- CO_2 plots) (Nakamura et al., 2012). Rice seedlings of both Koshihikari and Takanari were transplanted in experimental plots on May 24–25 in 2017. Heading occurred about 70 and 75 days after the transplanting for Koshihikari and Takanari, respectively (Hasegawa et al., 2019). Measurements were made at panicle initiation (July 11–15) stage and at grain-filling stage (August 13–25) in 2017 (Table 1).

Combined gas exchange and chlorophyll fluorescence measurements

The uppermost expanded leaves were targeted for gas exchange and chlorophyll fluorescence measurements with the LI-6800. The heading date varied among plants, particularly in Takanari, and flag leaves with a fully-emerged panicle were selected at the grain-filling stage. Leaf samples were exposed to photosynthetic photon flux density (PPFD) at $1,500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ for 15–20 min until the steady-state condition was achieved under $[\text{CO}_2]$ of $390 \mu\text{mol mol}^{-1}$ in A- CO_2 and $590 \mu\text{mol mol}^{-1}$ in E- CO_2 . When it was overcast, samples were first exposed to PPFD at $1,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ for several minutes and PPFD was increased to $1,500 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The fractions of red and blue lights were set at 0.9 and 0.1, respectively. Gas exchange data including net leaf photosynthesis rate (A_n) and g_{sc} were then logged at the steady-state condition. Once the steady-state condition was achieved, $[\text{CO}_2]$ in the reference cell was decreased and was kept at $200 \mu\text{mol mol}^{-1}$ for several minutes. Subsequently, $[\text{CO}_2]$ was further decreased to 150, 100, 55, and $20 \mu\text{mol mol}^{-1}$, and data were logged within 150 s at each CO_2 level. The chamber temperature and humidity were set at 30°C – 35°C and 60%, respectively. Other chamber conditions were: fan speed, 10,000 rpm;

flow rate to the sample cell, $500 \mu\text{mol air s}^{-1}$; and overpressure, 0.2 kPa.

Chlorophyll fluorescence measurements were conducted at the same time as the gas exchange measurements with a multiphase flash fluorometer. Genty, Briantais and Baker (1989) first reported that the electron transport rate (J) based on the fluorometry (J_F) was linearly related to the quantum yield of photosystem II. On the basis of their theory, J_F was estimated as follows:

$$J_F = \alpha_l \beta Q (F_m' - F_s) / F_m' \quad (1)$$

where Q is PPFD and F_m' and F_s are maximum and steady-state chlorophyll fluorescence under illumination, respectively. We assumed that leaf light absorptance ($\alpha_l = 0.843$) and the fraction of electron distributed to photosystem II ($\beta = 0.5$) are constant.

The multiphase flash (MPF) method was used to estimate F_m' at an infinitely high saturation light (Loriaux et al., 2013). A saturation light of $8,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ with 25% attenuation for 0.3 s was used at each of the three phases. The reasonability of the saturation light was examined in the fluorescence data during the MPF measurements (0.9 s). For measurements with low CO_2 , the fluorescence of Koshihikari seemed to decline continuously during the third phase of MPF, but we considered that the impact on J_F was minimal (Figure S1 in Supplementary Material). The MPF method increased J_F by 20% (Figure S1 in Supplementary Material), and both J and J_F were nearly identical when measured in the laboratory under saturated light levels (data not shown). We therefore assumed actual J equals J_F based on our protocol at least under high PPFD conditions.

Calculation of $V_{c,\text{max}}$ and g_m

The variable J method was used for calculating g_m from the gas exchange and chlorophyll fluorescence measurements (Harley, Loreto, Di Marco & Sharkey, 1992) using the following equation:

$$g_m = A_n / (C_i - \{ \Gamma^* [J + 8(A_n + R_d)] / [J - 4(A_n + R_d)] \}) \quad (2)$$

where Γ^* is CO_2 compensation point at C_c and R_d is daytime mitochondrial respiration, which was determined to be half of the dark respiration rate for Takanari and Koshihikari measured in both A- CO_2 and E- CO_2 in the study by Noguchi et al. (2018). The calculation of g_m based on the variable J method is sensitive to Γ^* and R_d (Centritto, Lauteri, Monteverdi & Serraj, 2009; Pons et al., 2009). We independently performed the so-called Laik's method for plant

samples within the experimental plot between the two stages (August 4–7) for Koshihikari and Takanari and found that CO₂ compensation point based on C_i (C_i^{*}) did not statistically differ between varieties (*n* = 7). Adachi et al. (2013) also reported that Γ^{*} did not differ between rice varieties. Instead of estimating Γ^{*} from measured C_i^{*}, we decided to estimate Γ^{*} following Bernacchi, Portis, Nakano, von Caemmerer and Long (2002) because of the following reasons: The temperature function of *g_m* used in this study (Scafaro, von Caemmerer, Evans & Atwell, 2011) is also based on Bernacchi et al. (2002) and the amount of our data was too limited to identify a temperature response of Γ^{*}.

Our pilot measurements in the laboratory for rice plants under saturated light levels showed that the values of *g_m* estimated by our protocol generally agreed with the online isotope method described in Evans and von Caemmerer (2013) using the isotope measurement system employed in Nishida, Kodama, Yonemura and Hanba (2015) ([*g_m* by variable J] = 0.99 [*g_m* by online isotope] + 0.02, R² = 0.66, *n* = 34). The good comparison was achieved when Γ^{*} for ambient O₂ concentration ([O₂] = 21%) was tuned at 30 μmol mol⁻¹, which is relatively lower than the values estimated from the temperature function. Appropriate parameterization for Γ^{*} awaits further investigation.

Maximum carboxylation rate (*V_{c,max}*) was calculated by fitting the FvCB model (Farquhar et al., 1980) to A_n and C_c for [CO₂] ranging from 20–200 μmol mol⁻¹ using the *lsqcurvefit* function of MATLAB (MATLAB R2015b, MathWorks, USA). CO₂ concentration at the carboxylation site (C_c) was estimated as follows:

$$C_c = C_i - A_n/g_m \quad (3)$$

Michaelis constant for CO₂ and O₂ (*K_c* and *K_o*, respectively) required for the calculation of *V_{c,max}* were based on Bernacchi et al. (2002). The values of *V_{c,max}* and *g_m* at 25°C (*V_{c,max25}* and *g_{m25}*) were estimated based on the temperature function, as described in Bernacchi, Pimentel and Long (2003) and Scafaro et al. (2011), respectively.

We used the same unit for both *g_{sc}* and *g_m* to compare their magnitude, although the physically more appropriate unit of *g_m* is molar flux per pressure because *g_m* in liquid phase is defined as the ratio of a net photosynthesis rate and the gradient of partial pressure of CO₂ (Sharkey, Bernacchi, Farquhar & Singaas, 2007). The LI-6800 generally outputs stomatal conductance for H₂O (*g_{sw}*), and *g_{sw}* was assumed to be 1.6 times greater than *g_{sc}*.

Leaf nitrogen content measurements

Leaf samples used for gas exchange and chlorophyll fluorescence measurements were sampled and dried at 80°C. Specific leaf nitrogen concentration (*N_i*) was calculated from nitrogen concentrations measured using an NC analyzer (Sumigraph NC-22, SCAS Ltd., Japan) and the weight of the dry samples.

Effects of leaf parameters on RuBP-saturated photosynthesis rate

To quantitatively compare the effects of each leaf parameter (*V_{c,max}*, *g_m*, and *g_{sc}*) on leaf photosynthesis, the rate of RuBP-saturated photosynthesis (*A_c*) (Farquhar et al., 1980) as a proxy of leaf photosynthesis was calculated by changing one of the parameters under the same [CO₂] of 390 μmol mol⁻¹ and in similar environmental conditions as when the measurements were taken (air and leaf temperature: 30°C; relative humidity: 60%; PPFD: 1,500 μmol m⁻² s⁻¹; and leaf boundary conductance for H₂O (*g_{bw}*): 3 mol m⁻² s⁻¹). The detailed calculation procedure is explained in Appendix A.

2016 field season data

To supplement our results with information from another field season, A-C_i curves obtained at the grain-filling stage in 2016 and that were used in the study by Ikawa et al. (2018) were analyzed to obtain *V_{c,max}* and *g_m* based on the curve-fitting method (Sharkey et al., 2007). Note that Ikawa et al. (2018) in their study calculated apparent *V_{c,max}* neglecting *g_m*. The Excel tool provided in the study by Sharkey et al. (2007) was modified so that respiration rate was fixed at the same rate as that of the grain-filling stage data in 2017, and the temperature function for *g_m* of *Oryza sativa* reported in Scafaro et al. (2011) was introduced. Further details about the measurements can be found in the study by Ikawa et al. (2018).

Statistical analysis

A split-plot approach was used with variety effect nested within [CO₂] and replicate blocks (*n* = 4). An R script used in the study by Mauritz et al. (2017) and Ikawa et al. (2018) was adapted for the analysis. Measurements were taken twice at two of the blocks on different days at the grain-filling stage, and the data were treated as subsets of each block. The overall variation of the simulated *A_c* was smaller than the variation

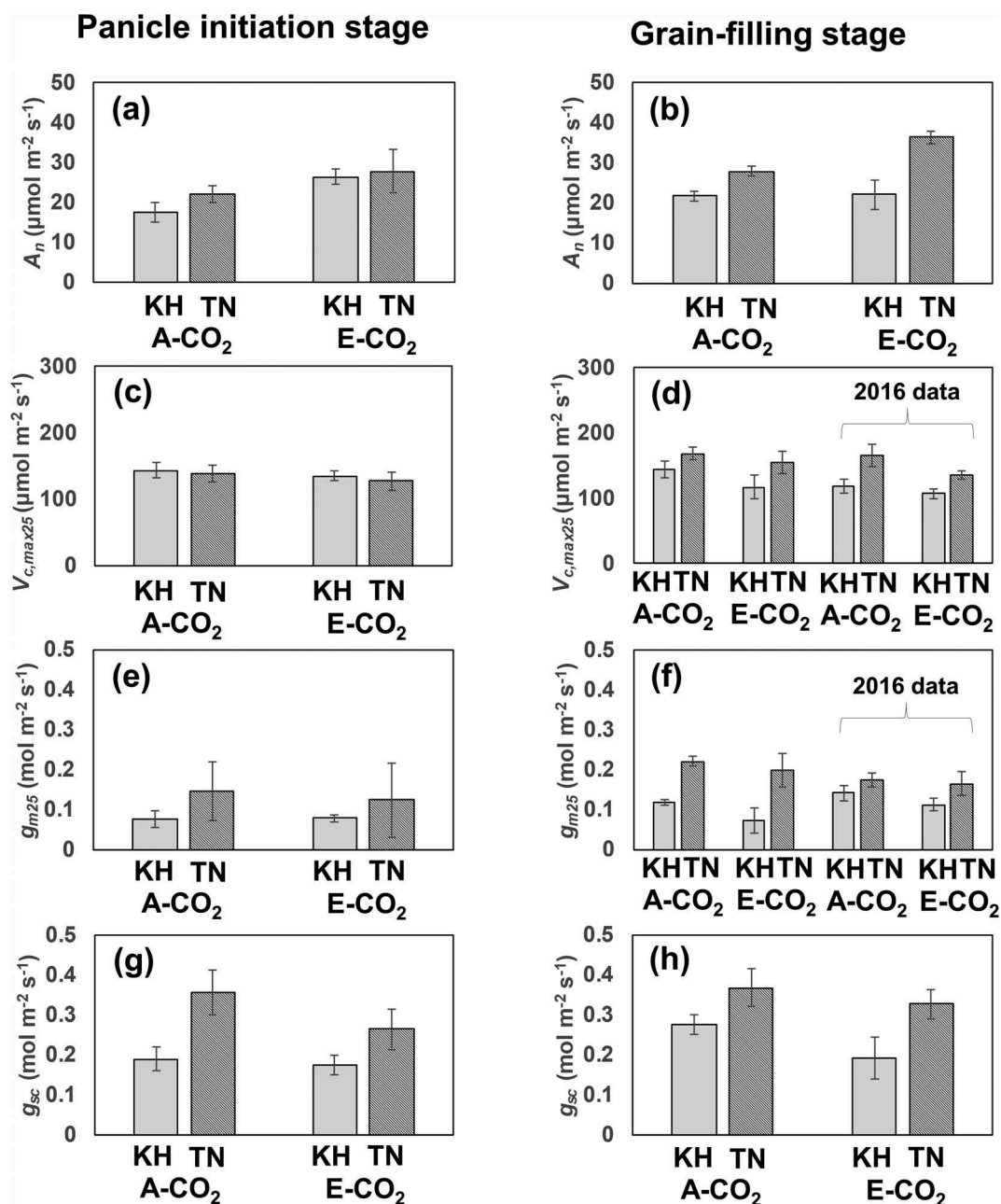


Figure 1. Net leaf photosynthesis rate (A_n) under PPF (photosynthetic photon flux density) at $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (a, b), maximum carboxylation rate at 25°C ($V_{c,max25}$) (c, d), mesophyll conductance at 25°C (g_{m25}) (e, f), and stomatal conductance for CO_2 (g_{sc}) (g, h) measured for Koshihikari (KH) and Takanari (TN) in current $[\text{CO}_2]$ (A- CO_2) and elevated $[\text{CO}_2]$ (E- CO_2) at the panicle initiation stage and grain-filling stage in 2017. Data from 2016 based on the curve-fitting method are included for $V_{c,max25}$ and g_{m25} . Measurements in 2017 for A_n , g_{m25} , and g_{sc} were conducted by setting the chamber $[\text{CO}_2]$ at $390 \mu\text{mol mol}^{-1}$ for plants grown in A- CO_2 and $590 \mu\text{mol mol}^{-1}$ for those grown in E- CO_2 . Four measurement blocks were treated as replicates ($n = 4$), and any subset samples were averaged within the block.

of other parameters, and the variety effect and CO_2 effect were tested separately.

Results

Takanari had a higher A_n than Koshihikari under both CO_2 treatments and in both panicle initiating and grain-filling stages (Figure 1(a,b) and Table 2). E- CO_2 increased A_n for

both varieties at both growth stages, but the increase for Koshihikari was not statistically significant at the grain-filling stage. At the grain-filling stage, the A_n of Takanari responded to CO_2 more strongly (30%) than did that of Koshihikari (2%), and the effect of CO_2 treatment and variety difference in A_n was significant.

There was not a clear varietal difference in $V_{c,max25}$ at the panicle initiation stage, but Takanari had a

Table 2. Statistical results (p values) of the mixed-effects model for variety (Koshihikari and Takanari) and CO₂ effects with respect to net leaf photosynthesis rate (A_n), maximum carboxylation at 25°C ($V_{c,max25}$), mesophyll conductance at 25°C (g_{m25}), stomatal conductance for CO₂ (g_{sc}), and the sensitivity of the three parameters to RuBP-saturated photosynthesis rate (A_c). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	A_n	$V_{c,max\ 25}$	g_{m25}	g_{sc}	$A_c (V_{c,max})$	$A_c (g_m)$	$A_c (g_{sc})$
Panicle initiation stage (2017)							
CO ₂	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Variety	*	n.s.	0.08	***	n.s.	0.05	***
CO ₂ x variety	n.s.	n.s.	n.s.	*	–	–	–
Grain-filling stage (2017)							
CO ₂	n.s.	*	*	*	n.s.	n.s.	n.s.
Variety	**	0.06	***	**	n.s.	***	n.s.
CO ₂ x variety	*	n.s.	n.s.	n.s.	–	–	–
Grain-filling stage (2016)							
CO ₂	–	n.s.	0.09	–	–	–	–
Variety	–	***	*	–	–	–	–
CO ₂ x variety	–	n.s.	n.s.	–	–	–	–

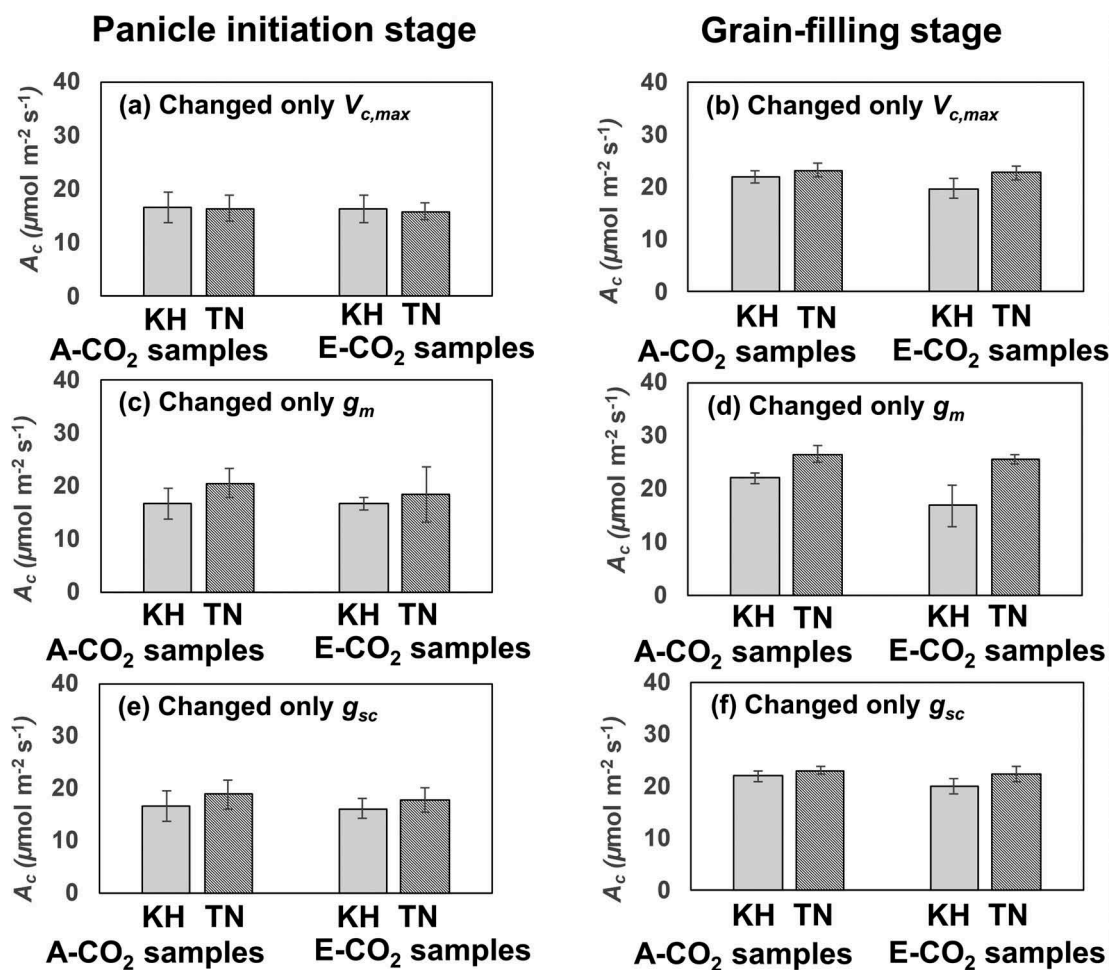


Figure 2. Simulated RuBP-saturated photosynthesis rate (A_c) of Koshihikari (KH) grown in current CO₂ (A-CO₂), and A_c with only one of the physiological parameters changed to that of KH grown in elevated CO₂ (E-CO₂) and that of Takanari (TN) grown in A-CO₂ and E-CO₂. Physiological parameters included maximum carboxylation rate at 25°C ($V_{c,max25}$) (a, b), mesophyll conductance at 25°C (g_{m25}) (c, d), and stomatal conductance for CO₂ (g_{sc}) (e, f). The simulation was conducted with air and leaf temperature at 30°C, relative humidity at 60%, PPFD at 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, leaf boundary conductance for H₂O at 3 $\text{mol m}^{-2} \text{s}^{-1}$, and [CO₂] at 390 $\mu\text{mol mol}^{-1}$.

greater $V_{c,max25}$ than did Koshihikari at the grain-filling stage, both in 2016 and 2017 (Figure 1(c,d) and Table 2). The downregulation of $V_{c,max25}$ under E-CO₂ was also evident at the grain-filling stage, although in

2016 it was not statistically significant. The varietal differences in g_{sc} and g_{m25} were more apparent than in the case of $V_{c,max25}$ at both panicle initiation stage and grain-filling stage (Figure 1(e,h) and Table 2). E-

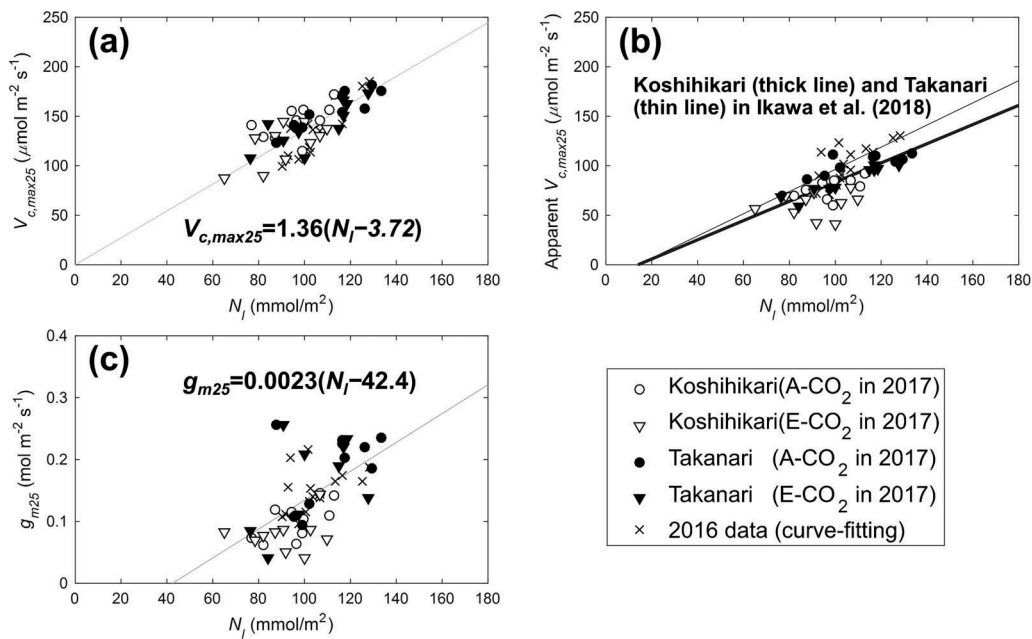


Figure 3. Maximum carboxylation rate at 25°C ($V_{c,max25}$) in relation to leaf nitrogen content (N_l) (a); $V_{c,max25}$ with mesophyll conductance (g_m) assumed to be infinite (apparent $V_{c,max25}$) in relation to N_l (b); g_m at 25°C (g_{m25}) in relation to N_l (c). ANCOVA (analysis of covariance) indicates that the relationship between $V_{c,max25}$ (or g_{m25}) and N_l was different for Koshihikari and Takanari in (b) and (c). No difference was found between CO₂ treatments (i.e. A-CO₂ and E-CO₂).

CO₂ also decreased both g_{m25} and g_{sc} at the grain-filling stage. The magnitude of g_{sc} was always greater than that of g_m , and their ratio was greater in A-CO₂ (~1.5) than it was in E-CO₂ (~1.3) at the panicle initiation stage, and greater in Koshihikari (~1.7) than in Takanari (~1.2) at the grain-filling stage.

The varietal difference in g_{sc} had a significant impact on the calculated A_c at the panicle initiation stage, and the varietal difference in g_m had a significant effect on A_c at the grain-filling stage (Figure 2 and Table 2). Although varietal differences in $V_{c,max25}$ and g_{sc} were found at the grain-filling stage, they did not lead to a statistically significant difference in A_c between the varieties (Table 2).

A clear correlation ($R^2 = 0.60$) was found between $V_{c,max25}$ and N_l , and the correlation was much clearer when $V_{c,max25}$ was calculated taking g_m into consideration rather than assuming g_m to be infinite (i.e. apparent $V_{c,max25}$) (Figure 3(a,b)). Analysis of covariance (ANCOVA) indicated that the relationship between $V_{c,max25}$ and N_l was not different between Koshihikari and Takanari, but that the relationship between apparent $V_{c,max25}$ and N_l significantly differed between varieties ($p < 0.0005$). Apparent $V_{c,max25}$ of each variety was also smaller than that estimated from N_l based on the data from 2016 and on the relationship reported in Ikawa et al. (2018) (Figure 3(b)). Interestingly, the relationship between $V_{c,max25}$ and N_l was comparable between 2016 and 2017 (Figure 3(a)), despite using different methods and instruments to estimate

$V_{c,max25}$. For the data in 2016, Ikawa et al. (2018) used a LI-6400 photosynthesis system, but we found no significant difference in the measured apparent $V_{c,max25}$ between LI-6400 and LI-6800 (Figure S2 in Supplementary Material).

A correlation was also found between N_l and g_{m25} ($R^2 = 0.37$), but the relationship within the variety was obscure ($R^2 = 0.20$ for Koshihikari and $R^2 = 0.24$ for Takanari). Takanari had greater N_l than did Koshihikari at the grain-filling stage. At the panicle initiation stage, E-CO₂ also decreased N_l for both varieties, but the decrease was not statistically significant ($p = 0.1$).

Discussion

Our results partly support the hypothesis that Takanari has greater $V_{c,max}$, g_m , and g_{sc} than does Koshihikari both in current and elevated [CO₂]. Both g_{m25} and g_{sc} were greater in Takanari than in Koshihikari, but $V_{c,max25}$ did not differ between varieties at the panicle initiation stage. There was no clear varietal difference in $V_{c,max25}$ at the panicle initiation stage and only a possible difference ($p = 0.06$) was detected at the grain-filling stage. This result might be related to N_l . Takanari had a greater N_l than did Koshihikari at the grain-filling stage, but N_l did not differ significantly at the panicle initiation stage (data not shown). In the same FACE experiment, Muryono et al. (2017) reported that N_l in the top canopy leaves was even lower in Takanari than

it was in Koshihikari at the panicle initiation stage. Generally, Takanari begins to show a high level of N_l in the top canopy leaves after heading (e.g. Taylaran et al., 2011), and it is likely that the $V_{c,max}$ of Takanari also follows the seasonal trend of N_l .

Chen et al. (2014) reported in their study that the smaller mesophyll conductance limitation (MCL) of Takanari resulted in a higher rate of photosynthesis than that of Koshihikari, and that the varietal difference in MCL was greater than that of stomatal conductance limitation (SCL), particularly at later growth stages. Our results at the grain-filling stage agree with the results of the study by Chen et al. (2014) and support our second hypothesis: the difference in g_m between $[CO_2]$ treatment and variety is greater than that of either $V_{c,max}$ or g_{sc} with respect to their effects on leaf photosynthesis. At the panicle initiation stage, however, our results suggested that the varietal difference in SCL was greater than it was in MCL. The small varietal difference in g_m at the panicle initiation stage might be due to the small varietal difference in N_l as compared with the grain-filling stage.

The third hypothesis: Takanari's high $V_{c,max}$ and g_m are explained by high leaf nitrogen contents, was also partly supported by our results. The greater N_l of Takanari correlated with the greater $V_{c,max25}$ and g_{m25} as compared with Koshihikari at the grain-filling stage. A high N_l is considered to indicate an anatomical advantage for g_m by increasing the effective surface of chloroplast (Cai et al., 2018). However, the correlation between N_l and g_{m25} was not as strong as the correlation between N_l and $V_{c,max25}$, and the relation was not clear within varieties, suggesting that other factors influence g_{m25} . Responses to environmental changes (Flexas et al., 2007; Mizokami, Noguchi, Kojima, Sakakibara & Terashima, 2015), anatomical structure (Adachi et al., 2013; Terashima, Miyazawa & Hanba, 2001), and aquaporins that possibly control not only H_2O transport but also the transport of other materials including CO_2 (Ferrio et al., 2012; Miyazawa, Yoshimura, Shinzaki, Maeshima & Miyake, 2008) could all influence g_m .

Our results indicate that g_m is significant for determining leaf photosynthesis; that its importance changes with the growth stage; and that its variation is not simply explained by N_l . Furthermore, apparent $V_{c,max25}$ given the same N_l was estimated to be higher in Takanari than Koshihikari, and was generally higher in 2016 measurements than in 2017 measurements (Figure 3(b)); however, such bias in $V_{c,max25}$ was no longer observed when g_m was considered in the calculation. These results emphasize the importance of separately considering the roles of $V_{c,max}$ and g_m for

accurately understanding the ecophysiological processes that control leaf photosynthesis.

The magnitudes of $V_{c,max25}$ and g_{m25} and their relation to N_l were comparable between 2016 and 2017, even though different instruments (LI-6400 in 2016 and LI-6800 in 2017) and different methods (curve-fitting method in 2016 and variable J method in 2017) were used (Figures 1 and 3). When the values of g_m reported in the study by Chen et al. (2014) were converted to 25°C according to the temperature function of Scafero et al. (2011), they ranged roughly from 0.1 to 0.15 mol m⁻² s⁻¹ for Koshihikari and 0.15 to 0.2 mol m⁻² s⁻¹ for Takanari in the mid grain-filling stage in 2012 and the heading stage in 2013. These values are comparable to those of our present study (Figure 1). Our results suggest that the curve-fitting method would be useful to recompute $V_{c,max}$ and g_m based on $A-C_i$ curves obtained in the past as long as careful comparisons between the measurement techniques are made.

Elevated CO_2 decreased $V_{c,max}$, g_m , and g_{sc} at the grain-filling stage. Early studies have reported that higher CO_2 decreases g_m (e.g. Flexas et al., 2007; Yin et al., 2009), but the validity of the short-term response of g_m to CO_2 is debatable, partly because the calculation of g_m requires C_i as an input (Gu & Sun, 2014; Tazoe, von Caemmerer, Badger & Evans, 2009). To examine whether the CO_2 response was the artefact, Mizokami, Noguchi, Kojima, Sakakibara and Terashima (2019) demonstrated that *Arabidopsis thaliana* with impaired stomatal regulation decreased g_m with increasing CO_2 , which supports a hypothesis that the short-term response of g_m to CO_2 does occur. It is also known that g_m decreases during long-term exposure to high CO_2 (Cai et al., 2018; Mizokami et al., 2019). To investigate whether the decrease of g_m in E- CO_2 in the grain-filling stage was due to the direct response to CO_2 or to acclimation effects, we calculated g_{m25} when $[CO_2]$ of the reference cell was set at 150 $\mu\text{mol mol}^{-1}$. The paired t -test found no statistically significant difference in g_{m25} between the growth CO_2 condition and $[CO_2]$ at 150 $\mu\text{mol mol}^{-1}$. However, g_{m25} was still lower in the plants grown in E- CO_2 than in those grown in A- CO_2 under the same $[CO_2]$ of 150 $\mu\text{mol mol}^{-1}$ ($p < 0.05$). We therefore consider that the decrease of g_m in E- CO_2 was due to acclimation rather than to a short-term response to CO_2 .

The effects of decreased $V_{c,max}$, g_m , and g_{sc} in E- CO_2 on A_c were small and not statistically significant. Cai et al. (2018) also reported that the CO_2 acclimation effect on photosynthesis was small in rice leaves. It should be noted, however, that E- CO_2 increased A_n of Takanari much more than that of Koshihikari at the grain-filling

stage (Figure 1(b) and Table 2), even though a variety \times CO₂ interaction effect was not found in any of the single parameter (Table 2). This implies the combined effects of CO₂ acclimation of physiological parameters were greater in Koshihikari than in Takanari at the grain-filling stage. If CO₂ acclimation did not occur and $V_{c,max}$, g_m , and g_{sc} were assumed to be invariant between CO₂ treatments, then further sensitivity analysis with the FvCB model showed that E-CO₂ benefits A_n of Koshihikari slightly more than that of Takanari, which agrees with the theory that high CO₂ benefits individuals with lower conductance (Centritto, Tognetti, Leitgeb, Štřelcová & Cohen, 2011).

We consider that the relevance of this study to crop production is limited by the fact that we conducted gas exchange and chlorophyll fluorescence measurements under a similar environment with a high level of PPFD. The performance of the MPF method under different light conditions has yet to be investigated. While rice physiology rapidly changes through the growth (Tatsumi, Kuwabara & Motobayashi, 2019), it was not possible to completely eliminate potential effects of different phenology between Koshihikari and Takanari. Another limitation of our study is that we did not consider the interaction between physiological effects and plants' energy balance (Ikawa et al., 2018; Yoshimoto, Oue & Kobayashi, 2005) that impact the thermal environment and thus photosynthesis and rice production (Usui et al., 2014, 2016).

Conclusions

$V_{c,max}$, g_m , and g_{sc} of the uppermost leaves of the high-yielding rice cultivar Takanari were quantified and compared with those in Koshihikari under current and elevated [CO₂] before and after heading. The effects of the differences in these physiological parameters between varieties and CO₂ treatments on A_c were then quantified, using A_c as a proxy of leaf photosynthesis rate. The greater A_n of Takanari than of Koshihikari was mainly supported by high g_{sc} at the panicle initiation stage and by high g_m at the grain-filling stage. Calculating $V_{c,max25}$ taking g_m into consideration eliminated the artifact of $V_{c,max25}$ in relation to N_l that was observed when g_m was assumed to be infinite. Although N_l may provide a good estimate of $V_{c,max25}$, other factors besides N_l play a role in the variations of g_m . Our results highlight the importance of considering g_m to accurately understand photosynthetic processes and the need to further explore the mechanisms regulating g_m . E-CO₂ decreased all three parameters ($V_{c,max}$, g_m , and g_{sc}) at the grain-filling stage, and the decrease in g_m is likely to be due to acclimation to high CO₂.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix A. Calculation of RuBP-saturated photosynthesis rate (A_c)

RuBP-saturated photosynthesis rate (A_c) in the FvCB model (Farquhar et al., 1980) was calculated by iteratively solving Equations (A-1) and (A-2). The quadratic equation (Equation A-1) was proposed by von Caemmerer and Evans (1991) and its solution is explicitly described in Ethier and Livingston (2004)

$$A_c^2 - A_c \left\{ g_m \left(C_i + K_c \left(1 + \frac{C_o}{K_o} \right) \right) + V_{c,max} - R_d \right\} + g_m \left\{ V_{c,max} (C_i - \Gamma^*) - R_d \left(C_i + K_c \left(1 + \frac{C_o}{K_o} \right) \right) \right\} = 0 \quad (\text{A} - 1)$$

$$C_i = \{(g_{tc} - E/2)C_a - A_c\} / (g_{tc} + E/2) \quad (\text{A} - 2)$$

where C_a is atmospheric CO_2 concentration ($\mu\text{mol mol}^{-1}$), C_o is O_2 concentration ($\mu\text{mol mol}^{-1}$). The total conductance for CO_2 (g_{tc} , $\text{mol m}^{-2} \text{s}^{-1}$) and transpiration rate (E , $\text{mol m}^{-2} \text{s}^{-1}$) are calculated as follows (von Caemmerer & Farquhar, 1981):

$$g_{tc} = \{1/g_{sc} + 1/(1.37g_{bw})\}^{-1} \quad (\text{A} - 3)$$

$$E = g_{tw}(w_i - w_a) / \{1 - (w_i + w_a)/2\} \quad (\text{A} - 4)$$

where w_i and w_a are H_2O molar fraction at the intercellular space and atmosphere, respectively (mol mol^{-1}). The total conductance for H_2O (g_{tw} , $\text{mol m}^{-2} \text{s}^{-1}$) was defined as follows:

$$g_{tw} = \{1/g_{sw} + 1/g_{bw}\}^{-1} \quad (\text{A} - 5)$$

Equations (A-1)–(A-5) were coded with MATLAB (MATLAB R2015b, MathWorks, USA).