



A High-Yielding Rice Cultivar “Takanari” Shows No N Constraints on CO₂ Fertilization

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Enhancing crop yield response to elevated CO₂ concentrations (E-[CO₂]) is an important adaptation measure to climate change. A high-yielding indica rice cultivar “Takanari” has recently been identified as a potential candidate for high productivity in E-[CO₂] resulting from its large sink and source capacities. To fully utilize these traits, nitrogen should play a major role, but it is unknown how N levels influence the yield response of Takanari to E-[CO₂]. We therefore compared grain yield and quality of Takanari with those of Koshihikari, a standard japonica cultivar, in response to Free-Air CO₂ enrichment (FACE, +200 μmol mol⁻¹) under three N levels (0, 8, and 12 g m⁻²) over three seasons. The biomass of both cultivars increased under E-[CO₂] at all N levels; however, the harvest index decreased under E-[CO₂] in the N-limited treatment for Koshihikari but not for Takanari. The decreased harvest index of Koshihikari resulted from limited enhancement of spikelet number under N-limitation. In contrast, spikelet number increased in E-[CO₂] in Takanari even without N application, resulting in significant yield enhancement, averaging 18% over 3 years, whereas Koshihikari exhibited virtually no increase in yield in E-[CO₂] under the N-limited condition. Grain appearance quality of Koshihikari was severely reduced by E-[CO₂], most notably in N-limited and hot conditions, by a substantial increase in chalky grain, but chalky grain % did not increase in E-[CO₂] even without N fertilizer. These results indicated that Takanari could retain its high yield advantage over Koshihikari with limited increase in chalkiness even under limited N conditions and that it could be a useful genetic resource for improving N use efficiency under E-[CO₂].

Keywords: chalky grains, climate change adaptation, CO₂ fertilization effects, FACE, grain appearance quality, grain yield, nitrogen nutrition, *Oryza sativa*

INTRODUCTION

Atmospheric CO₂ concentrations ([CO₂]) are rising at an unprecedented rate. Global [CO₂] surpassed a milestone level of 400 ppm in 2015 and the rate of increase is becoming faster (<https://www.esrl.noaa.gov/gmd/ccgg/trends/>). Increasing concentrations of CO₂ and other greenhouse gases will likely induce global environmental changes such as increases in temperatures, which are projected to have negative effects on food production of the major cereal crops (Zhao et al., 2017) or slow the rate of food production increase that is required to meet increasing demand (Iizumi et al., 2017). In contrast, increasing [CO₂] will have a direct positive effect on crop photosynthesis and thereby increase grain yield; however, the size of the CO₂ fertilization effect is one of the major sources of uncertainty in the projection of the global crop production (Rosenzweig et al., 2014; Deryng et al., 2016; Schleussner et al., 2018).

The uncertainty in projecting the CO₂ fertilization effect arises partly from our limited quantitative understanding of crops to elevated [CO₂] (E-[CO₂]) (Hasegawa et al., 2017); however, CO₂ fertilization effects themselves are variable in nature depending on various factors, including temperature, water, nutrition, and crop species or genotype (Kimball, 2016). To exploit the positive effects of E-[CO₂] on crop production in the future, the mechanisms by which the CO₂ fertilization effects vary must be better understood.

Application of nitrogen (N) fertilizers played a significant role in driving crop yields during the Twentieth century, together with genetic improvement for the suitability of a greater use of N (Evans, 1993; Cassman et al., 2003). Efficient use of N is required to reduce the negative impacts of agriculture on agricultural production not only for increasing farm economy but also for reducing environmental loads by agriculture via possible emission of N₂O and contamination of groundwater (Zhang et al., 2015b).

Nitrogen is a key nutritional element mediating plant responses to E-[CO₂]. Nitrogen often limits the enhancement of photosynthesis, biomass production, and yield by E-[CO₂] (Nakano et al., 1997; Kimball et al., 2002). In fact, previous rice FACE experiments showed that grain yield enhancement in E-[CO₂] decreased when N application was below 80 kg/ha (Hasegawa et al., 2017). Nutritional value and appearance quality of the grains are also vulnerable to change under E-[CO₂] (Yang et al., 2007; Myers et al., 2014; Usui et al., 2016). Therefore, it is likely that N management practices will need to be revised or redesigned to achieve optimal productivity and quality outcomes under climate change (Bloom, 2015).

Genetic improvement is another major driver of improved crop productivity and will be the major and promising countermeasure against any negative effects of climate change. There is growing evidence of large variation in the yield response to E-[CO₂] among cultivars. For instance, a rice FACE experiment in Japan revealed that a wide range of yield enhancement (3–36%) exists among cultivars and that higher-yielding cultivars with a large sink size had a greater [CO₂] response (Hasegawa et al., 2013). A more recent study showed that quantitative trait loci for increasing spikelet number were effective in increasing grain yield under E-[CO₂] (Nakano et al.,

2017). Another rice FACE experiment in China also showed a large yield response to E-[CO₂] with hybrid and inbred indica cultivars (Zhu et al., 2015).

These findings are encouraging in that improvement of yield potential can also be beneficial for positive yield response to E-[CO₂]; however, yield improvement as a result of greater biomass generally requires greater nutrient input (Wang and Peng, 2017). We would therefore expect that the yield advantage would be smaller where the nutrient resource is limiting. However, previous cultivar studies of yield responses to E-[CO₂] were conducted under conventional N conditions and the potential interactions among [CO₂], cultivar, and N might have been overlooked.

E-[CO₂] is known to reduce grain appearance quality (Yang et al., 2007), which is exacerbated by increases in temperature largely through the increased occurrence of chalky grains (CG) (Usui et al., 2016). The severity of quality degradation by E-[CO₂] is cultivar dependent (Usui et al., 2014), and N dependent (Usui et al., 2016); however, the combined effects of N and [CO₂] have not been tested to date.

In the present study, we focused on the following two cultivars with contrasting yield potential and appearance quality: Takanari, a high-yielding indica cultivar (Imbe et al., 2004) and Koshihikari, a standard japonica cultivar (Kobayashi et al., 2018). Takanari has several favorable traits under E-[CO₂], including greater photosynthetic capacity (Chen et al., 2014), biomass, and grain yield (Hasegawa et al., 2013), and better appearance quality (Zhang et al., 2015a) with little increase in water use (Ikawa et al., 2018) compared to Koshihikari. Koshihikari has been a dominant rice cultivar in Japan for more than 50 years, accounting for more than a third of the rice planted area of the country (Kobayashi et al., 2018). Koshihikari has an intermediate response to E-[CO₂] in key traits such as yield (Hasegawa et al., 2013), grain appearance quality (Usui et al., 2014) and grain nutritional quality (Myers et al., 2014) and so can serve as an appropriate check cultivar for the study. To design future N management for contrasting cultivars, field level responses to E-[CO₂] must be compared. FACE is currently the most reliable technology to compare the [CO₂] responsiveness of different cultivars in open fields. We therefore grew these cultivars under three different N levels for 3 years and compared yield and quality traits in response to E-[CO₂] at the Tsukuba FACE facility in Japan.

MATERIALS AND METHODS

Site Description and Weather Conditions

The experiments were conducted at the Tsukuba FACE facility, Tsukubamirai, Ibaraki, Japan (35°58'N, 139°60'E, 10 m above sea level) over 3 years (2012–2014). Details of the site are described by Nakamura et al. (2012) and Hasegawa et al. (2013). Briefly, the site was established in 2010 in the central region of Japan. The climate is humid subtropical with an average temperature of 13.8°C and annual precipitation of 1,280 mm. The soil is a Fluvisol, which is typical of alluvial areas with 23% clay and 40% silt contents. The total C and N concentrations were 21.4 and 1.97 mg g⁻¹, respectively.

plot differed year-to-year, but was >15 m² to accommodate at least two cultivars (see section Plant materials and cultivation methods). Each plot was puddled again for uniformity of fertilizer within each treatment.

In 2012, we collected soil samples from 0 to 10 cm of the soil surface at transplanting. After passing the samples through a 2-mm sieve, we extracted exchangeable ammonium in a 1 M KCl solution. Ammonium N was determined in the filtered solution by the indophenol colorimetric method. We also conducted field soil incubation to estimate N supply in the soil in 2012. Before flooding in late April, we took soil samples from the plow layer of 8 N plots and passed them through a 2-mm sieve without air-drying. We then put the sieved unfertilized wet soil (10 g on the dry weight basis) into a 120-ml vial and added distilled water to make up the total amount of water including soil water to 20 ml. We sealed the vials after purging the headspace of the vial with N₂ gas. The vials were then wrapped with aluminum foil to keep the contents dark and placed in the 8 N plot during the flooding period until late August. At harvest, we measured the exchangeable ammonium N. By subtracting the initial ammonium N at transplanting, we estimated N mineralized during the growing season.

Plant Materials and Cultivation Methods

We used a high-yielding indica cultivar, Takanari, and a standard japonica cultivar, Koshihikari. Pregerminated seed of the two cultivars were sown in seedling trays (Minoru Pot 448, Minoru Industrial Co. Ltd. Okayama, Japan) filled with sterilized soil. The seedlings were raised in the puddled open field for approximately 4 weeks, with a tunnel cloche or floating mulch for the first 2 weeks. In each CO₂ and N treatment plots, we manually transplanted these seedlings in late May at a spacing of 30 × 15 cm (22.2 hills m⁻², 3 seedlings per hill). Dates of sowing and transplanting each year are provided in Table 2. The size of each cultivar in each N treatment varied but was >3 m². The field was kept flooded until late August until the surface water was withdrawn for harvesting. The depth of the surface water was 5–10 cm. Herbicide, pesticide, and fungicide were applied as described previously (Hasegawa et al., 2013). In mid-July, just before the stem-elongation phase, we installed plant support netting made of polyethylene, with an aperture size of 30 cm, to prevent plants from lodging.

Growth, Yield, and Quality Measurements

We measured the number of tillers of six hills in each treatment in each block every week from 2 weeks after transplanting until the heading stage and the maximum tiller number was determined as the largest number of tillers observed in these measurements. Culm length was measured for three hills in each plot by measuring the length from the shoot base to the panicle neck node. Panicle emergence was recorded every 2 to 3 days and the heading date was determined as the date when the number of emerged panicles reached 50% of the total productive stems in each treatment. We made all these measurements for 8 N in all years; however, we did not consistently collect all the data for 0 or 12 N for all years, which are indicated as 'ND' (i.e., no data) in Table 2.

At harvest, 18–21 hills from an area of 0.81–0.95 m² in the middle of each plot were collected and air-dried under the rain shelter. We measured yield and yield components following the method described by Hasegawa et al. (2013). Briefly, we first measured air-dried aboveground biomass and moisture content for both grains and straws to estimate dry biomass at harvest. We counted the number of panicles and threshed spikelets to determine spikelet number and grain weight. A subsample of the unhulled spikelets were soaked in an ammonium sulfate solution with a specific gravity of 1.06 to determine the number of filled (sunken) or unfilled (floated) spikelets. Another subsample of spikelets were dehulled and the brown rice yield was determined. The yield was expressed on the 15% moisture content basis.

We also measured grain appearance quality using a subsample of 50 g of the unsieved brown rice with a grain quality inspector (RGH120A; Satake Corp., Hiroshima, Japan) equipped with image analysis software. The inspector classified each grain into such categories as undamaged, cracked, and chalked grains of different types depending on the position of chalk appearance on the grain. Because E-[CO₂] is known to decrease the appearance quality of various cultivars via increased percentage of CG (Usui et al., 2014, 2016), we tested the effects of CO₂, N, and varieties on the percentages of undamaged grains (UDG) and CG (sum of white belly, basal white, and milky white grains).

In addition to the 18–21 hills for yield determination, we collected 4–6 hills at the heading and maturity stages, which were separated by organs and oven-dried at 80°C to determine dry weights. The dried samples were ground and used for tissue N concentration measurements with an NC analyzer (Sumigraph NC-22F, Sumika Chemical Analysis Service, Tokyo, Japan). We did not measure panicle N concentration (PNC) of Takanari in 12 N in 2012 and 2013 or appearance quality in 2012.

Experimental Design and Statistical Analysis

The experimental plots were laid out in a split-split plot design with CO₂ as the main plot, N as the split plot, and cultivar as the split-split plot with four replicates. Analysis of variance (ANOVA) for the year, CO₂, N, and cultivar effects was conducted with the statistical package R, version 3.3.3, using a split-split-split procedure described by de Mendiburu (2017). For the traits with missing cells, we performed ANOVA only for the years when full datasets were available.

RESULTS

Soil N, Growth, and Development

Soil exchangeable ammonium N (EAN) at transplanting in the 0 N plot in 2012 was 1.53 ± 0.32 and 1.59 ± 0.28 g m⁻² in A-[CO₂] and E-[CO₂], respectively. Soil N mineralized during the flooding period in 2012 was 5–6 times greater than the initial EAN and not significantly different between the CO₂ treatments: 8.25 ± 0.73 g m⁻² in A-[CO₂] and 10.0 ± 1.45 g m⁻² in E-[CO₂].

Days to heading (DTH) were on average approximately 5 days longer for Takanari than for Koshihikari ($P < 0.001$, Variety main effect, Table 2). Elevated [CO₂] decreased DTH significantly by 1–3 days ($P < 0.001$), which was similar to previous observations

TABLE 2 | The effects of two levels of [CO₂] and three levels of nitrogen on heading date, maximum tiller number, and culm length ± standard deviation (n = 4) of Koshihikari and Takanari tested at Tsukuba FACE for three growing seasons.

Cultivar	Year	Sowing date	Trans planting date	N (g m ⁻²)	Days to heading ^a		Maximum tiller number (m ⁻²)		Culm length (cm)	
					A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]
Koshihikari	2012	24-Apr	23-May	0	71 ± 0.5	70 ± 1.0	377 ± 19	390 ± 38	77.8 ± 0.5	78.5 ± 1.3
				8	70 ± 0.0	70 ± 0.5	421 ± 61	556 ± 61	87.1 ± 3.1	89.1 ± 1.8
				12	71 ± 0.6	70 ± 0.5	601 ± 45	592 ± 69	96.4 ± 5.6	95.4 ± 3.7
	2013	23-Apr	23-May	0	72 ± 0.0	69 ± 1.8	357 ± 29	381 ± 16	83.1 ± 3.5	83.8 ± 1.7
				8	73 ± 0.6	72 ± 0.6	556 ± 22	584 ± 42	92.2 ± 1.9	97.2 ± 3.4
				12	ND ^b	ND	ND	ND	96.7 ± 5.1	99.3 ± 5.6
	2014	22-Apr	22-May	0	70 ± 1.0	66 ± 0.0	443 ± 40	404 ± 52	79.1 ± 1.7	76.7 ± 4.0
				8	71 ± 0.5	69 ± 1.0	671 ± 31	672 ± 26	92.2 ± 3.8	86.2 ± 1.1
				12	71 ± 1.0	68 ± 1.0	755 ± 49	771 ± 59	95.3 ± 1.9	89.8 ± 4.4
Mean			0	71	68	392	392	80.0	79.6	
			8	71	70	550	604	90.5	90.8	
			12	71	69	678	681	96.1	94.8	
Takanari	2012	24-Apr	23-May	0	ND	ND	ND	ND	63.0 ± 2.9	68.1 ± 3.1
				8	77 ± 0.6	77 ± 1.5	445 ± 50	457 ± 68	69.2 ± 3.9	68.7 ± 3.3
				12	ND	ND	ND	ND	ND	ND
	2013	23-Apr	23-May	0	77 ± 0.8	77 ± 1.0	290 ± 15	271 ± 15	64.2 ± 3.9	65.0 ± 1.6
				8	76 ± 0.5	76 ± 1.0	497 ± 28	456 ± 50	72.7 ± 1.6	75.7 ± 1.6
				12	ND	ND	ND	ND	80.6 ± 2.4	79.8 ± 3.9
	2014	22-Apr	22-May	0	76 ± 1.4	75 ± 1.5	304 ± 52	351 ± 40	60.5 ± 1.2	62.1 ± 1.2
				8	75 ± 1.5	73 ± 0.5	610 ± 32	676 ± 64	69.5 ± 0.9	71.0 ± 2.9
				12	74 ± 0.6	73 ± 0.5	629 ± 88	685 ± 123	71.3 ± 2.3	74.8 ± 2.9
Mean			0	77	76	297	311	62.6	65.1	
			8	76	75	518	530	70.5	71.8	
			12	74	73	629	685	76.0	77.3	

ANOVA FOR THE 2014 DATA^c

CO ₂	***	ns	ns
N	ns	***	***
N × CO ₂	ns	ns	ns
Variety (V)	***	***	***
V × CO ₂	***	*	***
V × N	***	*	ns
V × CO ₂ × N	ns	ns	ns

^aDays from transplanting to heading.^bNo data (not measured).^cANOVA was conducted for 2014 data only without missing cells.Statistically significant effects are indicated as ****P* < 0.001; **P* < 0.05. ns, not significant.

in other rice FACE experiments (Hasegawa et al., 2016); however, the effect differed between the two varieties with a significant interaction between CO₂ and variety (*P* < 0.001). DTH was decreased by as much as 4 days in Koshihikari but only by 1–2 days in Takanari. Maximum tiller number (MTN) also differed significantly between the two cultivars with Koshihikari having approximately 10% greater MTN than did Takanari (*P* < 0.001, **Table 2**). The effect of E-[CO₂] on MTN was not apparent in Koshihikari but was positive in Takanari, which resulted in a significant Variety × CO₂ interaction (*P* < 0.05).

Culm length at harvest was much longer in Koshihikari than in Takanari by as much as 20 cm (*P* < 0.001). The ANOVA result for culm length only showed a strong interaction

between CO₂ and cultivar for the 2014 data, where culm length of Koshihikari was decreased by E-[CO₂] but that of Takanari was increased (Variety × CO₂, *P* < 0.001, **Table 2**). This strong interaction was not observed in other years. The effect of N was significantly positive for MTN and culm length. DTH and MTN showed a significant variety × N interaction.

Yield and Yield Components

Elevated [CO₂] increased paddy (unhulled grain) yield by 78 g m⁻² (9.3%) and aboveground dry mass by 139 g m⁻² (10.0%) when averaged over years, N, and cultivars (*P* < 0.001, **Table 3**). In all treatments, Takanari had a significantly greater paddy yield

TABLE 3 | The effects of two levels of [CO₂] and three levels of nitrogen on paddy yield, aboveground biomass, and harvest index of Koshihikari and Takanari tested at Tsukuba FACE for three growing seasons.

Cultivar	Year	N (g m ⁻²)	Paddy Yield ^a (g m ⁻²)		Aboveground dry mass (g m ⁻²)		Harvest index ^b (%)	
			A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]
Koshihikari	2012	0	476	477	925	954	44	42
		8	756	851	1,323	1,471	49	49
		12	882	950	1,437	1,627	52	50
	2013	0	583	592	1,104	1,206	45	42
		8	817	885	1,532	1,660	45	45
		12	869	948	1,594	1,770	46	46
	2014	0	571	515	1,004	978	48	45
		8	817	836	1,448	1,484	48	48
		12	845	954	1,524	1,661	47	49
	Mean	0	543	528	1,011	1,046	46	43
		8	797	857	1,434	1,538	47	47
		12	865	950	1,518	1,686	49	48
Takanari	2012	0	608	691	912	1,035	57	57
		8	1065	1,132	1,541	1,676	59	57
		12	1156	1,263	1,797	2,022	55	53
	2013	0	698	780	1,076	1,231	55	54
		8	1061	1,183	1,621	1,844	56	55
		12	1058	1,245	1,734	1,953	52	54
	2014	0	623	802	951	1,198	56	57
		8	1102	1,167	1,644	1,735	57	57
		12	1192	1,311	1,816	1,979	56	56
	Mean	0	643	758	979	1,155	56	56
		8	1076	1,160	1,602	1,752	57	56
		12	1135	1,273	1,782	1,985	54	55

ANOVA

Year	*	**	***
CO ₂	***	***	ns
CO ₂ × Year	ns	ns	ns
N	***	***	***
N × CO ₂	ns	ns	ns
N × Year	*	*	*
N × Year × CO ₂	ns	ns	0.067
Variety (V)	***	***	***
V × Year	0.079	**	ns
V × CO ₂	***	*	0.068
V × N	***	***	***
V × Year × CO ₂	ns	ns	ns
V × Year × N	ns	ns	***
V × CO ₂ × N	0.080	ns	*
V × CO ₂ × N × Year	ns	ns	ns

^aExpressed on the 15% moisture content.

^bPaddy yield divided by aboveground biomass both on the 0% moisture.

Statistically significant effects are indicated as ****P* < 0.001; ***P* < 0.01; **P* < 0.05. The value indicates the probability between 0.05 and 0.1. ns, not significant.

than did Koshihikari (*P* < 0.001, Variety main effect) and the difference was greater in E-[CO₂] (*P* < 0.001, Variety × CO₂). The greatest yield enhancement of paddy yield by E-[CO₂] was observed in Takanari in the 0N plot (18.0% averaged over 3

years, **Table 3**), and the smallest enhancement was in Koshihikari in the same 0N plot, resulting in a significant CO₂ × N × Variety interaction (0% averaged over 3 years, *P* = 0.080). The enhancement of aboveground dry mass by E-[CO₂] was higher in

Takanari than in Koshihikari ($P < 0.05$, CO₂ × Variety) similarly across N treatments with no significant three-way interaction. The harvest index (HI) of Takanari was much greater than that of Koshihikari by as much as 9 points on average and was not affected by [CO₂] or N applications (Table 3). In contrast, the HI of Koshihikari was decreased by E-[CO₂] particularly in the 0N plot by 3 points. This resulted in a significant three-way interaction effect (CO₂ × N × Variety) on HI ($P < 0.05$).

The response of hulled grain (brown rice) yield to CO₂ and N was similar to that of paddy yield (Table 4). Takanari had a greater brown rice yield in E-[CO₂] than Koshihikari and the enhancement by E-[CO₂] was particularly high in the 0N plot (CO₂ × Variety × N, $P = 0.057$). Elevated [CO₂] increased sink-related yield components such as panicle density, spikelet per panicle, and spikelet density (CO₂ main effect, $P = 0.098$, < 0.05 and < 0.01 , respectively); however, the effects of E-[CO₂] on spikelet per panicle and spikelet density was different between cultivars (CO₂ × Variety, $P < 0.05$). These yield components were increased by E-[CO₂] by 9 and 11%, respectively, in Takanari; however, virtually no increase was found in Koshihikari. High N application did not moderate the response of these yield components to E-[CO₂]. In contrast, a significant three-way interaction was observed in the percentage of ripened spikelets ($P < 0.05$).

Relationship Between N Uptake and Yield Components

The yield enhancement of Koshihikari by E-[CO₂] was substantially decreased by the limited N supply because HI decreased with a decrease in N supply significantly in E-[CO₂]. In contrast, the HI of Takanari remained unchanged across the different N and CO₂ treatments, which resulted in a significant difference in the CO₂ × N response between the two cultivars. To explore the reason for this difference, we examined the relationship between HI and the sink-related traits. Spikelet number per unit aboveground biomass of Koshihikari was significantly smaller than that of Takanari (Figure 1, Table S1, Variety main effect, $P < 0.01$) and decreased under the reduced supply of N, particularly in E-[CO₂]; however, that of Takanari was unchanged by the N conditions, resulting in a significant Variety × N interaction ($P < 0.001$). The HI was significantly correlated with the spikelet number per biomass (Figure 2A, $P < 0.001$); however, it was not correlated with the percentage of ripened spikelets (Figure 2B).

Aboveground crop N (ACN) increased with N supply (Figure 3, Table S1, N main effect, $P < 0.001$). Takanari had a greater ACN than Koshihikari at all N levels (Variety main effect, $P < 0.01$). The effect of E-[CO₂] on ACN differed between cultivars; E-[CO₂] did not affect ACN of Koshihikari but increased that of Takanari at all N levels, as indicated by a significant Variety × CO₂ interaction ($P < 0.01$).

We observed a close relationship between spikelet density and ACN at heading and harvest; however, the relationship was clearly cultivar-dependent (Figure 4), with Takanari having a substantially greater spikelet production capacity per unit N uptake than Koshihikari. For each cultivar, the spikelet

density-crop N relationship was consistent across the different [CO₂] treatments. In contrast, spikelet density of Takanari increased in response to E-[CO₂] by increasing ACN even under N-limited conditions.

Panicle N Concentration and Grain Appearance Quality

Panicle N concentration (PNC) increased with N supply ($P < 0.001$, Table 5); however, PNC was generally higher in Koshihikari than in Takanari ($P < 0.001$). For PNC, the ANOVA was based on the 2014 data only because of missing observations in the other 2 years. E-[CO₂] decreased the PNC in most treatments; however, this decrease was larger in Koshihikari than that in Takanari (Variety × CO₂, $P < 0.01$). Elevated [CO₂] reduced the percentage of UDG severely, mainly owing to the increased CG ($P < 0.001$, Table 5). The two cultivars also showed a contrasting response to E-[CO₂] for UDG and CG (CO₂ × Variety, $P < 0.001$, Table 5). The decrease in UDG by E-[CO₂] averaged 13.9 points for Koshihikari and 3.1 points for Takanari (Table 5), although UDG of Takanari was generally poor because of the high percentage of malformed grains across different CO₂, N and years (data not shown). The decreases in UDG were mostly accounted for by the increases in CG (96%), which was mainly caused by the occurrence of white-base type CG (71% for Koshihikari and 33% for Takanari, Table S2). Nitrogen application significantly increased UDG and decreased CG ($P < 0.001$); however, the dependence on N application was larger in Koshihikari than in Takanari (Variety × N, $P < 0.001$, Table 5). Koshihikari had the poorest appearance quality in the 0 N plot, whereas Takanari showed the best appearance in the 0 N plot.

There was a significant year effect on both UDG and CG with the 2013 crop showing the poorest appearance quality (lowest UDG and highest CG) among the 3 years ($P < 0.001$, Table 5). The large year-to-year variations in UDG and CG were closely associated with the cumulative temperature above 26°C for 20 days after heading (early grain filling period), which was highest around 40°C d in 2013 and lowest around 17–19°C d in 2012 (Table 5). The effect of N on UDG and CG was largest in 2013 when the quality loss was largest in A-[CO₂]. However, in E-[CO₂], CG exceeded 50% in all three N treatments in Koshihikari. The differences in UDG and CG between the two cultivars were noted in 2013 (Variety × Year × N, $P < 0.05$), where CG of Koshihikari was larger than that of Takanari by as much as 40 points in the 0 N plot.

These significant interactions for the grain quality related traits are summarized in Figure 5. CG of Koshihikari varied significantly among years but significant negative relationships were observed within each year (Figure 5A). The regression lines were statistically different between A-[CO₂] and E-[CO₂] except for in 2013, suggesting that the reduction in PNC was not enough to fully explain the variation in CG due to different [CO₂]. In contrast, CG of Takanari varied little among years and no negative relationship was observed with PNC, with a slightly positive relationship observed on one occasion (E-[CO₂] in 2014, Figure 5B).

TABLE 4 | The effects of two levels of [CO₂] and three levels of nitrogen treatment on yield and yield components of Koshihikari and Takanari tested at Tsukuba FACE for three growing seasons.

Cultivar	Year	N (g m ⁻²)		Brown rice yield ^a (g m ⁻²)		Panicle density (m ⁻²)		Spikelet per panicle		Spikelet density (10 ³ m ⁻²)		Percentage of ripened spikelet		Single grain mass (mg)	
		A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]
Koshihikari	2012	0	375	372	256	263	77	77	19.8	20.1	86.5	84.2	22.1	22.0	
		8	604	679	379	407	407	86	86	31.1	35.1	87.2	86.5	22.1	21.7
		12	709	762	431	440	440	88	88	38.1	38.9	82.1	86.4	21.7	22.2
2013	0	453	453	290	305	305	84	83	24.5	25.3	85.0	77.9	19.8	19.6	
		646	697	387	415	415	88	91	33.9	37.7	83.7	80.6	20.4	19.9	
		686	746	409	423	423	91	95	37.1	40.3	78.6	81.7	20.4	20.0	
2014	0	462	405	462	278	252	84	86	23.4	21.6	85.9	80.5	21.4	20.9	
		656	673	396	368	368	90	95	35.7	35.0	76.7	81.4	21.4	21.3	
		678	769	451	456	456	91	94	41.2	42.9	66.8	73.5	21.1	20.8	
Mean	0	430	410	275	275	273	82	82	22.5	22.4	85.8	80.8	21.1	20.8	
		635	683	387	396	396	87	91	33.6	35.9	82.6	82.8	21.3	21.0	
		691	759	430	440	440	90	93	38.8	40.7	75.8	80.6	21.1	21.0	
Takanari	2012	0	448	511	211	222	131	135	27.4	30.2	84.4	87.1	19.1	19.8	
		807	853	285	289	289	159	172	45.4	49.6	86.3	85.2	19.8	19.9	
		889	967	260	269	269	195	205	50.7	54.9	82.4	81.6	20.4	20.7	
2013	0	514	572	228	228	239	134	147	30.6	35.0	83.9	80.9	19.2	19.1	
		804	900	272	285	285	163	175	44.5	49.8	82.6	81.6	20.4	20.3	
		784	949	292	304	304	166	179	48.3	54.6	69.6	79.0	20.5	20.5	
2014	0	476	616	196	196	222	143	158	28.1	35.1	84.2	84.5	19.0	19.5	
		848	896	259	270	270	189	193	49.1	52.2	74.5	75.8	20.3	20.2	
		917	1013	271	291	291	199	202	53.9	58.7	71.6	74.1	20.6	20.8	
mean	0	479	566	211	211	228	136	147	28.7	33.4	84.2	84.2	19.1	19.4	
		820	883	272	281	281	171	180	46.3	50.5	81.1	80.9	20.2	20.1	
		863	977	274	288	288	187	196	51.0	56.0	74.5	78.2	20.5	20.6	
ANOVA															
Year		*	ns	ns	***	***	***	**	***	***	***	***	***	***	***
CO ₂		***	0.098	ns	*	ns	*	**	ns	ns	ns	ns	ns	ns	ns
CO ₂ × Year		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.087
N		***	***	***	***	***	***	***	***	***	***	***	***	***	***
N × CO ₂		0.079	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
N × Year		*	**	**	**	**	**	**	**	**	**	**	**	**	**
N × Year × CO ₂		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Variety (V)		***	***	***	***	***	***	***	***	***	***	***	***	***	***
V × Year		0.056	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.055
V × CO ₂		***	***	***	***	***	***	***	***	***	***	***	***	***	***
V × N		***	***	***	***	***	***	***	***	***	***	***	***	***	***
V × Year × CO ₂		ns	**	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V × Year × N		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V × CO ₂ × N		0.057	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V × CO ₂ × N × Year		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

^aUnsieved brown rice yield expressed on the 15% moisture content. Statistically significant effects are indicated as ***P < 0.001; **P < 0.01; *P < 0.05. The value indicates the probability between 0.05 and 0.1. ns, not significant.

DISCUSSION

CO₂ Fertilization Effects Influenced by N and Cultivars

The limitation of CO₂ fertilization effects as a result of limited N availability has long been known at the leaf physiological level or crop production level (Stitt and Krapp, 1999; Kimball et al., 2002). The present study has shown that the N-limited CO₂ fertilization effect does not hold for Takanari, a high-yielding indica cultivar. Our 3-year FACE experiments with three N levels confirmed that yield enhancement of Koshihikari, a standard japonica rice cultivar, by E-[CO₂] was limited by the soil N availability. The 3-year average yield enhancement of Koshihikari was 0%, whereas that in the standard and high

levels of N was 10% (Table 3). We also confirmed that the N limited CO₂ fertilization effect on grain yield was mainly via reduced biomass allocation to the grains (i.e., HI) rather than limited biomass increase at E-[CO₂] as was found in previous rice FACE experiments (Kim et al., 2003; Kobayashi et al., 2006; Zhang et al., 2013). Ample evidence exists that the biomass response to E-[CO₂] is limited by soil N availability in various plant species (Reich and Hobbie, 2012; Feng et al., 2015), which are in agreement with the physiological observations that the enhancement of leaf photosynthesis or canopy light use efficiency was decreased owing to poor N status of the leaves (Nakano et al., 1997; Sakai et al., 2006). In the present study, a relatively large amount of N was taken up by the crops even when no N fertilizer was applied, averaging 6.6 g m⁻² for 100–110

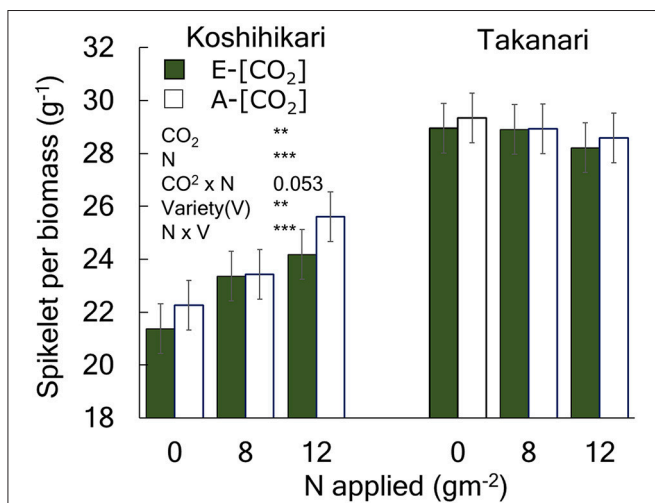


FIGURE 1 | The effects of N and [CO₂] on the spikelet number per unit aboveground biomass of Koshihikari and Takanari. The 3-year averages and a summary of ANOVA results with significant treatment and variety effects are shown in this figure. All data and the ANOVA results are provided in **Table S2**. *** and ** indicate significant differences at *P* = 0.001 and 0.01, respectively. The value indicates the probability between 0.05 and 0.1. Vertical bars are the standard errors of the residual of the ANOVA.

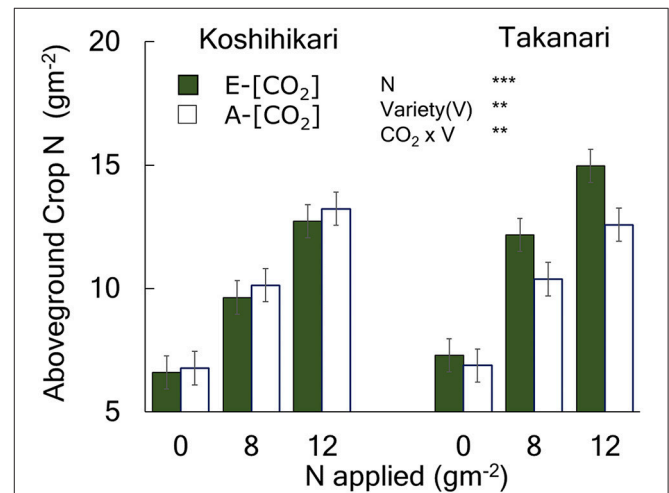


FIGURE 3 | The effects of N and [CO₂] on the aboveground crop N of Koshihikari and Takanari measured at maturity. The 2014 data with full data sets without missing cells are presented with a summary of ANOVA results with significant effects. All available data are presented in **Table S2** along with a summary of ANOVA using 0 and 8N over the 3-year period which agreed with that in 2014 without significant interactions with year. *** and ** indicate significant differences at *P* = 0.001 and 0.01, respectively. Vertical bars are the standard errors of the residual of the ANOVA.

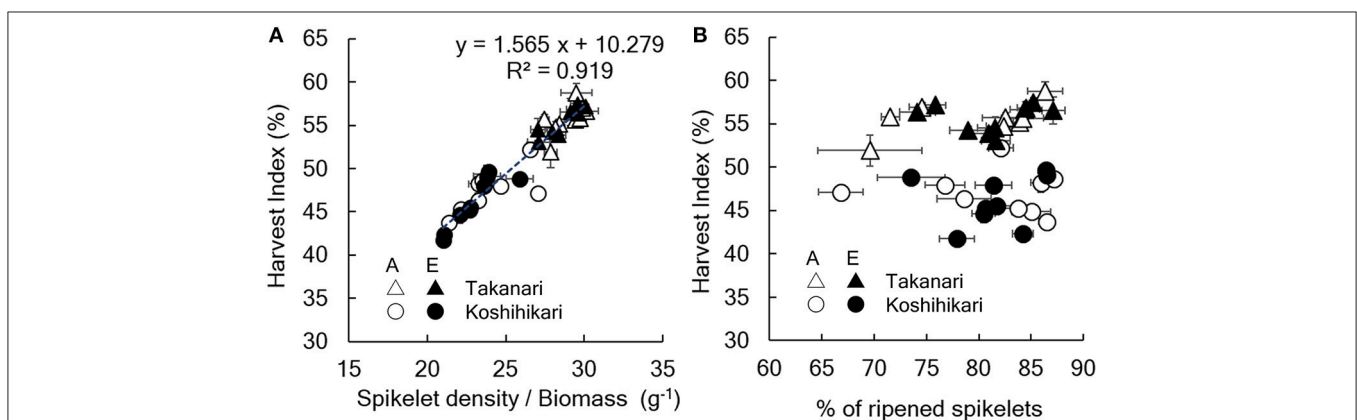
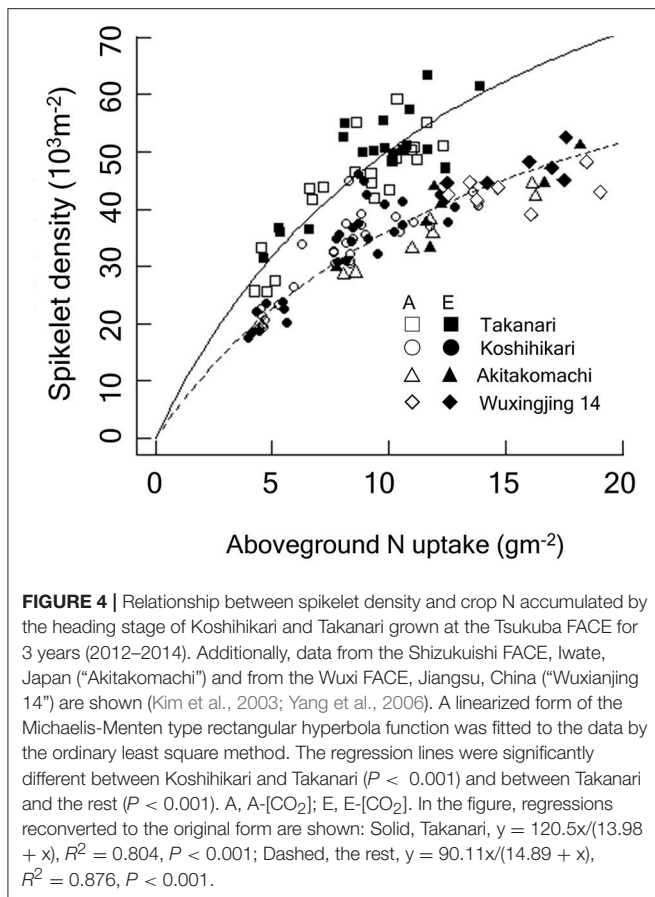


FIGURE 2 | Relationship between harvest index and (A) spikelet number/aboveground biomass or (B) % of ripened spikelets. A, A-[CO₂] = ambient [CO₂]; E, E-[CO₂] = elevated [CO₂]. Vertical or horizontal bars are the standard errors of the mean in each year.



days. This is similar to the typical rice paddy soil fertility in Japan (Toriyama, 2002) and the present N limitation might not have been so severe as to limit biomass enhancement in the E-[CO₂] treatment.

The HI of Koshihikari was decreased by E-[CO₂] under N-limited conditions because of the sink limitation. Elevated [CO₂] increased biomass even in the N-limited condition; however, it did not increase spikelet number, and thereby reduced the sink:biomass ratio (Figures 1, 2A). This sink limitation did not occur in Takanari even without N application. Consequently, Takanari had a significant yield increase of 18% by E-[CO₂] in the 0N plot averaged over 3 years, whereas Koshihikari had virtually no increase in yield.

The major determinant of spikelet density is crop N uptake (Wada, 1969). We also confirmed the close positive association between crop N at the heading stage and spikelet number. In Figure 4, we included data from previous FACE experiments conducted in Shizukuishi, Japan and in Wuxi, China with 3 N levels over 3 years (Kim et al., 2003; Yang et al., 2006), both of which used japonica cultivars: Akitakomachi in Shizukuishi and Wuxianjing 14 in Wuxi. Spikelet density of the three japonica-type cultivars fell on the same response function to crop N uptake regardless of the [CO₂] or N treatments, suggesting a strong limitation of spikelet production by crop N availability. The spikelet density of Takanari was also dependent on N but

was consistently greater than that of Koshihikari at the same crop N for the entire range of crop N. Takanari is known for its high N use efficiency (Taylaran et al., 2009), and this characteristics was maintained in E-[CO₂].

The large spikelet density of Takanari apparently resulted from a larger number of spikelets per panicle rather than from panicle density (Table 4). A previous study revealed that Takanari possess at least two genes that increase spikelet number per panicle: *AP01* and *GN1a* (Takai et al., 2014, 2018). These genes have proven to be effective under E-[CO₂] (Nakano et al., 2017). The present study showed that the advantage was retained under the limited N condition.

The greater yield or spikelet response of Takanari to E-[CO₂] compared to Koshihikari resulted from the enhanced crop N uptake even without N fertilizer application (Figure 3). Initially, we hypothesized that the advantage of high-yielding Takanari in response to E-[CO₂] over Koshihikari was pronounced with greater N supply because a limited availability of N would not meet the high N demand by Takanari. However, this hypothesis was not supported by our results; crop N uptake of Takanari was enhanced by E-[CO₂] even in the 0N plot, which in turn increased spikelet density (Figure 4), whereas that of Koshihikari was not. The source of additional N uptake by E-[CO₂] for Takanari in the 0N plot could not be identified in the present study. In an earlier study (Taylaran et al., 2009), showed that Takanari had significantly greater root length density than japonica cultivars, which might help to absorb N in the N-depleted soil. We speculate that E-[CO₂] could enhance the ability of Takanari to collect N from the subsoil. However, there is a concern about the long-term sustainability of the soil N supplying capacity if Takanari-type cultivars are repeatedly grown, although there is limited evidence that long-term CO₂ fumigation increases soil C and N accumulation (Guo et al., 2015). However, at least for the 3 years tested in the present study, the advantage of Takanari over Koshihikari was consistently evident.

There has been only limited evidence that E-[CO₂] affects N₂ fixation in the paddy (Hayashi et al., 2016); however, one previous study showed increased biological N₂ fixation activity by E-[CO₂] (Hoque et al., 2001). Free-living N₂ fixation was not affected by E-[CO₂] (Hayashi et al., 2014); however, there is a possibility that associative N₂ fixation might be enhanced by E-[CO₂]. Nitrification and denitrification processes are a large source of N loss in the paddy soil (Ishii et al., 2011; Bloom, 2015). There is growing evidence that genotypic difference in N use efficiency is related to the capacity to utilize or transport nitrate in the rhizosphere (Li et al., 2008; Hu et al., 2015) or to inhibit nitrification (Sun et al., 2016). Because we did not measure root morphology or rhizosphere chemistry of the two cultivars, we cannot discuss the relevance of the varietal difference in the N use efficiency in E-[CO₂] observed in our results, but this is an important subject in the future. The long-term response of the soil-plant system to E-[CO₂] is complex and uncertain (Reich and Hobbie, 2012; Reich et al., 2018). Better understandings of soil-plant dynamics that are affected by climatic and genetic factors are key to the effective development

TABLE 5 | The effects of two levels of [CO₂] and three levels of nitrogen treatment on panicle N concentration, % of undamaged grains, and chalky grains of Koshihikari and Takanari tested at Tsukuba FACE for three growing seasons.

Cultivar	Year	N (g m ⁻²)	Panicle N concentration		Undamaged grain		Chalky grain		Heatdose (>26) ^a	
			(mg g ⁻¹)		(%)		(%)		(°C d)	
			A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]	A-[CO ₂]	E-[CO ₂]
Koshihikari	2012	0	8.6	8.4	70.7	58.3	14.1	24.2	16.9	16.8
		8	9.6	9.0	74.7	68.4	8.7	14.6	16.9	16.8
		12	10.7	9.4	70.2	68.9	12.7	15.1	16.9	16.8
	2013	0	8.6	8.1	25.4	12.5	51.5	59.9	39.9	38.1
		8	9.6	8.9	46.1	29.4	33.3	50.1	39.9	39.7
		12	ND ^b	ND	45.2	26.1	33.8	55.4	(39.9)	(39.7)
	2014	0	8.3	8.1	59.5	34.1	28.4	52.2	25.9	23.8
		8	9.4	8.6	60.8	44.7	21.4	37.8	26.7	24.2
		12	10.3	9.1	61.0	46.6	17.6	35.4	26.7	24.2
Takanari	2012	0	8.7	8.4	55.9	52.5	4.6	4.8	(19.4)	(19.4)
		8	9.0	8.6	56.9	59.0	7.7	8.3	19.4	19.4
		12	ND	ND	ND	ND	ND	ND	(19.4)	(19.4)
	2013	0	8.6	8.2	45.1	44.0	13.1	15.6	35.5	37.2
		8	9.3	8.9	49.1	42.2	16.4	21.0	38.4	38.4
		12	ND	ND	46.4	43.4	12.3	19.9	(38.4)	(38.4)
	2014	0	8.7	8.0	48.5	42.5	8.8	17.4	14.9	21.9
		8	9.1	8.8	45.1	43.3	17.0	19.7	21.9	24.7
		12	9.3	9.2	47.0	42.9	16.7	23.9	24.7	24.7
ANOVA ^c			2014 data		2013–2014 data		2013–2014 data			
Year			**		**					
CO ₂			*		***		***			
CO ₂ × Year			ns		ns					
N			***		***		***			
N × CO ₂			ns		ns		ns			
N × Year			**		ns					
N × Year × CO ₂			*		**					
Variety (V)			ns		***		***			
V × Year			***		***					
V × CO ₂			0.098		***		***			
V × N			0.064		***		***			
V × Year × CO ₂			ns		ns					
V × Year × N			*		*					
V × CO ₂ × n			*		ns		ns			

^aCumulative temperature above 26°C for 20 days after heading. The values in the brackets are estimated heatdose assuming that heading dates are the same as those in 8N of the same [CO₂] and cultivar.

^bNo data (not measured).

^cANOVA was conducted for the years without missing cells.

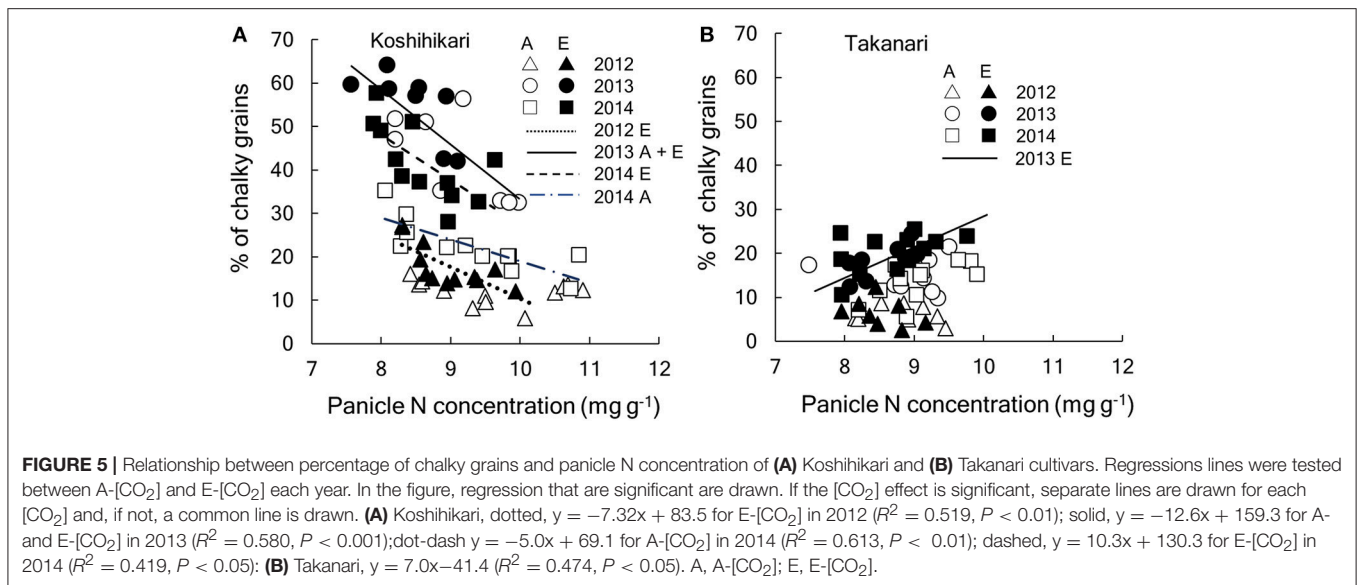
Statistically significant effects are indicated as ****P* < 0.001; ***P* < 0.01; **P* < 0.05. The value indicates the probability between 0.05 and 0.1. ns, not significant.

of long-term sustainable cultivars and management practices under climate change.

Grain Quality Degradation and Quality Related Responses

Grain appearance quality of Koshihikari was substantially decreased by E-[CO₂] by the increased occurrence of CG and quality degradation was particularly noted under the N limited condition (Table 5). The percentage of CG was higher in warmer years, suggesting that the quality degradation by E-[CO₂] was

similar to that frequently observed under heat during the grain filling period (Usui et al., 2016). Previous studies have shown that, under heat, the starch granules of endosperm cells become loosely packed and the air spaces between the granules create chalky appearances (Morita et al., 2016). It has been commonly observed that reduced N or protein content of the grain increases heat-induced CG (Morita et al., 2005; Wakamatsu et al., 2008), in particular, white-base and white-back type CG, which accounted for a majority (71 %) of the variation in the total CG observed in Koshihikari (Table S2). CG increased significantly with a decrease in PNC (Figure 5A). PNC was decreased not only



by reduced N fertilization but also by E-[CO₂], which has been commonly observed in many cereal crops (Myers et al., 2014). Consequently, the highest occurrence of CG was observed in the E-[CO₂] and 0N plot in the hottest summer in 2013 (Table 5). Elevated [CO₂] increased CG partly via the reduced PNC; however, as shown in the relation between CG and PNC (Figure 5), the CG-PNC relation was significantly different between E-[CO₂] and A-[CO₂]. CG of E-[CO₂] was larger than that of A-[CO₂] at the same PNC, suggesting that other factors are involved in the increase in CG by E-[CO₂].

Previous studies showed that E-[CO₂] increased panicle temperature as a result of decreased leaf stomatal conductance and transpirational cooling by E-[CO₂]; a typical increase in daytime panicle temperature was around 0.3–0.5°C (Yoshimoto et al., 2005). This might not be negligible for appearance quality when the temperature exceeds the threshold of approximately 26°C (Morita et al., 2016). However, it remains unclear how much panicle temperature and PNC account for the quality degradation under E-[CO₂].

In contrast to Koshihikari, Takanari showed consistently low CG in all treatments and years (Table 5) although UDG was generally poor mainly due to malshaped grains. Recently, breeding efforts are in progress to improve appearance quality traits under high temperatures (heat tolerance) and Takanari is known for its resistance to heat for CG occurrence and is currently used as a donor to heat tolerance (Ishimaru et al., 2016). In our previous FACE study, we showed that Takanari retained a good appearance quality under E-[CO₂] (Zhang et al., 2015a). Other studies revealed that Takanari had a consistently high stomatal conductance in both E- and A-[CO₂] (Chen et al., 2014) and a lower canopy temperature by 0.4°C than Koshihikari (Ikawa et al., 2018). Therefore, the difference in canopy or panicle temperature between the two cultivars might partially account for the difference in CG.

The present study showed that CG of Takanari did not reveal any decrease in grain appearance quality or increase in CG

under N limited conditions (Figure 5B). PNC of Takanari was decreased by E-[CO₂] (Table 5); however, unlike Koshihikari, this decrease in PNC did not increase CG even during the hot summer. Takanari had a much lower % of white-base CG than Koshihikari (Table S2), which is known to be sensitive to N nutrition (Morita et al., 2005); this reduced the sensitivity of CG to PNC. Increases in heat-induced white-base CG can be decreased by additional N fertilization to retain higher PNC (Morita et al., 2016); however, increases in PNC can have negative effects on eating quality (Okadome, 2005). The low CG of Takanari even under low N conditions is thus a favorable trait to make both appearance and eating quality traits compatible.

Mechanistic understanding of the negative correlation between PNC and CG for standard japonica cultivars is still limited. A recent genetic and molecular study revealed that *sucrose synthase 3 (Sus3)* derived from an indica cultivar Habataki, a sister variety of Takanari, increases under high temperature, which confers heat tolerance for appearance quality (Takehara et al., 2018). The relevance of sucrose synthase and N availability is unknown; however, lower PNC might reduce this enzyme and possibly exacerbate appearance quality. It is unclear how Habataki *Sus3* performs under limited N condition; however, this could be effective even under low N availability. Several other quantitative trait loci for appearance quality have also been identified on chromosomes 3, 5, 8, and 12 (Takeuchi, 2016); however, the functions of these remain unknown. Future studies are required to determine genetic and molecular backgrounds for the CO₂ × N × Variety interaction for appearance quality to effectively develop countermeasures against quality degradation under climate change.

CONCLUSIONS

Our 3-year FACE experiment combined with three levels of N fertilizers revealed that the CO₂ fertilization effect of a standard japonica cultivar, Koshihikari, diminished as N availability

decreased, mainly because of sink limitation. However, sink limitation did not occur in Takanari even without N application, resulting in a significant yield enhancement of 18% by E-[CO₂] in the 0N plot. Moreover, Takanari showed a consistently low CG in E-[CO₂], whereas appearance quality of Koshihikari was severely damaged by E-[CO₂], most notably in the 0N plot because of an increased occurrence of CG. These results indicated that Takanari could retain its yield advantage over Koshihikari even under limited N conditions and it could be a useful genetic resource for improving N use efficiency under E-[CO₂].

AUTHOR CONTRIBUTIONS

TH, HS, TT, and KH identified the research question and designed the field experiments. TH, HS, TT, YU, CC, HI, HW, GZ, HfN, and KH conducted the field experiments and determined growth and yield traits. HsN operated the FACE facility and collected environmental data. HS conducted the chemical analysis. MM, TT, and KH conducted soil analysis. TH, HW, and CC conducted the data analysis. TH wrote the manuscript. All commented and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2019.00361/full#supplementary-material>

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