

RESEARCH PAPER

The effects of free-air CO₂ enrichment (FACE) on carbon and nitrogen accumulation in grains of rice (*Oryza sativa* L.)

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Abstract

Rising atmospheric CO₂ concentrations will probably increase rice (*Oryza sativa* L.) yield but decrease grain nitrogen (GN) concentration. Grains attached to different positions in the panicles differ greatly in weight and quality, but their responses to elevated CO₂ (e[CO₂]) are poorly understood, which limits our understanding of the mechanisms of yield enhancement and quality degradation. Thus a free-air CO₂ enrichment experiment was conducted to examine the effects of e[CO₂] on grain mass (GM), grain carbon (GC), and GN accumulation in the spikelets attached to the upper primary rachis branch (superior spikelets; SS) and those attached to the lower secondary rachis (inferior spikelets; IS). e[CO₂] stimulated the rice yield by 13% but decreased the N concentration in the panicle by 7% when averaged over two levels of N fertilizations ($P < 0.01$). The responses of SS and IS to e[CO₂] were different particularly under higher N supply. For SS, e[CO₂] decreased GN by 24% ($P < 0.01$) but did not affect GM. For IS, e[CO₂] increased GM by 13% ($P < 0.05$) but GN was not affected. The reduction of GN due to e[CO₂] started to appear at the beginning of grain filling. These results suggest that future [CO₂] levels probably stimulate the grain growth of IS, most of which are not marketable due to limited size, at the expense of GN reduction in SS. Translocation of N from SS to IS may be a possible mechanism for reduction in GN of SS. This may degrade the grain quality of marketable rice under e[CO₂].

Key words: Dilution, free-air CO₂ enrichment, grain filling, grain mass, inferior spikelets, nitrogen, *Oryza sativa* L., protein, superior spikelets, translocation.

Introduction

A substantial increase in global food production will be required, as the world's population is expected to be >9 billion by 2050 (FAO, 2009). Rice (*Oryza sativa* L.) is a staple food for more than half of the world's current population. Although rice production dramatically increased through genetic improvement along with application of a greater amount of chemical fertilizer during the green revolution in the last half century (Zhang, 2007), by the beginning of the present century, the rice yield increase had started to slow (Horie *et al.*, 2005; Long, 2012).

Anticipated environmental changes, such as high concentrations of ground-level ozone, elevated temperatures, and drought negatively affect rice production (Long, 2012), but the expected increase in the atmospheric carbon dioxide concentration ([CO₂]) is predicted to have a positive effect on the grain yield of rice (Ainsworth, 2008). [CO₂] has been steadily rising, from ~315 $\mu\text{mol mol}^{-1}$ in 1959 to a current atmospheric average of ~390 $\mu\text{mol mol}^{-1}$, and the rise is projected to continue and to reach as high as 550 $\mu\text{mol mol}^{-1}$ by 2050 (Alley *et al.*, 2007). Although higher [CO₂] increases the grain yield

of rice (Kobayashi *et al.*, 2006; Long *et al.*, 2006; Hasegawa *et al.*, 2007), it reduces grain quality, especially in terms of protein content (Seneweera *et al.*, 1996; Lieffering *et al.*, 2004; Terao *et al.*, 2005; Yang *et al.*, 2007; Taub *et al.*, 2008; Seneweera, 2011). To maximize the yield enhancement and maintain grain quality in the future, a better understanding is needed of rice grain growth, yield stimulation, and grain quality degradation, especially the reduction in grain protein, under future elevated atmospheric [CO₂].

Grain yield stimulation by elevated [CO₂] is primarily and consistently achieved by increases in the number of grains (Baker, 2004; Ainsworth, 2008), whereas the response of individual grain weight to elevated [CO₂] has been inconsistent. It is apparently not affected (Baker and Allen, 1993; Ziska *et al.*, 1997), increased (Yang *et al.*, 2006), or has a minor effect on the overall yield increase (Kim *et al.*, 2003). It should be noted that the reported individual grain weight is the average across all grains harvested at maturity, but that grain weight, quality, and development vary widely depending on the grain position within the panicle (Iwasaki *et al.*, 1992; Jongkaewwattana *et al.*, 1993; Wang *et al.*, 2007).

In rice, one spikelet generally bears one grain. The earlier-flowering spikelets located on the upper primary rachis branches are referred to as superior spikelets, and the later-flowering spikelets located on secondary rachis branches of the lower primary rachis branches are referred to as inferior spikelets. The inferior spikelets usually fill more slowly and are smaller and lighter than the superior spikelets (Murty and Murty, 1982; Matsumoto and Yoshida, 1994; Yang and Zhang, 2006, 2010; Zhang *et al.*, 2012).

While elevated [CO₂] increases the carbohydrate supply to the panicle (Sasaki *et al.*, 2005; Madan *et al.*, 2012), the responses of superior and inferior grains to the elevated [CO₂] may be different. There are several possible ways in which elevated [CO₂] might affect the grain filling of superior and inferior spikelets. First, both the superior and inferior spikelets are better filled, producing heavier overall grains; secondly, the superior spikelets might be better filled and the inferior ones are unaffected, producing heavier superior grains; and, thirdly, the inferior spikelets are better filled and the superior ones are not affected, resulting in heavier inferior grains. It is yet to be determined which change actually occurs.

The distinction between the superior and inferior spikelets is important for understanding the mechanisms of reduction in nitrogen (N) concentrations in the grain due to elevated [CO₂], which are not fully understood (Taub *et al.*, 2008). The N and protein concentrations may be diluted by increased carbohydrates under elevated [CO₂] (Gifford *et al.*, 2000). Superior spikelets usually accumulate N earlier and faster than inferior spikelets (Iwasaki *et al.*, 1992, 1993). Therefore, under elevated [CO₂], a limited quantity of available N may be more likely to be allocated to the superior spikelets, causing the protein content of the inferior spikelets to be reduced. If too little N is supplied by fertilization, there may be less available N in the whole plant, which would allow reductions in N and protein to be more easily detected.

Grain growth is mainly accomplished by the accumulation of grain carbon (GC), grain N (GN), and grain mass (GM)

during grain filling. Because elevated [CO₂] alters both the rate and duration of grain filling (Li *et al.*, 2000; Hu *et al.*, 2007), the coordinated accumulation of GC, GN, and GM may be disturbed. Because the superior spikelets are favoured in the assimilate partitioning during grain filling, they fill early in the grain-filling period, and a reduction in protein may not be detected. The inferior spikelets, however, begin to fill rapidly ~20 days after heading (DAH) (Iwasaki *et al.*, 1992), after the period of rapid accumulation in the superior spikelets has ended and much of the available N has already been taken up. As a result, they may be more likely to show reduced protein.

Over the past three decades, various laboratory and chamber experiments have been conducted to examine plant responses to elevated [CO₂], but the need to test findings and hypotheses under field conditions has become increasingly apparent (Leakey *et al.*, 2009). Free-air CO₂ enrichment (FACE) experiments provide a unique platform for investigating how future ecosystems are likely to respond to higher [CO₂] within various ecosystem-scale interactions. Therefore, a FACE experiment was conducted with two N fertilization levels to answer the following questions. (i) Do grains located at different panicle positions respond differently to an elevated [CO₂]? (ii) Under an elevated [CO₂], if the average grain N concentration is decreased, is the reduction restricted to superior or inferior grains or is it observed in all grains? (iii) Will the N concentration in rice grains be reduced by an elevated [CO₂] more at a lower nitrogen fertilization level? (iv) When does the elevated [CO₂] start to decrease the GN concentration during grain filling, and is the response of the superior and inferior spikelets the same with respect to that timing?

The answers to these questions will improve our understanding of grain filling and growth responses to an increased source supply, and of the mechanisms of rice yield enhancement, under elevated [CO₂]. In addition, the results may suggest how grain yield might be increased and grain quality might be maintained in the future.

Materials and methods

Site description, [CO₂] control, and weather conditions

The experiment was conducted at the Tsukuba FACE site in Tsukubamirai city, Ibaraki Prefecture, Japan (35°58'N, 139°60'E; 10 m above sea level) in 2010 and 2011. The climate is humid subtropical with an average temperature of 13.8 °C and annual precipitation of 1280 mm. The soils are Fluvisols, typical of alluvial areas. Additional soil properties are given in Hasegawa *et al.* (2013).

Nakamura *et al.* (2012) have described the method used to control [CO₂]. Briefly, four blocks were established in paddy fields, with each block consisting of two octagonal plots (240 m², 17 m across): an ambient CO₂ (ambient) plot and an elevated CO₂ (FACE) treatment plot. The FACE plots were equipped with emission tubes on the perimeter, where CO₂ was released from the windward side to keep the [CO₂] measured at the central point at 200 μmol mol⁻¹ above the ambient control level. The season-long daytime average [CO₂] in the ambient plots and in the FACE plots was 386 μmol mol⁻¹ and 584 μmol mol⁻¹ in 2010, and 386 μmol mol⁻¹ and 560 μmol mol⁻¹ in 2011, respectively. The seasonal mean air temperature was 25.0 °C in 2010 and 24.1 °C in 2011, higher than that of the average year

of ~23 °C. The 2010 season was also sunnier and drier than was the 2011 season (monthly climate data are given in [Supplementary Table S1](#) available at *JXB* online).

Crop cultivation

Crop cultivation practices in 2010 were reported by [Hasegawa et al. \(2013\)](#), and the 2011 growing season followed essentially the same practices. Briefly, all plots received equal amounts of P and K in early April, before ploughing, when PK compound fertilizer was applied at a rate of 4.36 g m⁻² of P and 8.30 g m⁻² of K. The fields were kept submerged after late April. Each plot was divided into two areas by corrugated PVC boards. In one area, no N fertilizer was applied (0N), and in the other area three kinds of N fertilizer were applied just prior to puddling (SN): 2 g m⁻² as urea, 4 g m⁻² as one type of controlled-release fertilizer-coated urea (type LP100, JCAM Agri. Co. Ltd, Tokyo, Japan), and 2 g m⁻² as another type of controlled-release fertilizer (type LP140, JCAM Agri. Co. Ltd).

Seeds of the rice cultivar Koshihikari were germinated in water and sown into seedling trays, each with 448 circular cells (16 mm in diameter and 25 mm in depth, Minoru Pot 448, Minoru Industrial Co. Ltd, Okayama, Japan). Each cell was filled with sterilized soil amended with fertilizer at the rate of 0.4 g N, 0.35 g P, and 0.5 g K per 1 kg of soil. Three seeds were sown in each cell. After seedling emergence, the seedling trays were transferred to the puddled open field and protected with a tunnel cloche or floating mulch for the first 2 weeks. The seedlings were manually transplanted into hills (30 cm×15 cm) on 26 May in 2010 and 25 May in 2011, with a density of 22.2 hills m⁻² (three seedlings per hill). After the transplanting, the fields were kept flooded until 20 August, ~3 weeks after heading, when the ponding water was drained for harvesting. We applied flush irrigations on several occasions to keep the soil moist.

Sampling and analysis

At physiological maturity, we harvested 21 hills for each subplot, equivalent to an area of 0.945 m² in both years. The harvested plants were dried under a rain shelter, and then their total weight was determined and the number of panicles was counted. After threshing, the spikelets were weighed. The spikelets were de-hulled to determine the brown rice weight. The moisture content of the grains was measured with a grain moisture tester (Riceter f, Kett Electric Laboratory, Tokyo, Japan), and that of the rice straw was determined by the gravimetric method after oven-drying at 80 °C. Brown rice yield and single-grain mass were expressed on the basis of a 15% moisture content.

In 2011, 20–30 hills were monitored in each plot every day and the date of panicle emergence was recorded; the date was written with a marker pen on adhesive tape, which was wrapped around each panicle below the flag leaf sheath. Five panicles were sampled from each plot at 5, 10, 15, 20, 30, and 40 DAH, and oven-dried at 80 °C. The superior and inferior spikelets were then separated ([Supplementary Fig. S1](#) at *JXB* online) according to the method of [Peng et al. \(2011\)](#). After oven-drying, grains were de-husked, weighed, ground into powder, and then subjected to N and C analysis with an NC analyser (SUMIGRAPH NC-22F, Sumika Chemical Analysis Service, Ltd, Tokyo, Japan).

Fifteen panicles from each subplot were collected at maturity and stored in an 80% ethanol solution. From each panicle, the following information was collected: the number of primary and secondary branches, the numbers of spikelets on primary and secondary branches, the numbers of sterile spikelets on primary and secondary branches, and the numbers of superior and inferior spikelets ([Peng et al., 2011](#)).

Statistical analysis

A blocked split-plot design was used for the experiment, with the CO₂ treatment as the main plot and the N treatment as the subplot,

with four replicates. The statistical significance of each factor (FACE versus ambient, 0N versus SN) was tested by using the Mixed Model procedure of the SAS statistical analysis software package (SAS Add-In 4.3 for Microsoft Office, SAS Institute, Tokyo, Japan). For the GM data, the Richards' function was fitted to the time course of GM accumulation in the superior and inferior spikelets and then the active grain-filling duration and average grain-filling rate were estimated according to [Zhang et al. \(2012\)](#).

Results

Grain yield and C and N concentrations of the bulk sample

Compared with the ambient treatment, FACE significantly increased the brown rice yield by 13% averaged across years and N treatments ($P < 0.01$, [Table 1](#)), but the yield enhancement differed between the N treatments, as evidenced by the significant interaction between [CO₂] and N ($P < 0.05$): 11% at 0N and 16% at SN. Harvest index (HI), defined here as the grain dry mass divided by above-ground dry mass, was slightly decreased by elevated [CO₂] ($P < 0.10$), but this effect was only apparent in 0N where the HI decreased by 3.5%, and no reduction occurred in SN, resulting in a significant interaction between [CO₂] and N ($P < 0.05$): elevated [CO₂] did not affect panicle C concentration, but decreased N concentration significantly ($P < 0.01$). The reduction in the panicle N concentration was slightly larger in SN (8.5%) than in 0N (5.5%), with a marginally significant interaction ($P < 0.10$). Other yield components are shown in [Supplementary Table S2](#) at *JXB* online.

Panicle structure

Elevated [CO₂] had no significant effect on the number of primary and secondary branches and the number of spikelets within a panicle ([Table 2](#)). N treatment had significant effects on the numbers of secondary branches, spikelets, and fertile spikelets, which were higher at SN than at 0N. The number of primary spikelets was sensitive to both [CO₂] and the N levels, namely at 0N the number of primary spikelets was increased by FACE, while at SN it was decreased by FACE.

Elevated [CO₂] had no significant effects on the numbers of superior and inferior spikelets. N levels had a significant effect on the number of inferior spikelets; N fertilizer increased the number of inferior spikelets ([Table 3](#)). The effects of FACE on the number of fertile spikelets were dependent on the N level and the grain position: at 0N, the number of fertile superior spikelets was higher in FACE than in the ambient plots; at SN, the number of fertile superior spikelets in FACE was lower and the number of fertile inferior spikelets was higher than in ambient.

Effects of elevated [CO₂] and N levels on GM, GN, and GC contents in superior and inferior spikelets at maturity

Elevated [CO₂] affected the GM of superior and inferior spikelets differently depending on the N fertilization level

Table 1. Effects of [CO₂] (A, ambient; E, elevated) and nitrogen fertilization levels (0g m⁻² and 8g m⁻²) on the panicle number, grain yield, and harvest index of cultivar Koshihikari in 2010 and 2011 and the results of an analysis of variance (ANOVA)

N applied (g m ⁻²)	Year	Brown rice yield ^a (g m ⁻²)			Harvest index ^b (%)			Panicle			Panicle number (m ⁻²)					
		A	E	E/A	A	E	E/A	A	E	E/A	A	E	E/A			
0	2010	400	455	1.14	44.1	43.5	0.99	429	426	0.99	9.00	8.57	0.95	257	272	1.06
	2011	430	459	1.07	44.2	41.4	0.94	430	430	1.00	9.34	8.81	0.94	278	297	1.07
8	2010	552	642	1.16	46.1	46.7	1.01	427	426	1.00	10.10	8.96	0.89	321	351	1.09
	2011	604	698	1.16	45.7	45.5	0.99	433	433	1.00	10.61	9.95	0.94	347	403	1.16
ANOVA ^c																
Year		NS			†			**			*			*		
CO ₂		**			†			NS			**			*		
Year×CO ₂		NS			†			NS			NS			NS		
N		***			**			NS			***			***		
Year×N		†			NS			*			†			NS		
CO ₂ ×N		*			*			NS			†			†		
Year×CO ₂ ×N		NS			NS			NS			NS			NS		

^a Expressed on a 15% moisture content basis.

^b Brown rice yield divided by the above-ground mass expressed on the 0% moisture (dry mass) basis.

^c †P < 0.1; *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

Table 2. Effects of [CO₂] (A, ambient; E, elevated) and nitrogen fertilization levels (0g m⁻² and 8g m⁻²) on the panicle structure of cultivar Koshihikari in 2011 and the results of an analysis of variance (ANOVA)

N applied (g m ⁻²)	Branch number				Spikelet number						Fertile spikelet number						
	Primary		Secondary		Primary		Secondary		Total		Primary		Secondary		Total		
	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E	
0	9	10	16	16	52	55	42	44	93	99	49	52	38	40	87	92	
8	10	10	20	19	58	55	56	54	113	109	55	53	51	49	106	102	
ANOVA ^a																	
CO ₂		NS		NS		NS		NS		NS		NS		NS		NS	
N		NS		**		*		**		**		**		**		**	
CO ₂ ×N		NS		NS		*		NS		NS		*		NS		NS	

^a *P < 0.05; **P < 0.01; NS, not significant.

Table 3. Effects of [CO₂] (A, ambient; E, elevated) and nitrogen fertilization levels (0g m⁻² and 8g m⁻²) on the number of superior and inferior spikelets within a panicle of cultivar Koshihikari in 2011 and the results of an analysis of variance (ANOVA)

N applied (g m ⁻²)	Spikelet number				% of sterile spikelets (%)				Fertile spikelet number				% in total fertile spikelets				
	Superior		Inferior		Superior		Inferior		Superior		Inferior		Superior/total		Inferior/total		
	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E	
0	7.0	7.5	5.8	5.8	5.8	7.3	1.3	1.8	6.8	7.3	4.8	4.8	7.8	7.7	5.5	5.2	
8	7.5	7.5	8.3	8.5	7.0	5.8	2.0	1.8	7.0	6.8	7.0	7.3	6.7	6.9	6.5	6.8	
ANOVA ^a																	
CO ₂		NS		NS		NS		NS		NS		NS		NS		NS	
N		NS		***		NS		NS		NS		**		*		*	
CO ₂ ×N		NS		NS		NS		NS		†		NS		NS		NS	

^a †P < 0.1; *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

(Table 4). At 0N, the GM of superior spikelets in FACE was higher than that in ambient, whereas the GM of inferior spikelets in FACE was lower than that in ambient. At SN, the GM

of superior spikelets in FACE was lower than that in ambient, whereas the GM of inferior spikelets in FACE was higher than that in ambient. The GC content of superior spikelets

Table 4. Effects of [CO₂] (A, ambient; E, elevated) and nitrogen fertilization levels (0g m⁻² and 8g m⁻²) on grain mass, grain carbon, and grain nitrogen in superior and inferior spikelets of Koshihikari in 2011 and the results of an analysis of variance (ANOVA)

N applied (g m ⁻²)	Grain mass (mg kernel ⁻¹)				C concentration (mg g ⁻¹)				C content (mg kernel ⁻¹)				N concentration (mg g ⁻¹)				N content (mg kernel ⁻¹)			
	Superior		Inferior		Superior		Inferior		Superior		Inferior		Superior		Inferior		Superior		Inferior	
	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E	A	E
0	18.3	18.5	13.9	12.2	452	447	452	455	8.3	8.3	6.3	5.6	9.1	8.4	11.6	12.0	0.16	0.16	0.16	0.15
8	20.5	19.8	13.7	15.5	447	457	442	447	9.2	9.0	6.0	6.9	10.0	7.9	14.5	13.4	0.21	0.16	0.20	0.21
ANOVA ^a																				
CO ₂	NS		NS		NS		NS		NS		NS		*		NS		**		NS	
N	***		*		NS		*		***		†		NS		NS		*		†	
CO ₂ ×N	†		*		*		NS		NS		*		NS		NS		*		NS	

^a †*P* < 0.1; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, not significant.

was not affected by [CO₂], while GC of inferior spikelets was similar to the GM of inferior spikelets: at 0N, GC of inferior spikelets in FACE was lower than that in ambient, whereas the GC content of inferior spikelets in FACE was higher than that in ambient at SN. The GN content of superior spikelets was significantly decreased (by 24%) by FACE (*P* < 0.01), whereas GN in inferior spikelets was not affected by FACE. The N level had significant effects on the GM, GC, and GN content of superior and inferior spikelets: N fertilizer stimulated GM, GC, and GN in both the superior and inferior spikelets.

Time courses of GM, GN, and GC in superior and inferior spikelets

Grain-filling patterns differed between superior and inferior spikelets (Fig. 1). In the superior spikelets, GM accumulation started to increase immediately after panicle emergence (Fig. 1A), whereas in the inferior spikelets, rapid GM accumulation began at ~20 DAH (Fig. 1B). The final GM of inferior spikelets was lower than that of superior spikelets: at 0N, the GM of inferior spikelets was 52% lower in FACE and 32% lower in ambient compared with superior spikelets; and at SN, the GM of inferior spikelets was 28% lower in FACE and 50% lower in ambient compared with superior spikelets.

The effects of elevated [CO₂] on GM accumulation were more apparent in the inferior spikelets at SN (Fig. 1B). The duration of active grain filling estimated from the Richards' function fitted to each plot (Zhang *et al.*, 2012) was shorter at 0N, and the average grain-filling rate of superior spikelets was increased and that of inferior spikelets was decreased by the FACE treatment (*P* < 0.05; data not shown). In contrast, the duration of active grain filling was longer at SN, and the average grain-filling rate of superior spikelets was decreased and that of inferior spikelets was increased by the FACE treatment compared with ambient treatment.

Nitrogen levels had a significant effect on the accumulation of GM in both superior and inferior spikelets. In superior spikelets, a difference in GM accumulation between SN and 0N became apparent at ~20 DAH (Fig. 1A). In inferior spikelets, a difference became apparent at ~30 DAH, and the

inferior spikelets apparently continued to accumulate GM even after 40 DAH (Fig. 1B).

FACE and N levels modified the relationship between GN and GM accumulation (Fig. 1, lower panels). FACE decreased the GN concentration, and SN increased the GN concentration in both superior and inferior spikelets. The effects were more apparent before 15 DAH, and from 30 to 40 DAH the trend differed between superior and inferior spikelets (Fig. 1C, D). The GN concentration in the superior spikelets was apparently (*P* < 0.10) decreased by FACE at 5 DAH, 30 DAH (*P* < 0.01), and 40 DAH (*P* < 0.05) (Fig. 1C). SN significantly increased the GN concentration in superior spikelets at 10 DAH (*P* < 0.05) and 20 DAH (*P* < 0.05). The GN concentration in inferior spikelets was significantly decreased by FACE at 30 DAH (*P* < 0.05) and increased by SN at 10 DAH (*P* < 0.10) (Fig. 1D).

With the increase of GM from inferior spikelets to superior spikelets, the GN concentration was decreased (Fig. 2); the slope of the relationship between the GN concentration and GM differed significantly between FACE and ambient (*P* < 0.05). For superior spikelets, although the GM was not affected by FACE, the GN concentration was decreased by FACE (*P* < 0.05).

Discussion

Grain yield stimulation by elevated [CO₂]

Elevated [CO₂] significantly increased the grain yield by 13% (*P* < 0.05, Table 2), as was commonly observed in the previous studies for the rice FACE experiments (Kim *et al.*, 2003; Yang *et al.*, 2006). The grain yield increase was primarily due to the increase in the number of panicles (Tables 1, 2), which was also previously shown (Kim *et al.*, 2003; Yang *et al.*, 2006). The number of spikelets located on primary and secondary branches of each panicle, on the other hand, was not affected (Tables 2, 3). These results show that elevated [CO₂] did not affect the ratio between inferior and superior spikelets, indicating that variations among superior and inferior spikelets with respect to grain weight and grain quality have important effects on the average grain response to elevated [CO₂].

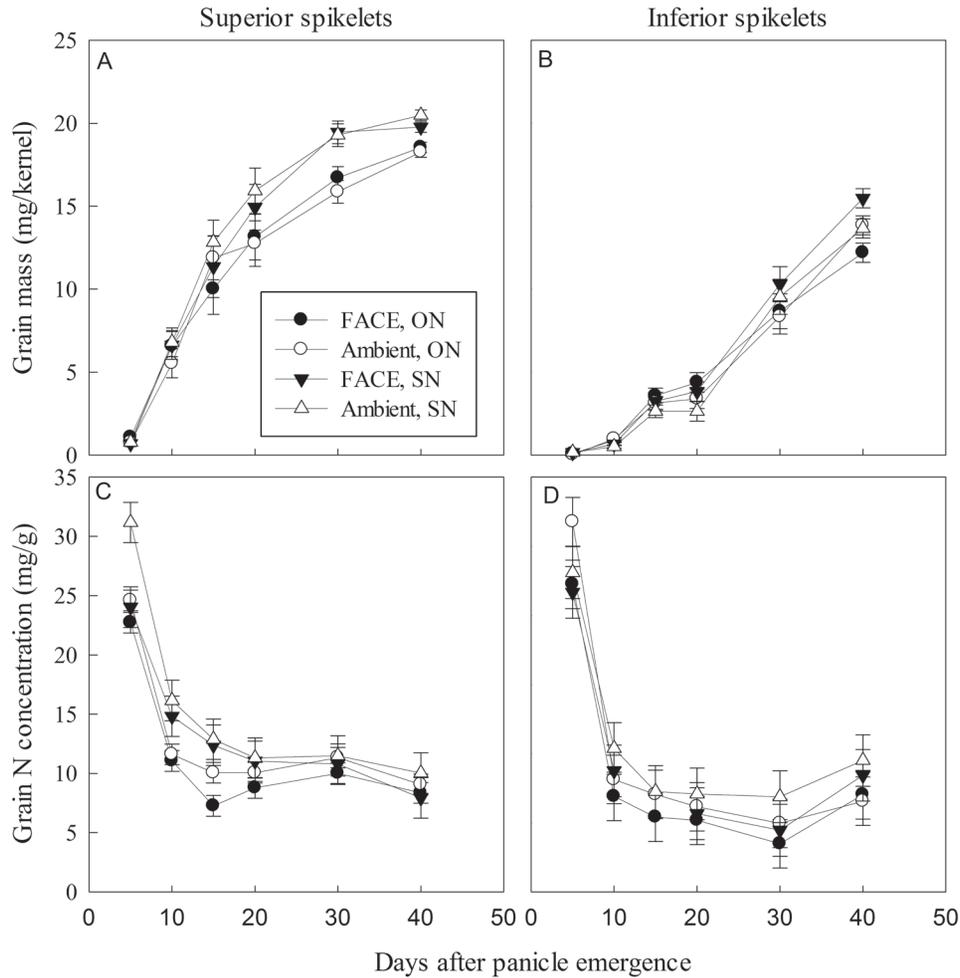


Fig. 1. Changes in grain mass (A, B) and N concentration (C, D) of superior and inferior spikelets grown under two CO₂ conditions (FACE and ambient) and two levels of N supply (ON and SN) in the 2011 FACE experiment. Bars indicate the SEM (*n*=4).

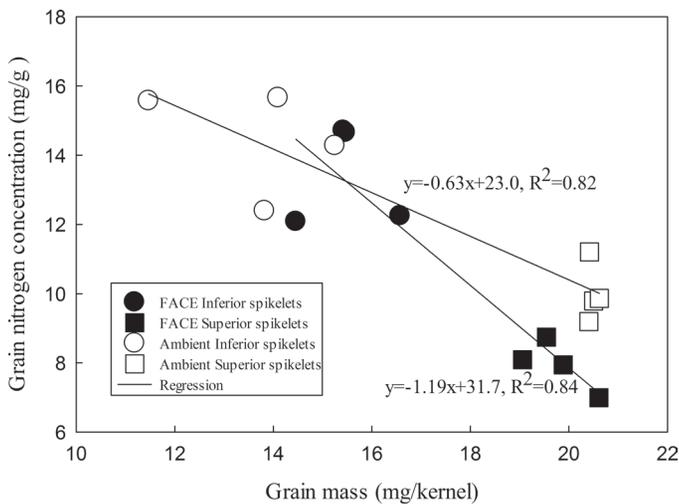


Fig. 2. Relationships between grain mass and N concentration for superior (square) and inferior (circle) spikelets, under two [CO₂] conditions (FACE, closed; ambient, open) under the ample N condition (SN). The regressions lines were drawn for FACE and ambient, respectively. The slopes between FACE and ambient were significantly different (*P* < 0.05).

Is a reduction of GN concentration by elevated [CO₂] a result of dilution?

It has been commonly reported that elevated [CO₂] decreases GN concentration in rice (Seneweera *et al.*, 1996; Lieffering *et al.*, 2004; Terao *et al.*, 2005; Yang *et al.*, 2007; Seneweera, 2011). A significant reduction in N concentration of the bulk panicle samples was also observed (*P* < 0.01, Table 2). Further analysis showed, however, that this decrease is apparent only in superior spikelets (Table 5). The mechanisms by which elevated [CO₂] decreases plant N and protein concentrations are not yet well understood, but one plausible mechanism is that the stimulated production of carbohydrates by elevated [CO₂] dilutes plant N (Gifford *et al.*, 2000; Taub *et al.*, 2008). A negative correlation was also found between the GM and GN concentration when superior and inferior spikelets were pooled (Fig. 2). When the effect of FACE in superior and inferior spikelets was examined separately, however, it was found that the FACE treatment decreased the GN concentration of the superior spikelets, but that their GM was not increased by elevated [CO₂]. Moreover, whereas the GM of inferior spikelets was significantly increased by elevated [CO₂], their GN concentration was not reduced. These results

suggest that the reduced GN concentration cannot be a simple result of dilution by increased GM. Rather, differential responses of C and N allocation to the grains in response to elevated [CO₂] must be a part of the mechanism that accounts for the reduced GN.

In previous experiments under current [CO₂], removal of superior spikelets induced allocation of N to inferior spikelets at various stages during grain filling (Iwasaki *et al.*, 1992, 1993; Kato, 2004). Seneweera (2011) showed that elevated [CO₂] greatly affects rice growth and N partitioning among different organs, and the present study demonstrated that elevated [CO₂] affects the partitioning of N between superior and inferior spikelets.

Reduction of GN under elevated [CO₂] occurred at early grain filling

GN accumulated differently in superior and inferior spikelets during the grain-filling period in response to elevated [CO₂], and the reduction in GN concentration occurred at the beginning of the grain-filling period (Fig. 1C, D). The superior spikelets started the rapid growth immediately after heading, whereas growth and N accumulation in inferior spikelets started only at ~20 DAH (Iwasaki *et al.*, 1992), when superior spikelets had already completed their rapid growth. In this study, under ambient [CO₂], N accumulation trends in superior and inferior spikelets were similar to those previously reported (Iwasaki *et al.*, 1992). They were, however, different from the previous report under elevated [CO₂] (Fig. 1C, D), which showed a reduced GN concentration in superior spikelets at both early (5 DAH) and late grain-filling stages (from 30 to 40 DAH). The mechanisms of these two reductions may be different. The late GN reduction in superior spikelets may be due to the preferential allocation of N to inferior spikelets whose growth has been stimulated by elevated [CO₂], with a greater need for N. In contrast, the early drop in GN concentration may reflect dilution by stimulated carbohydrate accumulation in elevated [CO₂] against lagging N accumulation. At this early stage, the growth of inferior spikelets is slow or has not yet begun, and they are not competing with the superior spikelets for N. No dilution effect was observed in the inferior spikelets at this early stage because of their slow growth. By the time the inferior spikelets began to grow rapidly, after 20 DAH, the rapid growth of superior spikelets had already slowed and much of the available N had been taken up. Therefore, the limited amount of available N was easily diluted by the stimulated carbohydrates. Thus, in inferior spikelets, the GN reduction by elevated [CO₂] occurred at a late stage (30 DAH). The translocation of N to inferior spikelets from superior spikelets from 30 DAH to 40 DAH may have ameliorated the GN reduction in inferior spikelets at maturity under elevated [CO₂], and, at the same time, caused a significant GN reduction in the superior spikelets. In addition to the N reduction, the GM of superior spikelets was also slightly decreased under elevated [CO₂], whereas the GM of inferior spikelets was significantly increased at SN (Table 4). Inferior spikelets are usually limited by the C supply (Murty and Murty, 1982; Fu *et al.*, 2011); thus, the

increased C supply by elevated [CO₂] stimulated the growth of inferior grains.

Limited N supply did not intensify the effects of elevated [CO₂] on GN

The different responses of superior and inferior spikelets to elevated [CO₂] were dependent on the N level (Table 4, Fig. 1A, B). The GM of superior spikelets was little affected by elevated [CO₂]. On the other hand, the GM of inferior spikelets was lower at 0N and higher at SN under elevated than ambient [CO₂]. Poor grain filling of inferior spikelets is reported to be attributed to a sink limitation due to poor activity of enzymes related to starch synthesis (Patel *et al.*, 1996; Liang *et al.*, 2001; Ishimaru *et al.*, 2005), whereas the present results showed that when the source supply was increased by elevated [CO₂], the grain filling of inferior spikelets was improved. Manderscheid *et al.* (2009) reported that grain growth of winter barley under elevated [CO₂] is strongly sink limited, whereas the present results showed that the GM of superior spikelets, which have a larger sink capacity than inferior spikelets, responded negatively to elevated [CO₂]. These results suggest that in rice, more than source supply and sink limitations, other mechanisms regulate the partitioning of assimilate and grain growth in response to environmental change.

Low N fertilization did not intensify N reduction in rice grains under elevated [CO₂] (Table 4). The stimulation of biomass and yield by elevated [CO₂] is usually small at low N levels (Amthor, 2001; Kim *et al.*, 2003; Manderscheid *et al.*, 2009). In this study, the grain yield enhancement and panicle N reduction by elevated [CO₂] were 11% and 5.5% at low N, and 16% and 8.5% at ample N (Table 1). At low N, the carbohydrate stimulation by elevated [CO₂] may not be enough to dilute the GN concentration significantly in well-filled superior spikelets or growth-depressed inferior spikelets. As inferior spikelets usually have a higher N content than superior spikelets (Matsue *et al.*, 1994), inferior spikelets may become an N source for superior spikelets when N is deficient. The translocation of N from inferior to superior spikelets may ameliorate some of the N reduction in superior spikelets at 0N.

The different responses in GM and GN accumulation between superior and inferior spikelets under elevated [CO₂] may be an indication of how rice adapts to environmental change. The present study suggests that under elevated [CO₂], the better growth of superior spikelets occurs at the expense of the depressed growth of inferior grains when N is deficient. On the other hand, when N is ample, the better growth of inferior grains occurs without the greater growth of superior grains.

Relevance of the position-dependent response of grains to elevated [CO₂] with rice quality

The differences in the translocation of N between superior and inferior grains may affect the grain quality response of rice to elevated [CO₂]. The ratio of the number of superior to inferior

grains was not affected (Tables 1–3), but the number of panicles was increased by elevated [CO₂]. Therefore, the numbers of inferior and superior grains increased simultaneously. Compared with superior grains, the cooking quality and appearance of inferior grains are usually lower (Chaudhry and Nagato, 1970; Iwasaki *et al.*, 1992; Jongkaewwattana *et al.*, 1993). Moreover, although the GM in inferior grains was significantly increased by elevated [CO₂], some inferior grains were still too small and thus would be discarded when the grain was sieved to evaluate the thousand grain weight and grain quality (Lieffering *et al.*, 2004), resulting in an overall protein loss. Some well-filled inferior grains might not be discarded during sieving. In fact, while inferior grains were 41% lighter than superior grains, inferior grain mass ranged from 11 mg to 17 mg, the heavier ones being close to the range of superior grain mass (17–21 mg). The reduced grain size would have a negative impact on milling quality (Jongkaewwattana and Geng, 2001). Head rice loss under elevated [CO₂] directly affects the market value of rice and the income of rice producers (Yang *et al.*, 2007). Thus, the protein reduction in the superior grains under elevated [CO₂] might affect the overall grain quality of the yield.

Implications for future experiments

There is an urgent need to understand how future elevated CO₂ concentrations will affect yield and grain quality so that rice yields and grain quality can both be maximized. Elevated [CO₂] stimulates the grain yield of rice mainly by increasing the spikelet number, and the spikelet number increment is mainly achieved by an increase in the number of panicles. The grain yield of cultivars with fewer spikelets within a panicle may also become higher under elevated [CO₂]. The translocation of assimilate among spikelets should be examined in cultivars with fewer spikelets per panicle or with spikelets of uniform size within a panicle. Such cultivars may be better able to adapt to future elevated [CO₂]. Moreover, the differences between superior and inferior grains are also dependent on panicle type and cultivar (Liu *et al.*, 2005; Wang *et al.*, 2007). Thus, cultivars with different panicle types should be tested to better understand the mechanisms of yield enhancement and protein reduction in rice grains under future elevated [CO₂].

Conclusion

Future elevated [CO₂] levels are likely to stimulate the growth of inferior grains at the expense of reductions in the protein content of superior grains. More than a dilution effect caused by the accumulation of more carbohydrates under elevated [CO₂], the differential allocation of N and assimilates between superior and inferior grains is responsible for this reduction in grain protein content, which thus affects the grain quality of the rice.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Superior and inferior spikelets within a rice panicle. The spikelets located on the upper primary branches (painted in black) are referred to as superior spikelets, and the

lower spikelets located on the lower secondary branches (also painted in black) are referred to as inferior spikelets.

Table S1. Weather conditions in the 2010 and 2011 growing seasons at the Tsukuba FACE site, Japan.

Table S2. Yield, yield components, and panicle C and N concentration of rice cultivar Koshihikari obtained in the 2010 and 2011 growing seasons at the Tsukuba FACE experiments.

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References

- Ainsworth EA. 2008. Rice production in a changing climate: a meta-analysis of responses to elevated carbon dioxide and elevated ozone concentration. *Global Change Biology* **14**, 1642–1650.
- Alley R, Berntsen T, Bindoff NL, *et al.* 2007. *Climate change 2007: the physical science basis. Summary for policymakers*. Geneva, Switzerland: IPCC Secretariat.
- Amthor JS. 2001. Effects of atmospheric CO₂ concentration on wheat yield: review of results from experiments using various approaches to control CO₂ concentration. *Field Crops Research* **73**, 1–34.
- Baker J, Allen L. 1993. Contrasting crop species responses to CO₂ and temperature: rice, soybean and citrus. *Plant Ecology* **104**, 239–260.
- Baker JT. 2004. Yield responses of Southern US rice cultivars to CO₂ and temperature. *Agricultural and Forest Meteorology* **122**, 129–137.
- Chaudhry F, Nagato K. 1970. Role of vascular bundles in ripening of rice kernel in relation to the locations on panicle. *Crop Science Society of Japan* **39**, 301–309.
- FAO. 2009. *Global agriculture towards 2050*. Rome: FAO.
- Fu J, Huang ZH, Wang ZQ, Yang JC, Zhang JH. 2011. Pre-anthesis non-structural carbohydrate reserve in the stem enhances the sink strength of inferior spikelets during grain filling of rice. *Field Crops Research* **123**, 170–182.
- Gifford RM, Barrett DJ, Lutze JL. 2000. The effects of elevated CO₂ on the C:N and C:P mass ratios of plant tissues. *Plant and Soil* **224**, 1–14.
- Hasegawa T, Sakai H, Tokida T, *et al.* 2013. Rice cultivar responses to elevated CO₂ at two free-air CO₂ enrichment (FACE) sites in Japan. *Functional Plant Biology* **40**, 148–159.
- Hasegawa T, Shimono H, Yang L, *et al.* 2007. Response of rice to increasing CO₂ and temperature: recent findings from large-scale free-air CO₂ enrichment (FACE) experiments. In: Aggarwal P, Ladha J, Singh R, Devakumar C, Hardy B, eds. *Proceedings of the 26th international rice conference*, 9–12 October 2006. New Delhi, India, 439–447.

- Horie T, Shiraiwa T, Homma K, Katsura K, Maeda S, Yoshida H.** 2005. Can yields of lowland rice resume the increases that they showed in the 1980s? *Plant Production Science* **8**, 259–274.
- Hu J, Yang L, Zhou J, Wang Y, Zhu J.** 2007. Effect of free-air CO₂ enrichment (FACE) on grain filling dynamics of rice. *Scientia Agricultura Sinica* **40**, 2443–2451. (in Chinese with English abstract)
- Ishimaru T, Hirose T, Matsuda T, Goto A, Takahashi K, Sasaki H, Terao T, Ishii R, Ohsugi R, Yamagishi T.** 2005. Expression patterns of genes encoding carbohydrate-metabolizing enzymes and their relationship to grain filling in rice (*Oryza sativa* L.): comparison of caryopses located at different positions in a panicle. *Plant and Cell Physiology* **46**, 620–628.
- Iwasaki Y, Mae T, Fukazawa C, Makino A, Ohira K, Ojima K.** 1993. Glutelin accumulation and changes in the levels of its messenger-RNA in the superior and inferior spikelets of rice ear during ripening. *Plant and Soil* **155**, 211–214.
- Iwasaki Y, Mae T, Makino A, Ohira K, Ojima K.** 1992. Nitrogen accumulation in the inferior spikelet of rice ear during ripening. *Soil Science and Plant Nutrition* **38**, 517–525.
- Jongkaewwattana S, Geng S, Hill J, Miller B.** 1993. Within-panicle variability of grain filling in rice cultivars with different maturities. *Journal of Agronomy and Crop Science* **171**, 236–242.
- Jongkaewwattana S, Geng S.** 2001. Inter-relationships amongst grain characteristics, grain-filling parameters and rice (*Oryza sativa* L.) milling quality. *Journal of Agronomy and Crop Science* **187**, 223–229.
- Kato T.** 2004. Effect of spikelet removal on the grain filling of Akenohoshi, a rice cultivar with numerous spikelets in a panicle. *Journal of Agricultural Sciences* **142**, 177–181.
- Kim H-Y, Liewfering M, Kobayashi K, Okada M, Mitchell MW, Gumpertz M.** 2003. Effects of free-air CO₂ enrichment and nitrogen supply on the yield of temperate paddy rice crops. *Field Crops Research* **83**, 261–270.
- Kobayashi K, Okada M, Kim HY, Liewfering M, Miura S, Hasegawa T.** 2006. Paddy rice responses to free-air [CO₂] enrichment. In: Nösberger J, Long SP, Norby RJ, Stitt M, Hendrey GR, Blum H, eds. *Managed ecosystems and CO₂: case studies, processes, and perspectives*. Berlin: Springer-Verlag, 87–104.
- Leakey ADB, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR.** 2009. Elevated CO₂ effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. *Journal of Experimental Botany* **60**, 2859–2876.
- Li AG, Hou YS, Wall GW, Trent A, Kimball BA, Pinter PJ Jr.** 2000. Free-air CO₂ enrichment and drought stress effects on grain filling rate and duration in spring wheat. *Crop Science* **40**, 1263–1270.
- Liang J, Zhang J, Cao X.** 2001. Grain sink strength may be related to the poor grain filling of indica-japonica rice (*Oryza sativa*) hybrids. *Physiologia Plantarum* **112**, 470–477.
- Liewfering M, Kim H-Y, Kobayashi K, Okada M.** 2004. The impact of elevated CO₂ on the elemental concentrations of field-grown rice grains. *Field Crops Research* **88**, 279–286.
- Liu ZH, Cheng FM, Cheng WD, Zhang GP.** 2005. Positional variations in phytic acid and protein content within a panicle of japonica rice. *Journal of Cereal Science* **41**, 297–303.
- Long SP.** 2012. Virtual Special Issue on food security—greater than anticipated impacts of near-term global atmospheric change on rice and wheat. *Global Change Biology* **18**, 1489–1490.
- Long SP, Ainsworth EA, Leakey ADB, Nosberger J, Ort DR.** 2006. Food for thought: lower-than-expected crop yield stimulation with rising CO₂ concentrations. *Science* **312**, 1918–1921.
- Madan P, Jagadish S, Craufurd P, Fitzgerald M, Lafarge T, Wheeler T.** 2012. Effect of elevated CO₂ and high temperature on seed-set and grain quality of rice. *Journal of Experimental Botany* **63**, 3843–3852.
- Manderscheid R, Pacholski A, Frühauf C, Weigel H-J.** 2009. Effects of free air carbon dioxide enrichment and nitrogen supply on growth and yield of winter barley cultivated in a crop rotation. *Field Crops Research* **110**, 185–196.
- Matsue Y, Odahara K, Hiramatsu M.** 1994. Differences in protein content, amylose content and palatability in relation to location of grains within rice panicle. *Japanese Journal of Crop Science* **63**, 271–277.
- Matsumoto M, Yoshida H.** 1994. Difference of quality of grains in primary and secondary rachis branches. *Japanese Journal of Crop Sciences* **61**, 182–183.
- Murty P, Murty K.** 1982. Spikelet sterility in relation to nitrogen and carbohydrate contents in rice. *Indian Journal of Plant Physiology* **25**, 40–48.
- Nakamura H, Tokida T, Yoshimoto M, Sakai H, Fukuoka M, Hasegawa T.** 2012. Performance of the enlarged rice-FACE system using pure CO₂ installed in Tsukuba, Japan. *Journal of Agricultural Meteorology* **68**, 15–23.
- Patel R, Mohapatra PK.** 1996. Assimilate partitioning within floret components of contrasting rice spikelets producing qualitatively different types of grains. *Australian Journal of Plant Physiology* **23**, 85–92.
- Peng T, Lv Q, Zhang J, Li JZ, Du YX, Zhao QZ.** 2011. Differential expression of the microRNAs in superior and inferior spikelets in rice (*Oryza sativa* L.). *Journal of Experimental Botany* **62**, 4943–4954.
- Sasaki H, Aoki N, Sakai H, Hara T, Uehara N, Ishimaru K, Kobayashi K.** 2005. Effect of CO₂ enrichment on the translocation and partitioning of carbon at the early grain-filling stage in rice (*Oryza sativa* L.). *Plant Production Science* **8**, 8–15.
- Seneweera S.** 2011. Effects of elevated CO₂ on plant growth and nutrient partitioning of rice (*Oryza sativa* L.) at rapid tillering and physiological maturity. *Journal of Plant Interactions* **6**, 35–42.
- Seneweera S, Blakeney A, Milham P, Basra AS, Barlow EWR, Conroy J.** 1996. Influence of rising atmospheric CO₂ and phosphorus nutrition on the grain yield and quality of rice (*Oryza sativa* cv. Jarrah). *Cereal Chemistry* **73**, 239–243.
- Taub DR, Miller B, Allen H.** 2008. Effects of elevated CO₂ on the protein concentration of food crops: a meta-analysis. *Global Change Biology* **14**, 565–575.
- Terao T, Miura S, Yanagihara T, Hirose T, Nagata K, Tabuchi H, Kim HY, Liewfering M, Okada M, Kobayashi K.** 2005. Influence of free-air CO₂ enrichment (FACE) on the eating quality of rice. *Journal of the Science of Food and Agriculture* **85**, 1861–1868.
- Wang F, Chen S, Cheng F, Liu Y, Zhang G.** 2007. The differences in grain weight and quality within a rice (*Oryza sativa* L.) panicle

as affected by panicle type and source–sink relation. *Journal of Agronomy and Crop Science* **193**, 63–73.

Yang J, Zhang J. 2010. Grain-filling problem in ‘super’ rice. *Journal of Experimental Botany* **61**, 1–5.

Yang JC, Zhang JH. 2006. Grain filling of cereals under soil drying. *New Phytologist* **169**, 223–236.

Yang L, Huang J, Yang H, Zhu J, Liu H, Dong G, Liu G, Han Y, Wang Y. 2006. The impact of free-air CO₂ enrichment (FACE) and N supply on yield formation of rice crops with large panicle. *Field Crops Research* **98**, 141–150.

Yang LX, Wang YL, Dong GC, Gu H, Huang JY, Zhu JG, Yang HJ, Liu G, Han Y. 2007. The impact of free-air CO₂ enrichment

(FACE) and nitrogen supply on grain quality of rice. *Field Crops Research* **102**, 128–140.

Zhang H, Li HW, Yuan LM, Wang ZQ, Yang JC, Zhang JH. 2012. Post-anthesis alternate wetting and moderate soil drying enhances activities of key enzymes in sucrose-to-starch conversion in inferior spikelets of rice. *Journal of Experimental Botany* **63**, 215–227.

Zhang Q. 2007. Strategies for developing green super rice. *Proceedings of the National Academy of Sciences, USA* **104**, 16402.

Ziska LH, Namuco O, Moya T, Quilang J. 1997. Growth and yield response of field-grown tropical rice to increasing carbon dioxide and air temperature. *Agronomy Journal* **89**, 45–53.