frontiers in Plant Science

ORIGINAL RESEARCH
published: 31 January 2017
doi: 10.3389/fpls.2017.00060

Fine Mapping of *Carbon Assimilation Rate 8*, a Quantitative Trait Locus for Flag Leaf Nitrogen Content, Stomatal Conductance and Photosynthesis in Rice

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Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *06 August 2016*
Accepted: *11 January 2017*
Published: *31 January 2017*

Citation:
Adachi S, Yoshikawa K, Yamanouchi U, Tanabata T, Sun J, Ookawa T, Yamamoto T, Sage RF, Hirasawa T and Yonemaru J (2017) Fine Mapping of Carbon Assimilation Rate 8, a Quantitative Trait Locus for Flag Leaf Nitrogen Content, Stomatal Conductance and Photosynthesis in Rice. Front. Plant Sci. 8:60. doi: 10.3389/fpls.2017.00060

Increasing the rate of leaf photosynthesis is one important approach for increasing grain yield in rice (*Oryza sativa*). Exploiting the natural variation in CO_2 assimilation rate (*A*) between rice cultivars using quantitative genetics is one promising means to identify genes contributing to higher photosynthesis. In this study, we determined precise location of *Carbon Assimilation Rate 8* (*CAR8*) by crossing a high-yielding *indica* cultivar with a Japanese commercial cultivar. Fine mapping suggested that *CAR8* encodes a putative Heme Activator Protein 3 (OsHAP3) subunit of a CCAAT-box-binding transcription factor called OsHAP3H. Sequencing analysis revealed that the *indica* allele of *CAR8* has a 1-bp deletion at 322 bp from the start codon, resulting in a truncated protein of 125 amino acids. In addition, *CAR8* is identical to *DTH8/Ghd8/LHD1*, which was reported to control rice flowering date. The increase of *A* is largely due to an increase of RuBP regeneration rate via increased leaf nitrogen content, and partially explained by reduced stomatal limitation via increased stomatal conductance relative to *A*. This allele also increases hydraulic conductivity, which would promote higher stomatal conductance. This indicates that *CAR8* affects multiple physiological aspects relating to photosynthesis. The detailed analysis of molecular functions of *CAR8* would help to understand the association between photosynthesis and flowering and demonstrate specific genetic mechanisms that can be exploited to improve photosynthesis in rice and potentially other crops.

Keywords: leaf nitrogen content, *Oryza sativa*, photosynthesis, quantitative trait locus, RuBP regeneration, stomatal conductance

INTRODUCTION

Rice (*Oryza sativa*) is one of the most valuable crops in the world, both in terms dollar value and contribution to the human food supply (FAO, 2015). Increasing its yield is a major challenge for improving global food security (Khush, 2013) and could be achieved by increasing the rate of net CO_2 assimilation rate in individual leaves (A) (Long et al., 2006; Murchie et al., 2009). While photosynthetic improvement often emphasizes improving specific known traits within the photosynthetic apparatus (Suzuki et al., 2007; Takahara et al., 2010; von Caemmerer and Evans, 2010), or through introducing novel photosynthetic pathways such as the C_4 pathway (Kajala et al., 2011; http://C4rice.irri.org), analysis of quantitative trait locus (QTL) through crossing experiments provide the opportunity to identify novel genetic elements that control photosynthetic performance in existing rice cultivars (Flood et al., 2011).

Most agronomic traits including A are controlled by multiple genetic factors, such traits are known as quantitative traits. QTL analyses can provide associations between quantitative traits and molecular markers (Tanksley, 1993). To conduct a QTL analysis, phenotypic values of interest are quantified in a segregating population whose genotypes have been determined by DNA markers. In rice, the complete genome sequence is available and many DNA markers have been identified (International Rice Genome Sequencing Project, 2005). Several advanced populations, including backcrossed inbred lines and chromosome segment substitution lines, have been developed to facilitate the QTL investigations in rice (Yamamoto et al., 2009). As a result, many genes associating with important agronomic traits have been identified using QTL methods (Yamamoto et al., 2014).

Wide variations in A among rice cultivars have been described (Takano and Tsunoda, 1971; Cook and Evans, 1983; Yeo et al., 1994; Kanemura et al., 2007; Jahn et al., 2011), and several QTL underlying this variation have been identified in populations derived from crosses between *japonica* and *indica* cultivars (Teng et al., 2004; Hu et al., 2009; Takai et al., 2010) and between *japonica* and *indica*/*japonica* cultivars (Gu et al., 2012). However, there is only one report that identified a causal gene controlling photosynthetic variation among rice cultivars (Takai et al., 2013). To understand the whole picture of the genetic control of A and to apply it in breeding aimed at increasing rice grain yield, it is necessary to identify the causal genes and understand their physiological aspects.

The CO_2 assimilation rate in C_3 species is considered to be limited by ribulose 1,5-bisphosphate (RuBP) carboxylation capacity of Rubisco or the RuBP regeneration capacity (Farquhar et al., 1980). Under low CO_2 concentration and light-saturated conditions, A is commonly limited by the RuBP carboxylation capacity, while it is limited by RuBP regeneration capacity under elevated CO_2 concentration and light-saturated conditions. The RuBP regeneration capacity reflects the capacity of electron transport, the Calvin cycle, and under high CO_2 concentration, the ability of starch and sucrose synthesis to release inorganic phosphate (Sharkey, 1985). The CO_2 diffusion from air into leaves is also important determinant of A (Farquhar and Sharkey, 1982). In healthy leaves, stomatal conductance (g_s) is regulated to track the value of A such that the intercellular CO_2 concentration (C_i) and the ratio of intercellular to ambient CO_2 (C_i/C_a) vary little as A increases (Farquhar and Sharkey, 1982). In contrast, Kusumi et al. (2012) shows that the increase in g_s relative to A can enhance A and C_i/C_a in a rice mutant with a defective anion channel in the guard cells. This suggested that we should consider both the stomatal control and the enzymatic control of the photosynthetic apparatus to know the physiological reasons relating to the difference in A.

During grain filling, the flag leaf is the most important leaf in the rice canopy because its position at the top of the canopy ensures maximum light availability and it has greater photosynthetic capacity than leaves lower in the canopy. In our previous research, we used chromosome segment substitution lines derived from "Habataki," a high-yielding *indica* cultivar with high A, and the *japonica* variety "Koshihikari," the most popular cultivar in Japan with lower A, to identify four QTLs affecting A in flag leaves (Adachi et al., 2011, 2014). One of the four QTLs was identified at ∼1.2 Mb region on the short arm of chromosome 8 (Adachi et al., 2011). According to the rice annotation database, 124 genes are predicted in this region (Sakai et al., 2013, http://rapdb.dna.affrc.go.jp). To determine gene responsible for the increase in A, fine-scale mapping is required. In this study, we examined a region in the QTL that correlates with the increase in A, which we term *Carbon Assimilation Rate 8 (CAR8)*. Our objective is to identify the gene underlying *CAR8* via fine mapping and to evaluate the physiological mechanism by which it increases A.

Abbreviations: A, CO_2 assimilation rate; C_a, ambient CO_2 concentration; *CAR8, carbon assimilation rate 8*; C_i, intercellular CO_2 concentration; C_p, hydraulic conductance from roots to leaves; dCAPS, derived cleaved amplified polymorphic sequence; DAS, days after sowing; *DTH8*, *days to heading 8*; *Ehd1*, *Early heading date1*; g_s, stomatal conductance; $\Gamma*$, CO_2 compensation point in the absence of day respiration; *Ghd8, grain number, plant height and heading date 8*; *GPS*, *Green for Photosynthesis*; HAP, heme activator protein; *Hd1*, *Heading date 1*; *Hd3a*, *Heading date 3a*; InDel, insertion-deletion; J_{max}, maximum rate of electron transport; K_c, Michaelis constants for CO_2; K_o, Michaelis constants for O_2; *LHD1*, *Late Heading Date 1*; L_p, hydraulic conductivity; LNC_a, leaf nitrogen content per leaf area; LNC_w, leaf nitrogen content per leaf dry weight; NIL, near-isogenic line; O, intercellular O_2 concentration; PPFD, Photosynthetic photon flux density; Ψ_l, leaf water potential; QTL, quantitative trait locus; R_d, day respiration rate; Rubisco, ribulose-1,5 bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; S_r, root surface area; T, transpiration rate; V_{cmax}, maximum rate of RuBP carboxylation; VPD, vapor pressure deficit.

MATERIALS AND METHODS

Growth Conditions

We grew rice plants in three conditions—paddy fields, outdoors in pots, and in a controlled-environment cabinet in pots. We used the plants grown in paddy fields for QTL mapping, the plants grown outdoors in pots for evaluating the physiological effect of *CAR8* on A, and the plants grown in a controlled environment cabinet in pots for evaluating the hydraulic conductance and root surface area. Plants in a paddy field were grown at the National Institute of Agrobiological Sciences in Tsukuba, Japan (36°03′N, 140°11′E). Seedlings at the fifth-leaf stage were transplanted

(one plant per hill) into the field (alluvial clay loam). Each line was planted in a single row of 12 hills (18 cm between hills and 30 cm between rows) and fertilized with 56 kg N, 176 kg P_2O_5, and 56 kg K_2O ha^{-1} with no top dressing was applied. Plants in pots were grown outdoors in 12-L pots filled with a 1:1 (v/v) mixture of paddy soil (alluvial clay loam) and upland soil (diluvial volcanic ash) at a density of three hills per pot (three plants per hill). Fertilizer (1.0 g each N, P_2O_5, and K_2O per pot) was applied at planting, and additional fertilizer (0.3 g N per pot) was applied at 69 and 85 days after sowing (DAS). Plants grown in a controlled-environment cabinet (14.5 h light/9.5 h dark; 28°C for 12 h and 24°C for 12 h) were in 3-L pots filled with a flooded, granular culture soil. The relative humidity was 60%; the photosynthetic photon flux density (PPFD) at the top of the canopy was 500 μmol photons m^{-2} s^{-1}. The soil contained 1.2 g N, 3.2 g P_2O_5, and 1.8 g K_2O per pot.

Plant Materials for QTL Mapping

CAR8 mapping was carried out using self-pollinated progenies derived from a BC_5F_4 population (912 plants) of a "Koshihikari" × "Habataki" cross with "Koshihikari" as the recurrent parent. They have a single heterozygous region in chromosome 8 and most other regions were homozygous for "Koshihikari" alleles. We selected 23 plants from the BC_5F_4 population and used homozygous BC_5F_6 generation for phenotyping (**Figure 1**). The near isogenic line NIL(*CAR8*) was also selected from the BC_5F_6 generation. Subsequently, fine mapping was carried out using self-pollinated progenies derived from a BC_5F_5 population (144 plants) of the same "Koshihikari" × "Habataki" cross. We selected 6 plants from the BC_5F_5 generation and used homozygous BC_5F_7 generation for the phenotyping (**Figure 2**). Molecular markers used for mapping are listed in Table S1. These plants were grown in the paddy field. For both experiments, a randomized block design (three

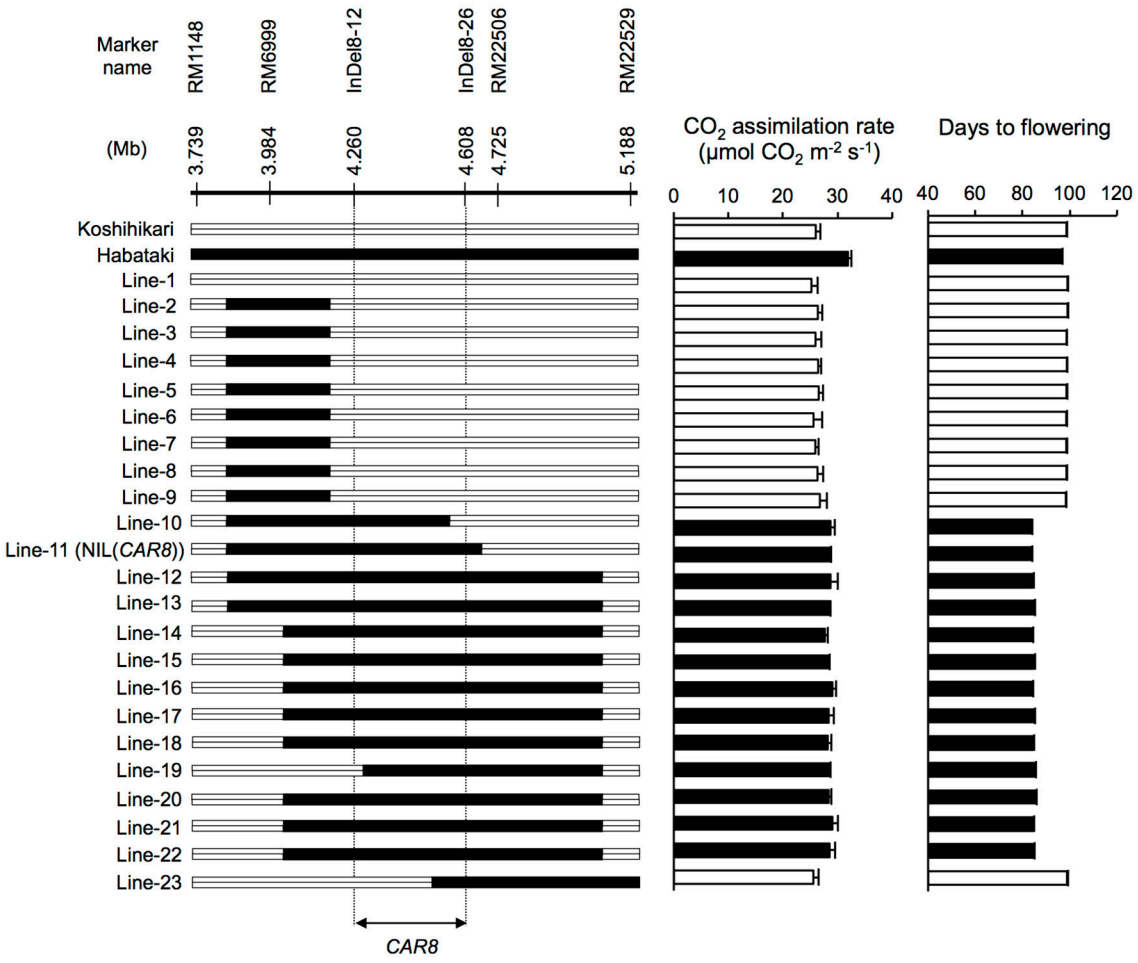

FIGURE 1 | Substitution mapping of *CAR8* using homozygous recombinant lines (BC_5F_6). Molecular markers are shown from the short arm **(left)** to the long arm **(right)** of chromosome 8. White segments, homozygous for "Koshihikari" alleles; black segments, homozygous for "Habataki" alleles. Field-grown plants were used. CO_2 assimilation rate of flag leaves was measured at an ambient CO_2 concentration of 370 μmol mol^{-1}, a PPFD of 2000 μmol photons m^{-2} s^{-1}, a leaf-to-air vapor pressure difference of 1.3–1.6 kPa, and an air temperature of 30°C. Black bars in graphs indicate significant difference from "Koshihikari" at the 5% level by Dunnett's test. Error bars indicate SD ($n = 3$).

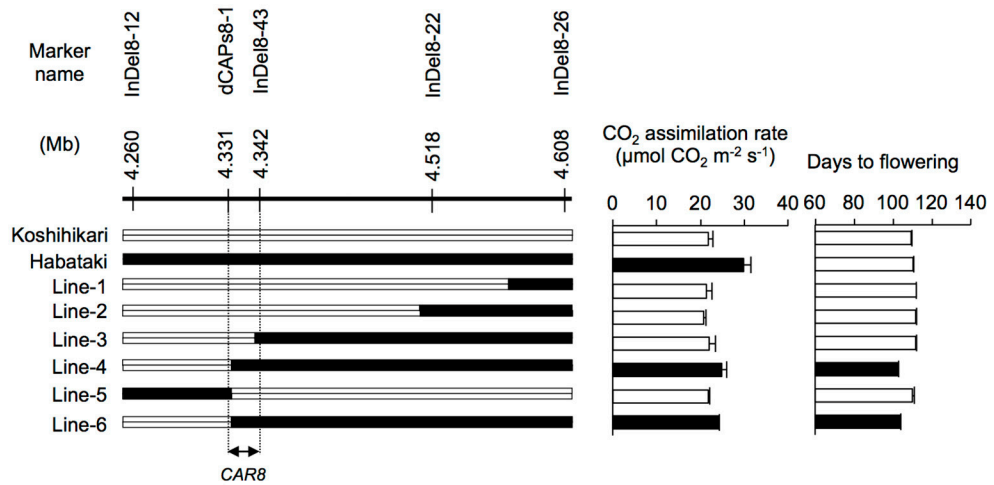

FIGURE 2 | Fine mapping of *CAR8* using homozygous recombinant lines (BC_5F_7). Molecular markers are shown from the short arm **(left)** to the long arm **(right)** of chromosome 8. White segments, homozygous for "Koshihikari" alleles; black segments, homozygous for "Habataki" alleles. Field-grown plants were used. CO_2 assimilation rate of flag leaves was measured at an ambient CO_2 concentration of 370 μmol mol^{-1}, a PPFD of 2000 μmol photons m^{-2} s^{-1}, a leaf-to-air vapor pressure difference of 1.3–1.6 kPa, and an air temperature of 30°C. Black bars in graphs indicate significant difference from "Koshihikari" at the 5% level by Dunnett's test. Error bars indicate SD ($n = 3$).

replicates) was used and 4∼6 plants were evaluated in each replicate.

Gas Exchange and Nitrogen Measurements

Leaf gas exchange was measured with a portable gas-exchange system (LI-6400; LI-COR, Lincoln, NE, USA) and 2 × 3 cm cuvette with an LED irradiation source (LI-6400- 02B; LI-COR). The uppermost fully expanded leaves were used for the measurements before heading, and flag leaves after heading. A and g_s were measured at an ambient CO_2 concentration of 370 μmol mol^{-1}, PPFD of 2000 μmol photons m^{-2} s^{-1}, a leaf-to-air vapor pressure difference of 1.3–1.6 kPa, and a leaf temperature of 30°C. Plants were examined from 08:30 to 11:30, when the photosynthetic rate was close to the daily maximum (Hirasawa and Ishihara, 1992). The CO_2 assimilation rate vs. C_i was examined at a light intensity of 2000 μmol photons m^{-2} s^{-1} and a leaf temperature of 30°C at full heading stage by changing the ambient CO_2 concentration. To prevent potential leaks, we sealed the gaskets with vacuum grease. Rubisco-limited photosynthesis (A_c) was calculated from Farquhar et al. (1980) as:

$$A_c = [V_{cmax}(C_i - \Gamma^*)]/[C_i + K_c(1 + O/K_o)] - R_d,$$

where Γ^* (μmol mol^{-1}) is the CO_2 compensation point in the absence of day respiration, K_c (μmol mol^{-1}) and K_o (mmol mol^{-1}) are the Michaelis constants for CO_2 and O_2 respectively, and R_d (μmol mol^{-1}) is the day respiration rate. Photosynthetic rate limited by RuBP regeneration capacity (A_r) is calculated as;

$$A_r = [J_{cmax}(C_i - \Gamma^*)]/(4C_i + 8\Gamma^*) - R_d,$$

The K_c, K_o, and Γ^* at 30°C were calculated from the data of Makino et al. (1988) using the Arrhenius function described by von Caemmerer (2000). To convert the K_c and K_o from concentrations to partial pressures, solubilities of 0.0334 mol L^{-1} bar^{-1} for CO_2 and 0.00126 mol L^{-1} bar^{-1} for O_2 were used (von Caemmerer, 2000). A/C_i response curves were analyzed using the mathematical model developed by Sharkey et al. (2007) and the data were automatically fitted with the model fitting utility based on a Microsoft Excel program (http://www.blackwellpublishing.com/plantsci/pcecalculation/).

Immediately after the measurements of photosynthesis, 30-mm-long segment was cut from the center of the leaf of measured plants and stored at −80°C. The leaves were then dried at 80°C for 24 h and the nitrogen content was assayed using with a CN analyzer (MT700 Mark II, Yanako, Kyoto, Japan).

Determination of Stomatal Density and Pore Length

The middle part of flag leaves was fixed in solution containing (v/v) 5% formalin, 5% acetic acid, and 45% ethyl alcohol in distilled water. Abaxial and adaxial surfaces of the fixed leaves were photographed under a scanning electron microscope (TM3030; Hitachi, Tokyo, Japan). Stomatal number was counted using a touch screen (Flexscan T2351W; Eizo, Ishikawa, Japan) connected to a computer that was installed with original computer software that senses the number of contacts. Length of stomatal pores was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Determination of Hydraulic Conductance and Hydraulic Conductivity of Plants

The hydraulic conductance from roots to leaves (C_p, 10^{-8} m^3 s^{-1} MPa^{-1}) was calculated as $U_w/(\Psi_s - \Psi_l$; Hirasawa and Ishihara, 1991), where U_w (10^{-8} m^3 s^{-1}) is the water uptake rate of the whole plant, Ψ_s (MPa) is the water potential of

the soil immediately outside the root, and Ψ_l (MPa) is the average water potential of the uppermost three leaves. Since plants were submerged the water potential of the soil solution, Ψ_s was regarded as 0. Plants grown in 3-L pots were used. Measurements were made in a controlled-environment cabinet [air temperature, 28°C; air vapor pressure deficit (VPD), 1.5 kPa; PPFD at the top leaves, 900 μmol m^{-2} s^{-1}]. U_w was determined from the rate of weight loss of the pot over 20 min after a steady state had been reached. To prevent evaporation from the surface of the pot, the top was covered with polystyrene foam and the gap between the foam and the stem was sealed with oil clay. After measurement of U_w, Ψ_l was measured in a pressure chamber (model 3005; Soil Moisture Equipment, Santa Barbara, CA, USA). The transpiration rate and g_s do not influence C_p when the transpiration rate is high (Fiscus, 1975; Hirasawa and Ishihara, 1991; Stiller et al., 2003). The U_w per leaf area was sufficiently high (>2.0 mmol m^{-2} s^{-1}) to eliminate the effect of the difference in water uptake rate on C_p. After roots had been washed gently in water, root surface area (S_r) was measured with an image analyzer (WinRHIZO REG V 2004b; Regent Instruments, Quebec, Canada). The hydraulic conductivity (L_p,10^{-8} m s^{-1} MPa^{-1}) was expressed as C_p per S_r (Steudle and Peterson, 1998).

Response to the Change of Vapor Pressure Deficit

Plants grown outdoors in 12-L pots until full heading stage were moved to a controlled-environment cabinet (KG-50HLA; Koito Manufacturing Co. Ltd, Tokyo, Japan) at a PPFD and temperature at the flag leaf surface of 900 μmol photons m^{-2} s^{-1} and 30°C. Air humidity was modified in steps to generate a range of VPD values inside the cabinet. The temperature and humidity near the flag leaf were monitored with a thermo-hygro sensor (Climomaster model 6531; Kanomax, Osaka, Japan). g_s of the flag leaf was measured with the LI-6400 portable gas-exchange system after a steady state had been reached; the leaf chamber conditions were similar to those in the cabinet. After gas exchange measurements, water potential of each leaf was determined with the pressure chamber.

Statistical Analysis

For the fine mapping, Dunnett's test was applied in the mapping population. For comparisons of physiological traits, we analyzed ANOVA and least significant difference (LSD) test. All analyses were tested with JMP v.12 software (SAS Institute, Cary, NC, USA).

RESULTS

Fine Mapping of *CAR8*

Using homozygous recombinant lines derived from a cross between "Koshihikari" and "Habataki," we conducted fine mapping of *CAR8* (**Figures 1–3**). These plants were grown in the paddy field. The *A* of the flag leaves was measured at full heading stage, which was 3–7 days after flowering, under light-saturated conditions and ambient CO_2 concentration. Using lines of BC_5F_6 generation, we narrowed down the *CAR8* region to 348.3 kb between insertion-deletion (InDel) marker InDel8-12 and InDel8-26 on the short arm of chromosome 8 (**Figure 1**). Among lines of BC_5F_7 generation, two of the six lines showed

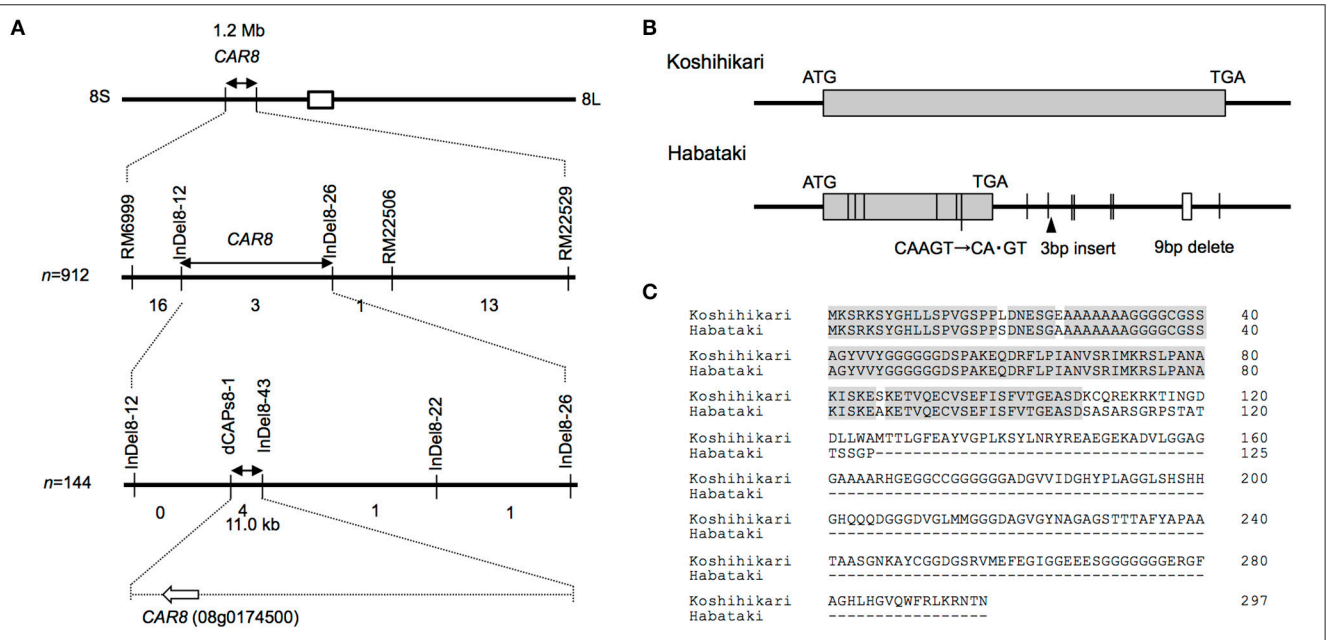

FIGURE 3 | Map-based cloning of *CAR8*. (A) Fine mapping of *CAR8*. The number of recombinants between molecular markers is indicated below the each line. **(B)** Structure of *CAR8*. The exon is shown as a gray box. Vertical lines without labels represent single-base substitutions between "Koshihikari" and "Habataki." Small open boxes represent deletions. **(C)** Alignment of *CAR8* amino acid sequences.

TABLE 1 | Photosynthetic parameters of flag leaves at the full heading stage.

		Koshihikari	NIL(*CAR8*)	Habataki	ANOVA
A	μmol CO_2 m^{-2} s^{-1}	21.7 ± 0.73c	25.2 ± 1.5b	30.2 ± 1.7a	***
LNC_a	g m^{-2}	1.54 ± 0.09c	1.66 ± 0.10b	1.88 ± 0.12a	**
LNC_w	mg g^{-1} DW	24.8 ± 1.3b	29.2 ± 3.4a	28.0 ± 0.6a	**
V_{cmax}	μmol CO_2 m^{-2} s^{-1}	176.2 ± 23.2b	222.4 ± 39.9a	266.5 ± 23.7a	**
J_{max}	μmol CO_2 m^{-2} s^{-1}	215.5 ± 32.2b	251.0 ± 23.3a	273.0 ± 24.0a	**
g_s	mol H_2O m^{-2} s^{-1}	0.55 ± 0.10c	0.74 ± 0.06b	1.06 ± 0.10a	***
C_i	μmol CO_2 mol^{-1}	289.4 ± 7.4b	297.4 ± 3.1a	303.3 ± 4.4a	**
C_i/C_a		0.78 ± 0.02b	0.80 ± 0.01a	0.82 ± 0.01a	**

*Plants were grown outdoors in 12-L pots. Leaf gas exchange was measured at an ambient CO_2 concentration of 370 μmol mol^{-1}, a PPFD of 2000 μmol photons m^{-2} s^{-1}, a leaf-to-air vapor pressure difference of 1.3–1.6 kPa, and a leaf temperature of 30°C. Values are mean ± SD ($n = 6$). **$P < 0.01$; ***$P < 0.001$. Values followed by the same letters indicate no significant difference among rice lines at $P < 0.05$ by LSD test. A, CO_2 assimilation rate; LNC_a, leaf nitrogen content per leaf area; LNC_w, leaf nitrogen content per leaf dry weight; V_{cmax}, maximum carboxylation rate; J_{max}, maximum electron transport rate; g_s, stomatal conductance; C_i, intercellular CO_2 concentration; C_i/C_a, ratio of intercellular to ambient CO_2 concentration.*

higher *A*-values than "Koshihikari" (**Figure 2**). This enabled us to delimit the *CAR8* region to 11.0 kb between the derived cleaved amplified polymorphic sequence (dCAPS) marker dCAPS8-1 and InDel8-43 (**Figure 3A**). A single gene, Os08g0174500, was predicted in this region using the RAP-DB. Os08g0174500 encodes a Heme Activator Protein 3 (OsHAP3) subunit of CCAAT-box-binding transcription factor called OsHAP3H. This gene was same gene to *DTH8*, *Ghd8,* and *LHD1* (*days to heading 8, grain number, plant height and heading date 8*, and *Late Heading Date 1*), which have been reported to regulate heading date (Wei et al., 2010; Yan et al., 2011; Dai et al., 2012). The time to heading was 7–10 days shorter in two homozygous recombinant lines with higher *A* than in "Koshihikari" (**Figures 1**, **2**). Sequence analysis of Os08g0174500 revealed that "Koshihikari" had a reading frame totaling 894 bp that encodes a protein of 297 amino acids. In "Habataki," a 1-bp deletion at 322 bp from the initiation codon caused a frameshift and premature termination of translation, resulting in a truncated protein of 125 amino acids (**Figures 3B,C**).

Photosynthesis Response

The near isogenic line NIL(*CAR8*) was selected from the BC_5F_6 generation derived from a cross between "Koshihikari" and "Habataki" with DNA marker assisted selection (**Figure 1**). NIL(*CAR8*) carries a single chromosome segment of "Habataki," which includes the *CAR8* region, in the genetic background of "Koshihikari" (Figure S1). The length of the substituted region in NIL(*CAR8*) was approximately 1.0 Mb.

Using plants grown outdoors in 12-L pots, we evaluated several traits that affect *A* (**Table 1**). At full heading stage, which was 2–4 days after flowering, *A* of the flag leaves in NIL(*CAR8*) at CO_2 concentration of 370 μmol mol^{-1} was 16% higher than that of the flag leaves in "Koshihikari." Leaf nitrogen content per leaf area (LNC_a) and leaf nitrogen content per leaf dry weight (LNC_w) in NIL(*CAR8*) were also higher than in "Koshihikari." V_{cmax} and J_{max} estimated from A–C_i responses (Sharkey et al., 2007) were higher in NIL(*CAR8*) than in "Koshihikari." g_s was higher in NIL(*CAR8*) than in "Koshihikari," such that C_i and C_i/C_a in NIL(*CAR8*) were also higher than those of "Koshihikari."

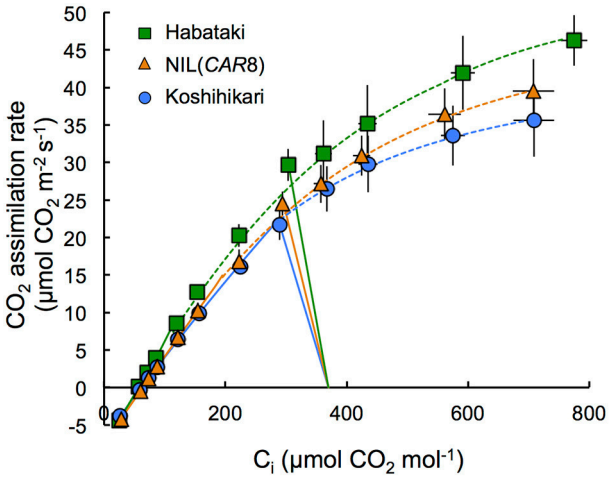

FIGURE 4 | Response of CO_2 assimilation rate of flag leaves at full heading to intercellular CO_2 concentration. Plants of "Koshihikari" (circles), NIL(*CAR8*) (triangles), and "Habataki" (squares) were grown outdoors in 12-L pots. Leaf gas exchange was measured at a PPFD of 2000 μmol photons m^{-2} s^{-1} and an air temperature of 30°C. CO_2 assimilation rate limited by RuBP carboxylation (solid line) and CO_2 assimilation rate limited by RuBP regeneration (dotted line) were shown. Curve fitting was described in the Materials and Methods Section. The straight lines represent the measurement at ambient CO_2 concentration of 370 μmol mol^{-1}. Error bars indicate SD ($n = 6$).

These values in "Habataki" were generally higher than those in NIL(*CAR8*), although the statistically significant differences were found in only *A*, LNC_a and g_s. We also found that NIL(*CAR8*) had slightly higher *A* regardless of C_i values than "Koshihikari" in the A-C_i curve (**Figure 4**).

Values of g_s are affected by stomatal density, pore length, and aperture (Maruyama and Tajima, 1990; Ohsumi et al., 2007). Stomatal densities in the adaxial and abaxial epidermis were similar between NIL(*CAR8*) and "Koshihikari" (**Figure 5A**). There was no significant difference in the pore length between NIL(*CAR8*) and "Koshihikari" (**Figure 5B**). These values in

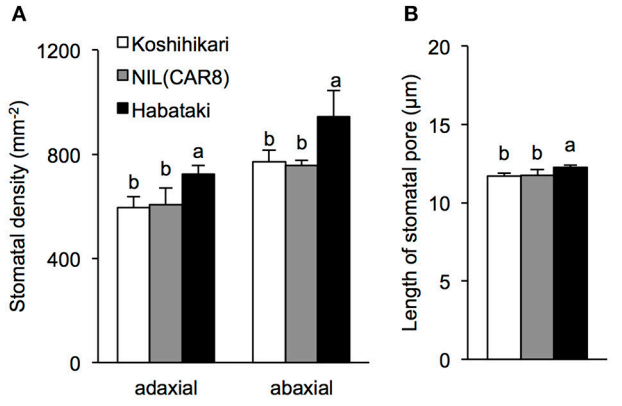

FIGURE 5 | Stomatal density (A) and stomatal pore length (B) in flag leaves of field-grown plants at full heading. Error bars indicate SD ($n = 3$). Values followed by the same letters indicate no significant difference among rice lines at $P < 0.05$ by LSD test.

"Habataki" were significantly higher than those in NIL(*CAR8*) and "Koshihikari."

We monitored leaf gas exchange and LNC throughout the growth period (**Table 2**). The number of days from sowing to flowering was 100 in "Koshihikari," 91 in NIL(*CAR8*), and 103 in "Habataki." There was no difference in A, g_s, LNC_a, and LNC_w between "Koshihikari" and NIL(*CAR8*) at 47 and 67 days after sowing (DAS). At 95 DAS, A, g_s, LNC_a, and LNC_w in NIL(*CAR8*) were higher than those of "Koshihikari," while only g_s was higher in NIL(*CAR8*) at 105 DAS. "Habataki" showed higher A than NIL(*CAR8*) from 67 to 105 DAS, which was accompanied by the higher g_s and in some cases higher LNC_a and LNC_w.

Hydraulic Conductance

It is suggested that g_s is influenced by the hydraulic conductance of a plant (Brodribb and Holbrook, 2003). When we compared the plants grown in 3-L pots in a controlled-environment cabinet at the full heading stage, C_p in NIL(*CAR8*) was significantly higher than in "Koshihikari" (**Table 3**). The C_p in "Habataki" was much higher than that in NIL(*CAR8*). C_p can be divided in root surface area (S_r) and hydraulic conductance per S_r, i.e., hydraulic conductivity (L_p) (Steudle and Peterson, 1998). NIL(*CAR8*) showed similar S_r but higher L_p in comparison with "Koshihikari," while "Habataki" showed higher S_r but similar L_p in comparison with "Koshihikari." We also determined that A and g_s in NIL(CAR8) were higher than those of "Koshihikari" (data not shown).

To assess relationship between leaf water status and g_s, we compared the responses of transpiration rate (T), g_s, and leaf water potential (Ψ_l) to vapor pressure deficit (VPD) at the full heading stage with the plants grown in 12-L pots (Figure S2). In all genotypes, T increased and g_s and Ψ_l declined with increasing VPD. In NIL(*CAR8*) and "Habataki," T and g_s were always higher than in "Koshihikari," whereas Ψ_l was similar in all three genotypes irrespective to VPD conditions. These results indicate that NIL(*CAR8*) keeps Ψ_l at a certain level even though their T are significantly higher than "Koshihikari."

Grain Yield

We examined the final grain yield of the plant grown in the paddy field (Figure S3). The brown rice yield in NIL(*CAR8*) was lower than that of "Koshihikari", while the yield of "Habataki" was significantly higher than the others.

DISCUSSION

The understanding of genetic factors and their physiological aspects that control the natural variation of rice photosynthesis are important for future rice breeding aimed at increasing grain yield. In this study, we narrowed down the genetic region of *CAR8* located in the short arm of chromosome 8 and evaluated the physiological aspects of *CAR8*.

The result of the fine mapping suggests that the protein encoded by *CAR8* is a putative OsHAP3 subunit of the HAP complex, OsHAP3H. HAP complex binds to CCAAT box and act either as a transcription activator or as a repressor (Laloum et al., 2013). The HAP complex consists of three subunits: HAP2, HAP3, and HAP5 (Mantovani, 1999). Each of the HAP subunits is encoded by a single gene in yeast (*Saccharomyces cerevisiae*) and mammals (Mantovani, 1999), while in rice, the genome encodes 10 OsHAP2, 11 OsHAP3, and seven OsHAP5 subunits (Thirumurugan et al., 2008). *CAR8* might be identical to *DTH8/Ghd8/LHD1*, which was reported to control rice flowering date (Wei et al., 2010; Yan et al., 2011; Dai et al., 2012). According to the classification of Wei et al. (2010), the "Koshihikari" allele corresponds to type 1 and the "Habataki" allele to type 8. Under long-day conditions, the type 1 allele of *DTH8* negatively influenced the expression of *Early heading date 1* (*Ehd1*) and *Heading date 3a* (*Hd3a*), resulting in repression of flowering (Wei et al., 2010). Recently, it is revealed that *DTH8* binds to *Heading date 1* (*Hd1*), which represses the expression of *Ehd1* and control the heading date (Chen et al., 2014). The "Koshihikari" allele might suppress the expression of these genes and delay the heading date, while the allele of "Habataki" might not. Although it is well known that OsHAP3H regulates rice flowering, the association of this gene with photosynthesis has been little noticed. The detailed analysis of molecular mechanisms including complementation tests would contribute to understand how *CAR8* controls both photosynthesis and flowering.

Two hypotheses can generally explain an increase in A in C_3 plants: (1) increase in the biochemical activity of the leaf photosynthetic machinery and (2) enhancement of CO_2 diffusion from air into leaves (Farquhar and Sharkey, 1982). While we didn't find any difference in A during vegetative stage (i.e., at 47 and 67 DAS in **Table 2**), we found the higher A by 16% in NIL(*CAR8*) than that in "Koshihikari" at full heading stage. A higher abundance of photosynthetic proteins is indicated by a corresponding increase in LNC (Makino et al., 1984). We found that NIL(*CAR8*) had higher LNC_a and LNC_w than "Koshihikari" at the full heading stage (**Table 1**). Biochemically, the rate of photosynthesis is generally limited by either RuBP carboxylation

TABLE 2 | CO_2 assimilation rate (*A*), stomatal conductance (g_s), and leaf nitrogen content (LNC) between 47 and 105 days after sowing (DAS).

	Lines	DAS (Day)				ANOVA		
		47	67	95	105	Line	DAS	Line × DAS
A μmol CO_2 m^{-2} s^{-1}	Koshihikari	33.5 ± 0.8a	18.4 ± 1.2b	21.7 ± 2.0c	21.7 ± 2.0b	***	***	***
	NIL(*CAR8*)	35.5 ± 1.6a	18.3 ± 1.6b	25.2 ± 1.5b	22.7 ± 1.5b			
	Habataki	33.6 ± 2.3a	22.2 ± 2.5a	29.8 ± 2.1a	29.5 ± 2.2a			
g_s mol H_2O m^{-2} s^{-1}	Koshihikari	0.67 ± 0.06b	0.42 ± 0.04b	0.55 ± 0.07c	0.55 ± 0.10c	***	***	**
	NIL(*CAR8*)	0.73 ± 0.07b	0.44 ± 0.08b	0.74 ± 0.06b	0.74 ± 0.03b			
	Habataki	0.88 ± 0.10a	0.67 ± 0.13a	1.02 ± 0.10a	0.98 ± 0.12a			
LNC_a g m^{-2}	Koshihikari	2.33 ± 0.09a	1.13 ± 0.03b	1.53 ± 0.08b	1.54 ± 0.09b	***	***	*
	NIL(*CAR8*)	2.36 ± 0.12a	1.13 ± 0.03b	1.66 ± 0.10a	1.43 ± 0.10b			
	Habataki	2.42 ± 0.14a	1.22 ± 0.05a	1.84 ± 0.13a	1.88 ± 0.12a			
LNC_W mg g^{-1} DW	Koshihikari	45.6 ± 5.2a	22.7 ± 1.1a	24.5 ± 3.1b	24.8 ± 1.3b	*	***	NS
	NIL(*CAR8*)	44.9 ± 6.1a	21.8 ± 2.3a	29.2 ± 3.4a	24.7 ± 1.4b			
	Habataki	45.7 ± 1.8a	23.5 ± 1.6a	29.0 ± 1.8a	28.0 ± 0.6a			

*Plants were grown in 12-L pots. Gas exchange was measured on the uppermost fully expanded leaves at an ambient CO_2 concentration of 370 μmol mol^{-1}, a PPFD of 2000 μmol photons m^{-2} s^{-1}, a leaf-to-air vapor pressure difference of 1.3–1.6 kPa, and a leaf temperature of 30°C between 08:30 and 11:30. LNC_a, leaf nitrogen content per leaf area; LNC_w, leaf nitrogen content per leaf dry weight. The number of days to heading was 100 for "Koshihikari," 91 for NIL(CAR8), and 103 for "Habataki." Nitrogen (0.3 g per pot) was applied at 69 and 85 DAS. Values are mean ± SD (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant at 0.05 probability level. Values followed by the same letters indicate no significant difference among rice lines at P < 0.05 by LSD test.*

capacity, or RuBP regeneration capacity in the broad sense (which would include Calvin cycle capacity and P_i regeneration in addition to electron transport rate; Farquhar et al., 1980; Sharkey, 1985). Yamori et al. (2011a) reported that the value of *A* in rice (cv. Notohikari) at an ambient CO_2 concentration of 380 μmol mol^{-1} was limited by RuBP regeneration rate. We applied the theoretical analysis of our results using the Farquhar and von Caemmerer model (as modified by Sharkey et al., 2007) and found the *A* of NIL(*CAR8*) and "Kohishikari" at 370 μmol CO_2 mol^{-1} tended to be limited by RuBP regeneration rate, although it is close to the limiting region of V_{cmax} (**Figure 4**). The increase of RuBP regeneration rate corresponds to increase of ∼2.0 μmol m^{-2} s^{-1} of *A* at the ambient CO_2 concentration when we calculated from the A-C_i curve. Hence, we conclude that the higher *A* in NIL(*CAR8*) than "Koshihikari" is mainly due to an enhanced J_{max} (**Table 1**). "Habataki" had even higher J_{max} and *A*, indicating "Habataki" includes additional QTL for enhanced J_{max}.

A number of possibilities could explain how J_{max} is enhanced in NIL(*CAR8*) and "Habataki." The simplest is that higher LNC_a and LNC_w in these lines provides more photosynthetic protein in both leaf area and leaf weight basis. This is probably the best explanation since both the V_{cmax} and J_{max} were increased, indicating an across the board enhancement of photosynthetic protein. With respect to J_{max}, it has been suggested that the electron flow through the Cytochrome b_6/f complex is a rate-limiting step for RuBP regeneration (Yamori et al., 2011b). Therefore, the increased LNC in NIL(*CAR8*) likely increases the cytochrome b_6/f, but could also the photosystems, quinones, and plastocyanin components of whole chain-electron transport. Enhanced Calvin cycle protein may also contribute to higher *A* should it share in the limitation of J_{max} (Raines, 2011). Enzymes of starch and sucrose synthesis probably do not, as the CO_2 responsiveness apparent in the A/C_i curve at 370 μmol mol^{-1} is much greater than would be expected under a P_i regeneration limitation (Sage, 1990). The higher LNC might be explained by the higher net accumulation of aboveground nitrogen and/or the higher rate of distribution of nitrogen to leaves (Mae and Ohira, 1981). This should be elucidated in future study. It is known that *HAP3* genes are associated with chloroplast biosynthesis and photosynthesis. In rice, an RNA interference construct silencing *OsHAP3A*, *OsHAP3B,* and *OsHAP3C* resulted in reduced expression of nuclear-encoded photosynthesis genes and degenerated chloroplast (Miyoshi et al., 2003). Recently, Alam et al. (2015) showed the overexpression of *OsHAP2E* increased the leaf chlorophyll content and *A* in rice. These suggest that HAP members redundantly affect the leaf photosynthesis in rice. It is also reported that the overexpression of *TaNF-YB3*, a member of *HAP3*, led to increases in the leaf chlorophyll content

TABLE 3 | Hydraulic conductance from roots to leaves (C_p), root surface area (S_r), and hydraulic conductivity (L_p) of plants grown in a controlled-environment cabinet.

	C_p	S_r	L_p
	10^{-8} m^3 s^{-1} MPa^{-1}	m^2	10^{-8} m s^{-1} MPa^{-1}
Koshihikari	0.128 ± 0.011c	0.079 ± 0.016b	1.66 ± 0.33b
NIL(*CAR8*)	0.191 ± 0.022b	0.081 ± 0.013b	2.39 ± 0.45a
Habataki	0.241 ± 0.006a	0.152 ± 0.024a	1.62 ± 0.24b
ANOVA	***	**	*

*The measurements were conducted at the vapor pressure deficit (VPD) of 1.5 kPa. C_p and S_r are expressed per stem. Values are mean ± SD (n = 4). *P < 0.05; **P < 0.01; ***P < 0.001. Values followed by the same letters indicate no significant difference among rice lines at P < 0.05 by LSD test.*

and photosynthesis in wheat (*Triticum aestivum*, Stephenson et al., 2011). These reports imply the association to the increased J_{max} in this study.

We then considered the second hypothesis that higher *A* results from enhancement of CO_2 diffusion from air into leaves. NIL(*CAR8*) showed higher g_s than "Koshihikari" at full heading stage (**Table 1**). While much of the g_s response could reflect the regulation of g_s to track *A* (Wong et al., 1979), there was a slight increases in C_i and C_i/C_a ratio in NIL(*CAR8*) relative to "Koshihikari" (**Table 1**). The higher C_i/C_a in NIL(*CAR8*) demonstrate a greater proportional increase in g_s than *A*, such that the stomatal control over *A* has been relaxed. The 3% higher C_i in NIL(*CAR8*) than "Koshihikari" at the ambient CO_2 concentration corresponds to increase of 1.5 μmol m^{-2} s^{-1} of *A* calculated from the A/C_i curve. These results indicate that *CAR8* enhances g_s independently of *A*.

In rice, g_s is determined by stomatal density, pore length, and aperture (Maruyama and Tajima, 1990; Ohsumi et al., 2007). Our results show that *CAR8* increases g_s by increasing stomatal aperture rather than stomatal density (**Figure 5**). The increase in stomatal density may also increase g_s in rice because stomatal density is higher in "Habataki" than in "Koshihikari" (**Figure 5**). This indicates that "Habataki" has alleles that enhance stomatal density, and a combination of these alleles and *CAR8* may further enhance g_s.

Stomatal conductance responds to changes in plant water status (Schulze and Hall, 1982), and several studies have shown that it is closely related to C_p (Meinzer and Grantz, 1990; Hirasawa and Ishihara, 1992; Hubbard et al., 2001; Cochard et al., 2002; Brodribb et al., 2007). NIL(*CAR8*) had higher C_p than "Koshihikari" due to higher L_p (**Table 3**). We also found that Ψ_l in NIL(*CAR8*) was similar to that of "Koshihikari" while g_s in NIL(*CAR8*) remained high regardless of VPD conditions (Figure S2). This suggests that the higher water uptake of the root in NIL(*CAR8*) keeps Ψ_l high and decreases the risk of water stress even though *T* in NIL(*CAR8*) is significantly higher than "Koshihikari." Therefore, the higher g_s in NIL(*CAR8*) would be partially explained by the higher L_p. In contrast, Sakurai-Ishikawa et al. (2011) suggested the increase of water demand of shoots enhances root hydraulic conductivity via increase in gene expression of several aquaporins in the plasma membrane intrinsic protein family. This might explain the concomitant increases of g_s and L_p in NIL(CAR8). To our knowledge, there has been no report that shows association between HAPs genes and stomatal conductance. The identification of molecular network of *CAR8* would help to understand the regulations of stomatal conductance in rice. Kanemura et al. (2007) reported a weak negative relationship between g_s of flag leaves and days to heading using the rice diversity research set of germplasm. This suggests that flowering time affects photosynthesis of flag leaves and the allelic variation of *CAR8* would explain in part the natural variation of photosynthesis. This also implies the necessity to determine the association between flowering genes and photosynthesis, comprehensively.

The final grain yield in NIL(*CAR8*) was inferior to that in "Koshihikari" (Figure S3). This might be resulted from the short growth duration due to the "Habataki" allele of *CAR8* gene. To enhance the grain yield in rice, we should extend the growth duration of NIL(*CAR8*) by adding genes which delay heading date or modifying the growth conditions such as planting time.

In conclusion, the "Habataki" allele of *CAR8* associates to LNC under the 12-L pot condition. The higher LNC in NIL(*CAR8*), which relates to the higher RuBP regeneration rate, would mainly explain the enhanced *A* of the flag leaves. The "Habataki" allele of *CAR8* also associates hydraulic conductivity and hydraulic conductance at full heading stage under the 3-L pot condition. This could allow for a higher g_s in NIL(*CAR8*), which would partially explain the enhanced *A*. The fine mapping suggested that *CAR8* encodes a putative OsHAP3 subunit of a CCAAT-box-binding transcription factor and is identical to *DTH8/Ghd8/LHD1*, which has been reported to regulate flowering date. Identification of its molecular function would help understanding the association between photosynthesis and flowering and demonstrate specific genetic mechanisms that can be exploited to improve photosynthesis in rice and potentially other crops.

AUTHOR CONTRIBUTIONS

SA, TY, RS, and JY designed the experiments. SA, KY, UY, and JS performed the experiments. TT built the stomata counting system. SA, TO, RS, TH, and JY wrote the manuscript.

FUNDING

This work was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science (Postdoctoral Fellowship to SA), Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology to SA, the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics-based for Agricultural Innovation, RBS-2006 to TH), and the Institute of Global Innovation Research in TUAT to SA and TH.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Wu and Dr. Y. Katayose for help in genome sequence analysis, Ms. H.J. Zhu, Ms. N. Iioka, Ms. M. Takahashi, Ms. M. Iizumi, Ms. Y. Shimazu, and Ms. E. Abe for their excellent technical support, and Dr. K. Hori, E. Yamamoto, and H. Omori for helpful advice on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00060/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AS and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.