博士論文

論文題名　**Effects of elevated CO$_2$ concentration on grain yield and quality of rice (*Oryza sativa* L.)**

（高 CO$_2$ 濃度がイネの収量と品質に及ぼす影響）

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Effects of elevated CO$_2$ concentration on grain yield and quality of rice (*Oryza sativa* L.)

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THANK YOU

*A*lthough I am going to earn my PhD degree soon, there is still a long, long way for me to go, to become a real doctor of philosophy. As we known the "philosophy" comes from the ancient greek: philosophia (philo + sophia), which literally means "love of wisdom". I think it is my addiction to wisdom, which has reached to a degree of doctor plus the efforts that I have paid in chasing wisdom made me deserve the doctor degree of philosophy. I wish to express my sincere respect and appreciation to my supervisor: Prof. Kazuhiko Kobayashi for his continuous encouragement and scientific guidance. Special thanks are extend to the other members of my thesis review committee, Prof. Kensuke Okada, Prof. Takashi Yamakawa, Dr. Toshihiro Hasegawa, Dr. Motohiko Kondo for their valuable comments to improve my dissertation.

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Abstract

Atmospheric concentration of carbon dioxide ($[CO_2]$) is projected to exceed 500 μmol mol^{-1} by 2050 (IPCC, 2007), when the world population will reach 9 billion or higher demanding a substantial increase in food production. Rice (*Oryza sativa* L.) is the most important food crop in the world, providing energy and nutrients for more than a half of the world's population. Despite the greater demand for rice production by the growing population, the grain yield increase had started to slow down at the beginning of this century.

The elevated $[CO_2]$ will stimulate grain yield of rice. The yield stimulations by elevated $[CO_2]$ vary depending on field managements and rice cultivars. In order to maximize the beneficial effect of elevated $[CO_2]$ on grain yield, we need to understand the mechanisms better, to develop suitable field management practices, and to select and breed better adapted cultivars. Despite the beneficial effects, elevated $[CO_2]$ could deteriorate the grain quality, which is becoming more important as rice serves as a palatable food component more than just as the source of food energy. Therefore, in order to help the farmers to take advantages of elevated $[CO_2]$ and provide the larger population in the future with sufficient amount and quality of rice, it's urgent to understand the mechanisms that elevated $[CO_2]$ changes grain yield and quality of rice (**Chapter 1**).

Developing efficient field management practices which will fit the future environment may be one of the strategies to take the advantages of elevated $[CO_2]$. As the growth of rice depends on various environmental factors such as water availability, air temperature and soil fertility, which may have interacting effects with elevated $[CO_2]$ on rice. Understanding the interactions and the underlying mechanisms can help us to develop suitable management practices so that we can maximize the benefit from rising $[CO_2]$. Nitrogen fertilizer is one of the most important factors, affecting the grain yield and quality of rice, while studies on the interactions between nitrogen fertilization and elevated $[CO_2]$ on rice have been

limited. Therefore, I studied the interactive effects of elevated $[CO_2]$ and nitrogen fertilization levels on the grain yield and quality of a japonica rice cultivar Koshihikari in a free-air CO_2 enrichment in Tsukubamirai city, Japan (**Chapter 2**).

Like previous studies, we found that nitrogen fertilizers regulated the effects of elevated $[CO_2]$ on grain yield of rice. Rice gained larger yield stimulation by elevated $[CO_2]$ under ample nitrogen condition than low nitrogen condition. Elevated $[CO_2]$ increased the nitrogen fertilizer efficiency on grain yield stimulation. While, elevated $[CO_2]$ degraded the grain quality of rice in terms of increasing the occurrence of various types of chalky kernels, nitrogen fertilizer application failed to counteract the negative effects.

Chalkiness of rice grain is caused by loosely packed starch granules in the endosperm, which is closely related with the grain growth processes. And both grain yield and quality are tightly linked with the grain growth processes, which may be also affected by the elevated $[CO_2]$ and nitrogen fertilizer application. The grain growth is not uniform among grains located at different positions within a panicle, inducing very large differences in grain weight and quality at maturity. Therefore, the responses of grain growth to elevated $[CO_2]$ were compared between superior and inferior spikelets of a rice cultivar Koshihikari grown under different nitrogen fertilizer levels in the free-air CO_2 enrichment in Tsukuba Mirai **(Chapter 3)**.

The effects of elevated $[CO_2]$ was only significant at ample nitrogen fertilizer level, in which the grain growth in inferior spikelets was significantly stimulated by elevated $[CO_2]$, whereas the grain growth in superior spikelets was unaffected. At maturity, the grain weight of inferior spikelets was significantly increased by elevated $[CO_2]$ while that of superior spikelets was unchanged. Elevated $[CO_2]$ reduced the grain nitrogen of the superior spikelets, but did not increase the individual grain mass. Moreover, whereas the grain mass of inferior spikelets was significantly increased by elevated $[CO_2]$, their grain nitrogen was not reduced.

The results showed that when the source supply was increased by elevated $[CO_2]$, especially under ample nitrogen fertilizer conditions, the grain filling of inferior spikelets was improved but not in superior spikelets which have a larger sink capacity than inferior spikelets. Differential responses of carbon and nitrogen allocation to superior and inferior grains in response to elevated $[CO_2]$ may be a part of the mechanisms that accounts for the grain protein reduction. The grain nitrogen reduction should be able to explain the increase of grain chalkiness. The increase of grain chalkiness should also be explained by alterations of source-sink interactions as shown in the disturbed grain growths.

These changes in rice grain growth, however, could vary greatly among cultivars that differ in panicle structures and sink capacity. The grain yield responses to elevated $[CO_2]$ differ among rice cultivars, and a large sink capacity may be the trait that confers a greater yield advantage under elevated $[CO_2]$. The increased sink capacity can be achieved through changes in panicle structures for either greater grain size or a larger number of grains. The both traits had been found to be effective for achieving higher productivity in cultivars Akita 63, a large-grained japonica cultivar, and Takanari, an indica cultivar bearing numerous spikelets within a panicle. Both Akita 63 and Takanari had indeed shown greater yield enhancements under elevated $[CO_2]$ than other cultivars. In order to better understand the grain growth response to elevated $[CO_2]$ and resultant changes in the grain yield and quality, three rice cultivars: Koshihikari, Akita 63 and Takanari were subjected to the free-air CO_2 enrichment in the field (**Chapter 4**).

If elevated $[CO_2]$ preferentially supports grain mass and nitrogen accumulation in inferior spikelet as shown in Koshihikari (**Chapter 3**), reduction in grain protein could be more pronounced in superior spikelet than in inferior spikelet. A cultivar having proportionately more inferior spikelets than Koshihikari with a larger number of secondary rachis branches, as seen in Takanari, could show larger increment of grain yield under elevated $[CO_2]$, since the inferior spikelet exhibit the greater grain growth and less nitrogen reduction than the superior

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spikelets. To the contrary, the increment of grain yield in Akita 63 may be smaller and the grain nitrogen reduction in superior spikelet of Akita 63 may be not as great as that in Koshihikari, because Akita 63 bears fewer secondary spikelets. Thus, we hypothesised that elevated [CO_2] would increase the grain yield and degrade the grain quality of rice most in Takanari followed by Koshihikari and Akita 63 (**Chapter 4**). The results of the study conformed to the expected order of the cultivars in their changes of grain growth in inferior spikelet and grain nitrogen in superior spikelet, and the stimulation of the grain growth of inferior spikelet was indeed largest in Takanari. Nevertheless, the grain nitrogen degradation under elevated [CO_2] was less in Takanari than Koshihikari and Akita 63. Takanari being a high yielding cultivar with high nitrogen use efficiency showed the largest grain yield enhancement and smallest grain quality degradation pointing to a strategy of breeding for varieties better adapted to elevated [CO_2] in the future.

The above findings in the elevated [CO_2] effects on grain yield and quality of rice can be synthesized as follows (**Chapter 5**). Firstly, nitrogen applications will increase the yield stimulation by elevated [CO_2] but additional application of nitrogen fertilizer would not gain proportional yield enhancement. Secondly, nitrogen application cannot counteract the negative effects of elevated [CO_2] on grain quality of rice. This is probably caused by the low efficiency of traditional nitrogen application in meeting the plant nitrogen demand under elevated [CO_2]. The plant nitrogen conditions may have to be improved by a greater nitrogen application after panicle initiation. Thirdly, the grain yield stimulation by elevated [CO_2] is mainly accomplished by the increment in spikelet number with the individual grain weight on the average across the entire spikelets being often unaffected. Nevertheless, the grain growth of inferior spikelets is enhanced by elevated [CO_2] with no change in that of superior grains, which may contribute to a greater yield enhancement. Fourthly, the enhanced grain growth of inferior spikelets is supported by allocating less nitrogen to superior grains, which caused the grain quality degradation in elevated [CO_2]. These insights may be used to

design field management practices and rice cultivars that can take better advantages of elevated [CO_2] in the future. Ensuring the plant nitrogen conditions by extra nitrogen fertilizer application especially after panicle initiation may help rice to increase sink capacity to make use of the advantages of elevated [CO_2]. Rice grain yield will be increased more with the grain quality being maintained with cultivars that have more inferior spikelets accompanied by a greater capacity of sustaining nitrogen supply to the growing grains.

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RICE FUTURE

Facing the rising carbon dioxide concentration

Chapter 1

1.1 Rising atmospheric carbon dioxide concentration

Global warming is mainly the result of rising carbon dioxide concentration ($[CO_2]$) in the earth's atmosphere. Carbon dioxide is the chief greenhouse gas that results from human activities such as: fossil fuel combustion, cement production and land use change. About half of the anthropogenic CO_2 remained in the atmosphere and the rest has been absorbed by the land and oceans (Le Quéré *et al.*, 2009). The increasing CO_2 emission and decline in the efficiency of CO_2 uptake by land and oceans accelerate the increase of atmospheric $[CO_2]$ which causes global warming and climate change (Canadell *et al.*, 2007; Le Quéré *et al.*, 2009).

Atmospheric $[CO_2]$ has stayed higher than the upper safety limit for more than twenty years (Rockstrom *et al.*, 2009), inducing instability of the earth's environment. According to NOAA (the National Oceanic and Atmospheric Administration in USA), the atmospheric $[CO_2]$ has risen from a pre-industrial level of 270 ppm (parts per million) to the current level of 395 ppm (Figure 1.1), 13% higher than the upper safety limit for atmospheric $[CO_2]$- 350 ppm, which can ensure the continued existence of the large polar ice sheets (Rockstrom *et al.*, 2009). As the atmospheric $[CO_2]$ has gone beyond 350 ppm since early 1988, the direct impacts on global warming and extreme whether has started to show evidences. The loss of ice sheet and rising sea level have shown that the earth is becoming unstable compared with the past 10,000 years (Cazenave, 2006; Hansen *et al.*, 2013; Nicholls and Cazenave, 2010). Moreover, both the rise in atmospheric $[CO_2]$ and climate change are accelerating (Figure 1.2).

One of the most direct and profound impacts of climate change on human society can be found in agriculture and global food security (Beddington, 2010). The challenge of maintaining food security is to keep the agricultural production growing as fast as the growing demand, especially under a changing climate and booming population. The world's population

will exceed 9 billion by this mid-century (FAO, 2009), and a substantial increase in global food production will be required. Unfortunately, the climate change is likely to reduce the growth rate of agricultural production in spite of the increasing demand for food, thus jeopardizing the food security (Lobell *et al.*, 2008; Lobell and Gourdji, 2012; Wheeler and von Braun, 2013).

Unlike other environmental changes such as increasing concentration of ground level ozone, higher temperatures, rising sea level, increasing frequency and/or intensity of flooding and drought, that negatively affect agricultural production, the rising atmospheric [CO_2] will stimulate global yields of crop plants (Lobell and Gourdji, 2012; Long *et al.*, 2006b). The atmospheric [CO_2] is predicted to rise to more than 500 ppm (IPCC, Intergovernmental Panel on Climate Change) (Alley *et al.*, 2007), and taking advantage of the rising atmospheric [CO_2] will be one of the most opportune strategies to maintain food security. To this end, selection and breeding crop varieties better suited to the elevated atmospheric [CO_2] may provide a simplest and direct strategy for increasing global yields and maintaining food security under changing climate (Ainsworth *et al.*, 2008a; Ziska *et al.*, 2012).

1.2 The effects of elevated atmospheric [CO_2] on crop

Rising atmospheric [CO_2] is likely to increase global crop yields, especially the major C_3 species (Kimball *et al.*, 2002; Lobell and Gourdji, 2012; Long *et al.*, 2004). In the C_3 photosynthetic pathway, Rubisco, the primary CO_2-fixing enzyme, is catalyzing inefficiently at current atmospheric [CO_2] condition (von Caemmerer *et al.,* 2012). The elevated [CO_2] hence accelerates carbon fixation while alleviating the photo respiratory carbon loss in the C_3 photosynthesis pathway, and thereby exerts the *fertilization effects* for C_3 cereals such as wheat, rice and most vegetable crops. Reduction in the stomatal conductance can also enhance the water use efficiency in both C_3 and

C_4 crops (Ainsworth and Long, 2005). The yield stimulation by elevated $[CO_2]$ varies widely among different studies and varieties (Long *et al.*, 2006a), suggesting a chance to identify and develop cultivars that can maximize the benefit from higher $[CO_2]$.

Despite the beneficial effects, the rising atmospheric $[CO_2]$ may reduce the nutritional quality of crops. Although the rising atmospheric $[CO_2]$ stimulates crop production through providing extra carbon, other elements that are essential for the crops may not be provided sufficiently at the same time. If this is the case, some elements will be diluted by the stimulated carbohydrate supply (Gifford *et al.*, 2000), inducing elemental imbalance in the crops (Loladze, 2002). Reductions in the nutritional quality of crops under rising atmospheric $[CO_2]$ may exacerbate the deficiencies in macro- and micro-nutrients, increasing the "hidden hunger" (Taub *et al.*, 2008). In order to supply a sufficient energy and nutrients for the increasing global population in the future, we need to know how we can take advantages of the elevated atmospheric $[CO_2]$ by increasing crop yield without deteriorating crop quality.

1.3 The effects of elevated atmospheric $[CO_2]$ on rice production

Rice (*Oryza sativa* L.) is a staple food for more than a half of the world's population. Rice yield has dramatically increased through genetic improvement along with greater amount of chemical fertilizer application during the Green Revolution in the latter half of the 20th century (Zhang, 2007). By the beginning of this century, however, the rice yield increase had started to slow down (Horie *et al.*, 2005; Long, 2012).

Elevated $[CO_2]$ is likely to stimulate the grain yield of rice like other C_3 species (Ainsworth, 2008), but the yield stimulation is often compromised by photosynthetic acclimation, limited sink capacities and nitrogen supply constraint (Kant *et al.,* 2012). There may be rooms for better utilizing the extra carbon fixed by the enhanced photosynthesis to get a greater grain yield increase. The yield stimulation by elevated $[CO_2]$ can be enhanced by means

of improved field management, e.g. nitrogen fertilizer application adjusted to the increased nitrogen demand, and the use of rice cultivars better adapted to elevated $[CO_2]$.

Nitrogen application has been reported to increase the growth and yield stimulation by elevated $[CO_2]$ in rice, but the results varied by rice cultivars, research methods and locations (Ziska *et al.,* 1996, Weerakoon *et al.,* 1999; Weerakoon *et al.,* 2000; Kim *et al.,* 2001; Kim *et al.,* 2003; Yang *et al.,* 2006; Zhang *et al.,* 2013). Both the Japan and China FACE (free-air CO_2 enrichment experiment) found that the yield stimulation by elevated $[CO_2]$ and nitrogen fertilization was related with the increment in the fertile spikelet. But there were some inconsistences on the mechanisms of the yield enhancement, for example, in Japan FACE the spikelet number per panicle was increased by elevated $[CO_2]$, while, in China FACE, the spikelet number per panicle was decreased by elevated $[CO_2]$. Whether the inconsistences were caused by cultivar differences or variations in the experimental site are not understood yet, limiting our understanding of the effects of nitrogen application on rice yield stimulation by elevated $[CO_2]$. Studies on the nitrogen application on the effects of elevated $[CO_2]$ on rice grain quality was also limited (Yang *et al.*, 2007).

Cultivar selection and breeding is another main strategy for farmers to take advantages of elevated $[CO_2]$ as the yield responses of rice to elevated $[CO_2]$ were cultivars dependent. And the mechanisms for the yield enhancement were not consistent (Kim *et al*., 2003; Yang *et al*., 2006; Hasegawa *et al*., 2013). Understanding the inconsistences and the physiological mechanisms that elevated $[CO_2]$ stimulate grain yield of rice are urgently needed.

1.4 Effects of elevated $[CO_2]$ on grain quality of rice

Grain quality affects market value and the adoption of new cultivars (Fitzgerald *et al.*, 2009; Fitzgerald and Resurreccion, 2009). Quality traits

encompass physical appearance, processing and milling, cooking and sensory properties and nutritional value.

Elevated $[CO_2]$ reduces grain quality, especially in terms of protein content, which will affect the nutrient intake of hundreds of millions of people (Borrell *et al.*, 1999; Lieffering *et al.*, 2004; Seneweera, 2011; Seneweera *et al.*, 1996; Taub *et al.*, 2008; Terao *et al.*, 2005; Yang *et al.*, 2007a). The reduced grain protein or nitrogen concentration has been explained by the dilution effect, that is, stimulated carbohydrate accumulation dilutes the nitrogen and protein concentrations in the plant (Gifford *et al.*, 2000; Lam *et al.*, 2012). While the dilution cannot explain all the observed decreases in nitrogen concentrations under elevated $[CO_2]$, as the increment of the nonstructural carbohydrates was not proportionally related with the nitrogen reduction. For example, the starch increment was reported to only dilute less than one-third of the nitrogen reduction in wheat grain (Wu *et al.*, 2004), and Donnelly *et al.*(2001) found that the nitrogen reduction in potato tubers was not related with tubers starch or sugar concentration, there must be some other mechanisms that account for the nitrogen reduction.

Among the various quality traits in rice, grain appearance often plays a major role, since rice is predominantly sold in markets as grains rather than flours. Chalkiness, for example, is a major appearance trait that affects milling quality and rice price (IRRI, 2006). In Japan, chalky grains have conventionally been classified into categories based on the position of opaque spots in the endosperm: milky white, white back, basal white, white core, and white belly kernels (Yoshioka *et al.*, 2007), and generally ranked lower than the translucent ones.

Grain chalkiness is a result of anomalous grain development and loosely packed starch granules, and is frequently found under high temperatures during the grain-filling stage (Tashiro and Wardlaw, 1991; Wang *et al.*, 2008). Various studies have been conducted to better understand the genetic and physiological mechanisms that underlie the chalkiness (Cheng *et al.*, 2003;

Ishimaru *et al.*, 2009; Patindol and Wang, 2003; Tabata *et al.*, 2005). In addition to the temperature effects during the grain-filling stage, the occurrence of white back and basal white kernels are more likely to be related to plant nitrogen conditions, whereas the milky white kernels are more likely to be related to source–sink interactions (Kondo, 2011). Elevated $[CO_2]$ has been reported to increase rice grain chalkiness (Yang *et al.*, 2007b), but the mechanisms are not fully understood.

1.5 Grain growth of rice

Grain growth is the key process that determines both grain yield and quality, and is sensitive to the changes in climatic factors, such as temperature, elevated concentrations of ozone and CO_2 (Asseng *et al.*, 2011; Hu *et al.*, 2007; Kobata and Uemuki, 2004; Li *et al.*, 2000a; Liu *et al.*, 2012; Manderscheid *et al.*, 2009; Mulholland *et al.*, 1998). In rice, however, the responses of grain growth to elevated $[CO_2]$ remain unclear. Grain growth is the result of series of processes including floret initiation and development, pollination, and the accumulation of carbon and nitrogen. Elevated $[CO_2]$ could potentially affect all these processes. As under elevated $[CO_2]$ concentrations, the stimulated photosynthesis followed by the changes in nitrogen nutrition (Leakey *et al.*, 2009) would alter carbon and nitrogen accumulation in grains, which could, in turn, influence the quality of rice grains.

Growth rates are not uniform among grains located at different positions within a panicle of rice. The duration of flowering of an entire panicle is normally 4 to 7 days. The different timing of flowering and grain filling leads to great variation in the weight and quality of grains borne on superior spikelets and inferior spikelets, referred to as superior and inferior grains, respectively (Cheng *et al.*, 2007; Liu *et al.*, 2005; Matsue *et al.*, 1994; Wang *et al.*, 2007).

Grain growth is mainly accomplished by the accumulation of grain carbon, grain nitrogen, and grain mass during grain filling. Because elevated $[CO_2]$ alters both the rate and duration of grain filling (Hu *et al.*, 2007; Li *et al.*, 2000b), the coordinated accumulation of grain carbon, grain nitrogen, and grain mass may be disturbed. Because the superior spikelets are favored in the assimilate partitioning during grain filling, they fill early in the grain-filling period, and a protein reduction may not be detected. The inferior spikelets, however, begin to fill rapidly around 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 nitrogen has already been taken up. As a result, the inferior spikelets may be more likely to show reduced protein under elevated $[CO_2]$. These mechanisms will improve our understandings in the grain growth response to elevated $[CO_2]$, but have not been studied.

1.6 Methodologies for CO_2 enrichment

Over the past few decades, various laboratory, greenhouse and chamber experiments have been conducted to examine plant responses to elevated $[CO_2]$, but the need to test findings and hypotheses under field conditions has become increasingly apparent (Leakey *et al.*, 2009). Early studies on the effects of elevated $[CO_2]$ were conducted in precisely controlled closed systems, such as: leaf cuvettes, whole plant growth chambers and greenhouses. Closed systems may amplify down regulation of photosynthesis and production (Morgan *et al.,* 2001), and may produce a 'chamber effect' that exceeds the effect of elevating $[CO_2]$ through environmental modification. The grain yield stimulation by elevated $[CO_2]$ was higher projected by chamber studies (Long *et al.,* 2006). Chambers may also have limited capacity to allow investigators to follow crops to maturity within a valid experimental design (McLeod & Long, 1999). The plants rooting volume may also be restricted in pots, suppressing plant responses to elevated $[CO_2]$ (Arp, 1991).

Free-air CO_2 enrichment (FACE) experiments provide a unique platform for investigating how future ecosystems are likely to respond to higher $[CO_2]$ within various ecosystem-scale interactions (Ainsworth *et al.*, 2008b). The plants in FACE grow as they do in the farmers' fields, and hence their responses to elevated $[CO_2]$ should be very close to what they will exhibit in the real world of the future of higher atmospheric $[CO_2]$ (Long *et al.*, 2006; Ainsworth *et al.*, 2008)

1.7 This study

In order to better understand the mechanisms of the effects of elevated $[CO_2]$ on the grain yield and quality of rice, studies in this dissertation was conducted.

1.7.1 Research questions

Better understandings in the effects of elevated $[CO_2]$ on rice grain yield and quality are required for us to be able to help the farmers to adapt to the future elevated $[CO_2]$. In particular, we need to understand the mechanisms of the interacting effects of field management practices and choice of cultivars on the responses of grain yield and quality in rice to elevated $[CO_2]$. In this study, I posed the following questions:

1. How does nitrogen fertilizer applications affect the effects of elevated $[CO_2]$ on grain yield and quality of rice?
2. How does grain growth of rice respond to elevated $[CO_2]$ and how does the grain growth response to elevated $[CO_2]$ affect grain yield and quality at maturity?
3. Do different rice cultivars show the same response in grain growth to elevated $[CO_2]$?

1.7.2 Approaches of this study to answering the research questions

In an effort to answer the first question as listed above, I studied the efficiency of nitrogen application on the yield enhancement under difference $[CO_2]$ and to understand the mechanisms for the yield enhancement (Chapter 2). I compared the grain yield, yield formation and panicle structure of rice grown at zero nitrogen fertilizer application and ample nitrogen fertilizer applications under ambient and elevated $[CO_2]$.

In Chapter 2, I also studied the occurrence of various types of chalky kernels in rice cultivar Koshihikari that was grown under two levels of nitrogen fertilizer applications under ambient and elevated $[CO_2]$.

I tried to answer the second question by studying the effects of elevated $[CO_2]$ on the grain growth of rice (Chapter 3). As the grain growth are not uniform among panicle positions (Iwasaki *et al.,* 1992; Iwasaki *et al.,* 1993), it is important to know whether grains located at different panicle positions show similar responses to elevated $[CO_2]$ or not. Moreover, the variation in grain growth among panicle positions was dependent on cultivars (Cheng *et al.*, 2003; Cheng *et al.*, 2007).

In Chapters 3 and 4, the grain protein response to elevated $[CO_2]$ were studied, trying to answer the second and the third questions. I checked the grain nitrogen, grain carbon and grain mass accumulations in different spikelets that located on different panicle positions. I particularly focused on the interaction between nitrogen fertilization and elevated $[CO_2]$ in Chapter 3, and on that between cultivar and elevated $[CO_2]$ in Chapter 4.

The studies on the responses of three cultivars, deferring in their panicle structures can help us to better understand the relation between grain growth and grain yield under elevated $[CO_2]$ (Chapter 4).

And in Chapter 4, I also compared the occurrence of chalky kernels in different rice cultivars under ambient and elevated $[CO_2]$, to see whether there are cultivar and $[CO_2]$ interactions which is important for cultivar selection and breeding in future.

In Chapter 5, I synthesized my findings in the efforts to answer the research questions, and gave thoughts to their implications for helping the adaptation of rice farming to the future high $[CO_2]$ environment.

I did these studies in Tsukuba FACE (Fig. 1.3) (Zhang *et al*., 2013), since the rice grain response to elevated $[CO_2]$ are modulated by various many processes, e.g. carbon and nitrogen supply and demand in the spikelets, and, hence, has to be studied in the real field environment. Some details of the FACE experiment are given in Chapter 2.

See Fig.1.4 for the entire structure of this dissertation.

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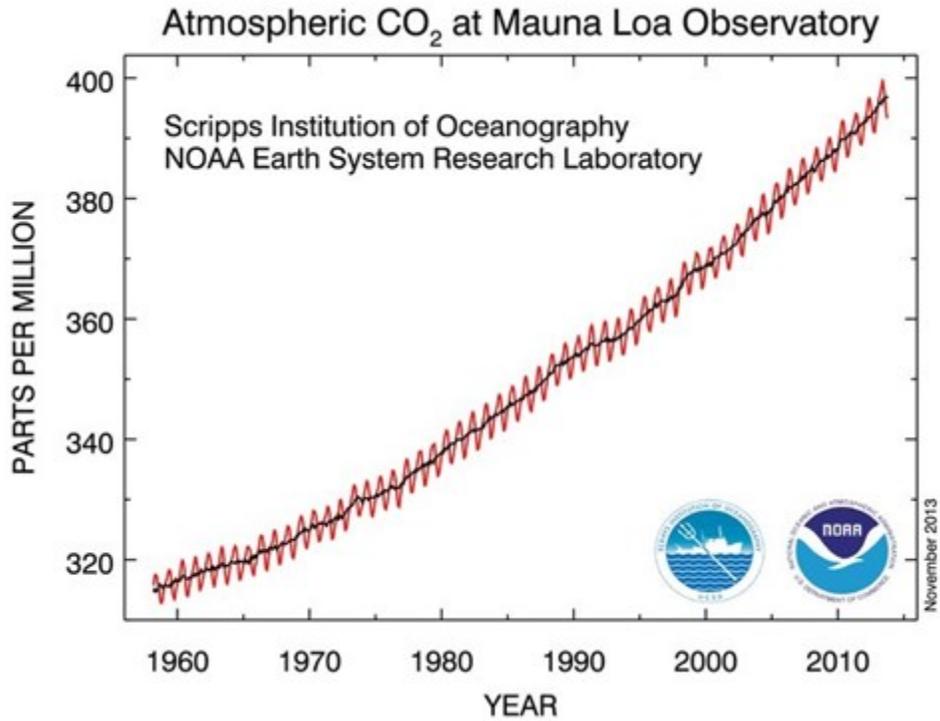
Figure 1.1 The concentration of atmospheric CO_2, [Dr. Pieter Tans, NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/) and Dr. Ralph Keeling, Scripps Institution of Oceanography (scrippsco2.ucsd.edu/).]
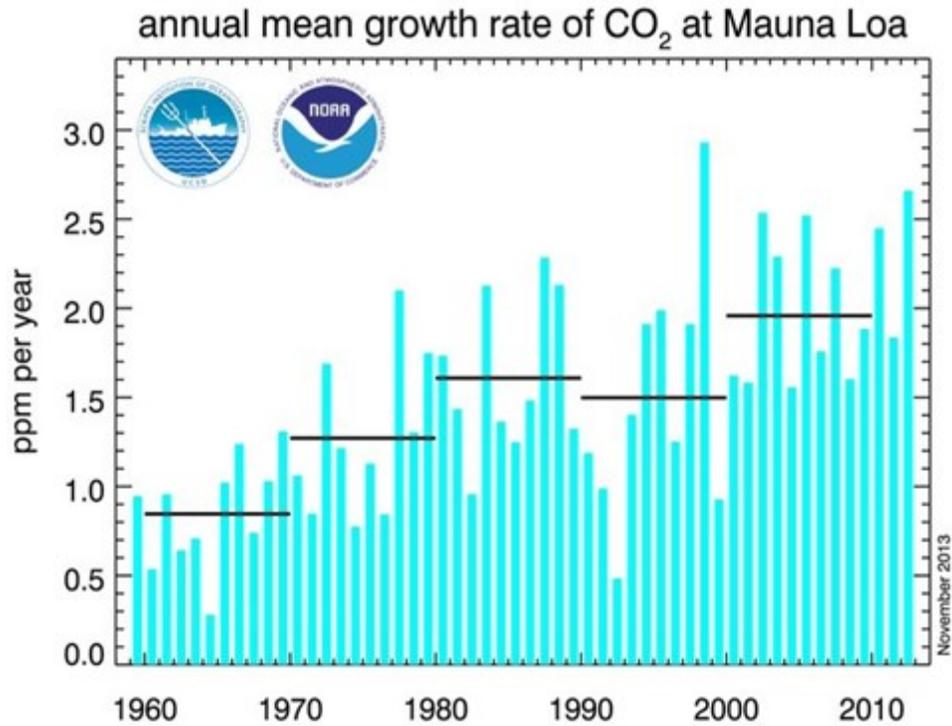
Figure 1.2 Growth rate of the concentration of atmospheric CO_2, [Dr. Pieter Tans, NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/) and Dr. Ralph Keeling, Scripps Institution of Oceanography (scrippsco2.ucsd.edu/).]
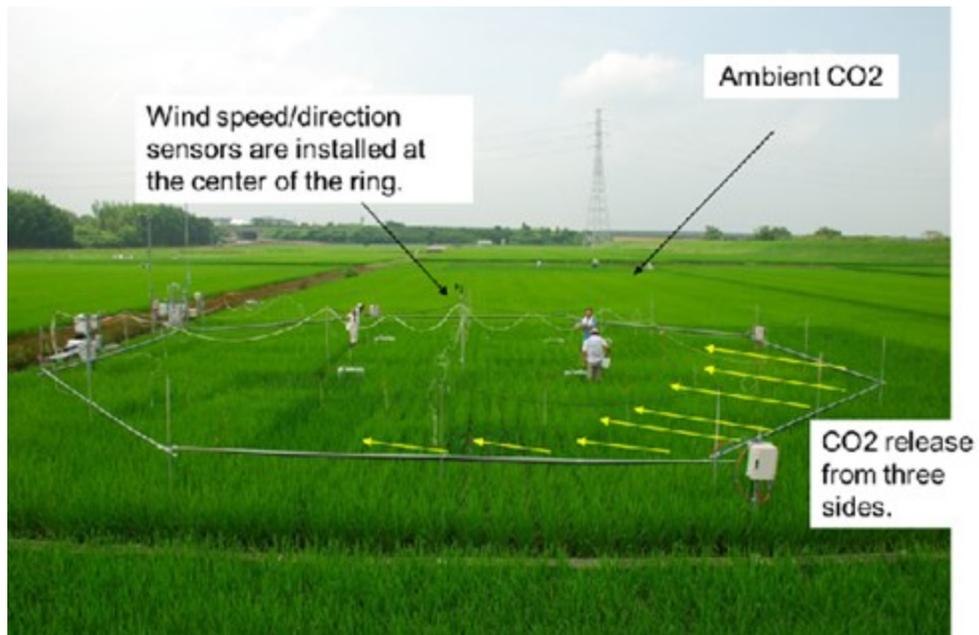
Figure 1.3 Tsukuba FACE (free-air carbon dioxide enrichment experiment), (http://www.niaes.affrc.go.jp/outline/face/english/outline/s7.html)

Structure of the thesis

Chapter 1 Rise future: facing the rising carbon dioxide concentration

Chapter 2 Yield or quality: how will the effects of elevated [CO_2] on grain yield and quality of rice be affected by nitrogen applications?

Chapter 3 A closer look into panicles: grain growth of rice under elevated [CO_2] at different nitrogen application rates

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Figure 1.4 Structure of the dissertation

CHAPTER 2

YIELD OR QUALITY

How will the effects of elevated [CO_2] on grain yield and quality of rice be affected by nitrogen application?

Chapter 2

2.1 Introduction

Rice (*Oryza sativa* L.) is a staple food for more than a half of the world's current population. A substantial increase in rice will be required, as we are going to have 2 billion more people to be fed in 2050 than we do now (FAO, 2009). Nevertheless, the dramatic increase in rice production during the latter half of the 20th century (Zhang, 2007) has yielded to the stagnation of the yield increase by the beginning of this century (Horie *et al.*, 2005; Long, 2012). Furthermore, rice provision in the future will be challenged by the environmental changes, such as high concentrations of ground-level ozone, elevated temperatures and drought, all of which will negatively affect rice production (Long, 2012).

The expected increase in the atmospheric carbon dioxide concentration ($[CO_2]$) is predicted to have a positive effect on the grain yield of rice (Ainsworth, 2008). The steady rise in $[CO_2]$ from ca. 315 μmol mol^{-1} in 1959 to a current global mean of ca. 390 μmol mol^{-1} is projected to continue, and $[CO_2]$ will become as high as 550 μmol mol^{-1} by 2050 (Alley *et al.*, 2007). As rising atmospheric $[CO_2]$ stimulates grain yield of rice, making full use of the advantages may help us to gain more yield and maintain the food security in future.

Developing efficient field management practices which will fit the future environment may be one of the strategies to maximize the yield stimulation by elevated $[CO_2]$. The production and growth of rice depend on environmental factors such as: water availability, air temperature and soil fertility. These factors may have interactive effects with elevated $[CO_2]$ on rice (Kim *et al.*, 2003; Roy *et al.*, 2012; Shimono *et al.*, 2008). Understanding the interactions and the underlying mechanisms can help us to develop suitable management practices so that we can best benefit from the rising $[CO_2]$.

Nitrogen fertilizer affects the grain yield and quality of rice, while studies on the interactions between nitrogen fertilization and elevated $[CO_2]$ on rice were limited (Kim *et al.*, 2001; Weerakoon *et al.*, 1999; Weerakoon *et al.*, 2000). Using glasshouses and open top chambers, Weerakoon *et al.* (1999, 2000) found that increasing soil nitrogen positively enhanced the stimulation of elevated $[CO_2]$ on rice growth and radiation use efficiency of indica cultivars (IR72, KDML 105 grown at IRRI, Philippines, 15^oN, 121^oE). In Japan FACE (free-air CO_2 enrichment experiment at Shizukuishi, Japan, $39^o38'N$, $140^o57'E$), it was found that the yield stimulations by elevated $[CO_2]$ on japonica rice cultivar (Akitakomachi) were dependent on nitrogen fertilization levels, and that the yield stimulation by elevated $[CO_2]$ under medium nitrogen fertilization level was almost as twice as that under low nitrogen fertilization level (Kim *et al*., 2001, 2003). In another FACE experiment in China, nitrogen fertilization was also reported to increase the yield stimulation by elevated $[CO_2]$, but the effects was not significant (Yang *et al.,* 2006). These results suggest that the nitrogen application more than a enough level will not help to enhance the yield stimulation by elevated $[CO_2]$. While how will the omission of nitrogen application affect the yield stimulation by elevated $[CO_2]$ are not clear, limiting our understanding on the nitrogen efficiency on the yield stimulation by elevated $[CO_2]$. Much more effort should be put into establishing the nitrogen fertilizer application strategies which can maximize the rice yield under future elevated $[CO_2]$ (Weerakoon *et al.*, 2005).

On the other hand, although elevated $[CO_2]$ increases the grain yield (Long *et al.*, 2006; Pleijel and Uddling, 2012), it reduces grain quality (Taub *et al.*, 2008; Terao *et al.*, 2005; Yang *et al.*, 2007), which will affect the rice market price as well as the nutrient intake of hundreds of millions of people (Borrell *et al.*, 1999). Better understanding on the mechanisms for the grain quality degradation under elevated $[CO_2]$ are needed, in this study I analysed the interaction of N application and elevated $[CO_2]$ on grain quality and checked the grain appearance quality further.

Among the various quality traits in rice, grain appearance often plays a major role, since rice is predominantly sold in markets as grains rather than flours. Chalkiness, for example, is a major appearance trait that affects milling quality and rice price (IRRI, 2006). In Japan, chalky grains have conventionally been classified into categories based on the position of opaque spots in the endosperm: milky white, white back, basal white, white core, and white belly kernels (Yoshioka et al., 2007), and generally ranked lower than the translucent ones. Grain chalkiness is a result of anomalous grain development and loosely packed starch granules, and is frequently found under high temperatures during the grain-filling stage (Tashiro and Wardlaw, 1991; Wang et al., 2008).

Various studies have been conducted to better understand the genetic and physiological mechanisms that underlie the chalkiness (Cheng et al., 2003; Ishimaru et al., 2009; Patindol and Wang, 2003; Tabata et al., 2005). In addition to the temperature effects during the grain-filling stage, the occurrence of white back and basal white kernels are more likely to be related to plant nitrogen conditions, whereas the milky white kernels are more likely to be related to source–sink interactions (Kondo, 2011). Elevated $[CO_2]$ has been reported to increase rice grain chalkiness (Yang et al., 2007), and there was no significant interactions between nitrogen fertilizer applications and elevated $[CO_2]$ on the occurrence of chalky grain, but the mechanisms are not fully understood.

In order to maximize the yield enhancement and maintain grain quality in the future, developing suitable nitrogen fertilizer application strategies is needed. To this end, a better understanding in the effects of nitrogen fertilizer application on the effects of elevated $[CO_2]$ on rice will be helpful. Thus we conducted a free-air CO_2 enrichment (FACE) experiment in Tsukuba, Ibaraki, Japan. A standard japonica type cultivar, Koshihikari was grown in two levels of nitrogen fertilizer application under ambient and elevated (ambient plus 200 ppm V) $[CO_2]$ conditions. The objectives of this study were to answer the following questions:

1) Whether the low soil nitrogen conditions will lessen the effects of elevated [CO_2] on the yield of rice?

2) What is the efficiency of nitrogen fertilizer on the stimulation of elevated [CO_2] on rice yield?

3) How will elevated [CO_2] affect the occurrence of various types of chalky kernels in rice?

4) Will nitrogen fertilizer applications affect the effects of elevated [CO_2] on rice chalkiness?

2.2 Materials and methods

2.2.1 Tsukuba FACE

The Tsukuba FACE site is located in Tsukubamirai city, Ibaraki Prefecture, Japan (35°58'N, 139°60'E; 10 m above sea level) under a humid subtropical climate with an average temperature of 13.8 °C and annual precipitation of 1280 mm (http://www.niaes.affrc.go.jp/outline/face/english/). Pure CO_2 injection type system was adopted to increase the concentration of CO_2 by 200 ppm in the FACE ring, which covers an area of 240 m^2 (octagonal plot, 17m across). CO_2 was released through the tubes settled along with the rings in the elevated [CO_2] (E-[CO_2]) plots and no tubes were installed in the ambient plots. There were in total four blocks. Details of the Tsukuba FACE can be found in Hasegawa *et al.* (2013) and Nakamura *et al.* (2012). Temperature, solar radiation and precipitations in 2010 and 2011 growing seasons can be found in Table 2.1.

2.2.2 Field management

The agronomic practices for Tsukuba FACE were similar with those used typically by the local farmers. Equal amounts of P and K were applied to all plots on early April through compound PK fertilizer, before ploughing. The rate of P was 4.36 g m^{-2} and the rate of K was 8.30 g m^{-2}. After late April, the fields were kept submerged. Different areas of nitrogen fertilizer treatments were separated by corrugated PVC boards. In the low nitrogen treatment, 0N area, no nitrogen fertilizer was applied. In the other area, which is the ample nitrogen treatment, 8 g m^{-2} of nitrogen was applied: 2 g m^{-2} of urea, 4 g m^{-2} of controlled-release fertilizer with coated urea (type LP100, JCAM Agri. Co. Ltd., Tokyo, Japan), and 2 g m^{-2} of another type of controlled release fertilizer (type LP140, JCAM Agri. Co. Ltd.).

A japonica type cultivar: Koshihikari was used in this study. Seeds were germinated in water and sown into seedling trays. Each tray has 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 own in each cell. The seedling trays were transferred to the puddled open field after seedling emergence, and protected with a tunnel cloche or floating mulch for the first 2 weeks. Then the seedlings were 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} (3 seedlings per hill) manually. The fields were kept flooded until 20 August, about 3 weeks after heading, when the ponding water was drained in preparation for harvesting. Flush irrigations were applied on several occasions to keep the soil moist after the drainage.

2.2.3 Harvesting and analysis

We harvested 21 hills for each subplot at physiological maturity, equivalent to an area of 0.945 m^2 in both 2010 and 2011. 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 ºC.

Brown rice yield and single-grain mass were expressed on the basis of a 15% moisture content. Panicle structure was analysed by fifteen panicles which were collected at maturity and stored in an 80% ethanol solution from each subplot. The number of primary and secondary branches, the numbers of spikelets on primary and secondary branches was collected from each panicle. Grain chalkiness of mature grains was evaluated visually (Tashiro and Wardlaw, 1991).

2.2.4 Statistical work

The experiment was on a blocked split-plot design, with the CO_2 treatment as the main plot and the nitrogen treatment as the sub-plot, with four blocks. The statistical significance of each factor (E-[CO_2] versus A-CO_2, 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).

2.3 Results

2.3.1 Grain yield enhancement

In comparison with the ambient treatment, E-[CO_2] significantly increased the brown rice yield by 13% averaged across years and nitrogen treatments ($P < 0.01$, Table 2.3), but the yield enhancement differed between the nitrogen treatments as evidenced by the significant interaction between [CO_2] and nitrogen ($P < 0.05$): 11 % at 0N and 16% at SN. Harvest index, defined here as the paddy grain yield divided by aboveground dry mass, was slightly decreased by elevated [CO_2] ($P < 0.10$), but this effect was only apparent in 0N where HI decreased by 3.5%, and no reduction occurred in SN, resulting in a significant interaction between [CO_2]

and nitrogen ($P < 0.05$). The aboveground biomass was increased by elevated [CO_2] by 14.8% at 0N, 15.4% at SN ($P < 0.01$), nitrogen significantly stimulated the aboveground biomass ($P < 0.001$), but there was no interaction effect between [CO_2] and nitrogen.

2.3.2 Grain yield component

Elevated [CO_2] significantly increased the number of spikelet by 9.95% at 0N and 15.59% at SN respectively averaged across two years (Table 2.4, $P < 0.05$), and there was a slight interaction between [CO_2] and nitrogen ($P < 0.1$). The spikelet number was significantly higher in SN than 0N conditions ($P < 0.001$). The spikelet number was higher in 2011 than 2010 ($P < 0.1$), the increment of spikelet number by elevated [CO_2] was 11.51% in 2010 and 14.03% in 2011 across two nitrogen levels ($P < 0.05$).

The ratio of ripened spikelet was significantly affected by nitrogen levels: 88.96% at 0N and 84.91% at SN across ambient and elevated [CO_2] and two years (Table 2.4, $P < 0.01$).

Single grain mass was not affected by [CO_2] or nitrogen level, while, it was significantly larger in 2011 than in 2010 (Table 2.4, $P < 0.001$).

The number of spikelet within a panicle was slightly increased by elevated [CO_2] (Table 2.4, $P < 0.1$). Nitrogen fertilizer increased the spikelet number within a panicle by 12.8% from 0N to SN across ambient and elevated [CO_2] and two years ($P < 0.001$).

Panicle number was significantly increased by elevated [CO_2] (9.50%, $P < 0.05$) and nitrogen fertilizer (28.75%, $P < 0.001$) across two years. The increment of panicle number by elevated [CO_2] was higher at SN than that at 0N condition ($P < 0.1$).

2.3.3 Panicle information

Elevated [CO_2] did not affect the number of primary or secondary branch, but nitrogen level significantly increased the number of secondary branches by 21.9% (Table 2.5, $P < 0.01$). Interactions between [CO_2] and nitrogen were found in the numbers of primary spikelet and fertile primary spikelet ($P < 0.05$): elevated [CO_2] increased the numbers of primary spikelet and fertile primary spikelet by 5.77% and 6.12% at 0N nitrogen condition, while, decreased the numbers of primary spikelet and fertile primary spikelet by 5.17% and 3.64% at SN nitrogen condition. Nitrogen increased the number of spikelet and fertile spikelet: primary spikelet (5.61%, $P < 0.05$), secondary spikelet (27.9%, $P < 0.01$), fertile primary spikelet (6.93%, $P < 0.01$), fertile secondary spikelet (28.2%, $P < 0.01$).

2.3.4 Efficiency of nitrogen on grain yield enhancement under elevated [CO_2]

The efficiency of nitrogen on grain yield enhancement was calculated by the net yield enhancement for every 1g m^{-2} of applied nitrogen fertilizer (Table 2.6). Elevated [CO_2] increased the efficiency of nitrogen by 23.2% in 2010 and 37.8% in 2011 ($P < 0.05$). The efficiency of nitrogen was 21.7% higher in 2011 than 2010 averaged across both ambient and elevated [CO_2] ($P < 0.1$).

2.3.5 Occurrence of various types of chalky kernels

Elevated [CO_2] increased the ratios of chalky kernels (Table 2.7, $P < 0.05$), and the effects of [CO_2] depend on nitrogen levels: for 0N, the effects of [CO_2] on the ratio of chalky sum was not apparent, in SN, however, the ratio of chalky sum was increased by 54.8% ($P < 0.01$). On the other hand, nitrogen decreased the ratio of chalky sum by 34.1% at ambient [CO_2] ($P < 0.05$), but had no apparent effect at elevated [CO_2] condition.

Elevated [CO_2] increased the ratios of milky white kernels by 59.3% averaged across two nitrogen levels (Table 2.7, $P < 0.001$). Nitrogen decreased the ratio of milky white kernel by 17.8% ($P < 0.05$) averaged across the both [CO_2] levels. No interactions between [CO_2] and nitrogen was found in the occurrence of this kind of chalky kernels.

Interactions between [CO_2] and nitrogen were found in the occurrence of basal white kernels ($P < 0.001$). Nitrogen decreased the ratio of basal white kernels by 38.3% in ambient [CO_2], but increased it by 134% in elevated [CO_2] condition.

Elevated [CO_2] increased the ratios of white back kernels by 87.9% averaged across two nitrogen levels (Table 2.7, $P < 0.001$). Nitrogen decreased the ratio of white back kernel by 36.5% ($P < 0.01$) averaged across both [CO_2] levels. No interaction was found between [CO_2] and nitrogen in the occurrence of this kind of chalky kernels.

Elevated [CO_2] did not affect the ratio of white belly kernels, while SN increased the ratio by 568.9% ($P < 0.05$), the ratio of opaque kernel was decreased by elevated [CO_2] by 45.8% ($P < 0.1$).

2.4 Discussion

2.4.1 Grain yield stimulation by elevated [CO_2]

Elevated [CO_2] significantly increased the grain yield by 13% averaged across two nitrogen levels and two years ($P < 0.05$, Table 2.3), as was commonly observed in the previous studies for the rice FACE experiments (Kim *et al.*, 2003; Yang *et al.*, 2006). Positive regulations of nitrogen fertilization on the effects of yield stimulation by elevated [CO_2] was found in a japonica rice cultivar, Akitakomachi (Kim *et al.*, 2003). Using another standard japonica type rice cultivar, Koshihikari, here, we found that the yield stimulation by elevated [CO_2]

was 5% larger in ample nitrogen conditions (16%) than in low nitrogen conditions (11%) ($P < 0.05$). On the other hand, elevated [CO_2] stimulated the nitrogen fertilization efficiency on the yield enhancement by 30.39% averaged across two years (Table 2.6, $P < 0.05$). This suggest that with proper nitrogen fertilizer application, we can enhance the yield stimulation of rice by elevated [CO_2].

The aboveground biomass was stimulated by elevated [CO_2] in both low and ample nitrogen conditions. The harvest index (HI) was decreased by 3.5% in low nitrogen ($P < 0.05$). In the low nitrogen conditions, although elevated [CO_2] increased the source supply, especially the carbohydrates (Sasaki *et al.*, 2005a; Sasaki *et al.*, 2005b), the yield enhancement failed to reach the level that relative to the increment in aboveground biomass, thus induce a reduction in HI. This suggest that the grain yield under elevated [CO_2] was sink limited at low nitrogen conditions. However, when the nitrogen was ample, elevated [CO_2] stimulated the grain yield by the same extent to that in the aboveground biomass, as shown in constant HIs. This suggest that the grain yield under elevated [CO_2] was source limited in ample nitrogen condition. The yield limitation thus shifted from source-limited to sink-limited, which was probably caused by the differences in the sink capacity under different nitrogen applications (Table2.4). As nitrogen applications can enhance the sink capacity by increasing panicle number and spikelet number (Chandler Jr, 1969; Hasegawa *et al.*, 1994; Kamiji *et al.*, 2011; Kim *et al.*, 2003).

The grain yield stimulation by elevated [CO_2] was primarily due to the increase in the number of panicles (Tables 2.4), which has also previously been reported (Kim *et al.*, 2003; Yang *et al.*, 2006). The increment in the panicle number per square meter by elevated [CO_2] was larger in ample nitrogen compared with low nitrogen conditions ($P < 0.1$), and the same trends were found in the spikelet number per square meter. The spikelet number per panicle was reported to be increased (Kim *et al.*, 2003), decreased (Yang *et al.*, 2006) or unaffected (Baker and Allen, 1993) by elevated [CO_2]. Here we only found slight increase (Table 2.4, $P < 0.1$), suggesting that the response of spikelet number per panicle to

elevated $[CO_2]$ is cultivar dependent. Further surveys on the panicle structure shown that elevated $[CO_2]$ had limited effect on the panicle structure, while, nitrogen significantly increased the spikelet number per panicle, mainly by increment in the secondary spikelet (Table 2.5). Nitrogen application enhances the sink capacity, which can load the increased source supply by elevated $[CO_2]$, thus the key to gain larger yield stimulation under elevated $[CO_2]$ is to have a larger sink capacity (Hasegawa *et al.*, 2013). High nitrogen applications did not gain proportional yield enhancement (Kim *et al.*, 2003; Yang *et al.*, 2006), as enhancement of sink capacity by nitrogen application may be cultivar dependent, as shown in the efficiency in the production of spikelet by applied nitrogen (Kamiji *et al.*, 2011). Thus besides nitrogen application, the selection of cultivars that has larger sink capacity is another practical strategy to maximize the grain yield of rice under future elevated $[CO_2]$ (Ainsworth *et al.*, 2008).

2.4.2 Grain quality degradation by elevated [CO₂]

Elevated $[CO_2]$ is reported to increase the whiteness and chalkiness of rice grain kernels (Terao *et al.*, 2005; Yang *et al.*, 2007). We also found the stimulate effect of elevated $[CO_2]$ on the occurrence of various types of chalky kernels (Table 2.7), while the effect were dependent on nitrogen level, with a significant interaction between $[CO_2]$ and nitrogen ($P < 0.01$).

The occurrence of chalky kernels were reported to be related with the plant nitrogen conditions and nitrogen application can suppress the occurrence of chalky kernels (Borrell *et al.*, 1999; Kondo, 2011; Qiao *et al.*, 2011). In this study, the suppress effect of nitrogen application on the occurrence of chalky kernels was only apparent in ambient $[CO_2]$. When the plant were grown under elevated $[CO_2]$, there was no difference in the ratio of chalky kernels between different nitrogen applications. Why did not the nitrogen application suppress the occurrence of chalky kernels under elevated $[CO_2]$?

One of the possible mechanisms of the increased occurrence of grain chalkiness under elevated $[CO_2]$ is the grain nitrogen reduction. Terao *et al.*(2005) found a close negative relationship between the nitrogen concentration and grain whiteness of rice cultivar Akitakomachi under elevated $[CO_2]$. Elevated $[CO_2]$ is well known to dilute plant nitrogen (Gifford *et al.*, 2000; Taub *et al.*, 2008). Moreover, the dillution effects may be greater in the high rate of nitrogen application, causing more nitrogen reductions in the plant grown under ample nitrogen conditions compared with low nitrogen supply (Zhang *et al.*, 2013). Thus, nitrogen application did not suppress the occurrence of chalky kernels under elevated $[CO_2]$ because the limited amount of plant nitrogen was diluted by stimulated carbohydrate supplys.

Further analysis showed that the ratio of milky white kernels were significantly increased by elevated $[CO_2]$, as the occurrence of milky white kernels is closely related with source-sink interactions during the grain growth processes (Tsukaguchi and Iida, 2008; Kondo, 2011). As elevated $[CO_2]$ disturbs grain filling process (Hu *et al.*, 2007; Li *et al.*, 2000). Thus the occurrence of milky white kernels here maybe related with the grain growth processes, which should be tested in future.

2.5 Conclusion and implication

Elevated $[CO_2]$ stimulated the grain yield of rice but degraded the grain quality in terms of increment in the ratio of various types of chalky kernels. The effects of elevated $[CO_2]$ was weaker in low nitrogen application than the ample nitrogen applications. The efficiency of nitrogen application on the grain yield enhancement was increased by elevated $[CO_2]$. Although the nitrogen application can enhance the effects of elevated $[CO_2]$ on the grain yield stimulation, it did not counteract the negative effect of elevated $[CO_2]$ on the grain appearance quality. The grain quality degradation under elevated $[CO_2]$ may be caused by the dilution effect of elevated $[CO_2]$ on plant nitrogen concentrations and the disturbed grain growth processes, which should be further studied in future.

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Table 2.1 Weather conditions in the 2010 and 2011 growing seasons at the Tsukuba FACE site, Japan.

Year	Month	Air temperature (°C)			Solar radiation	Precipitation
		Mean	Max	Min	$(MJ\ m^{-2})$	(mm)
2010	May (26)	15.7	19.6	12.3	16.0	6
	Jun	22.2	26.2	18.8	19.2	101
	Jul	26.2	30.1	23.0	18.5	74
	Aug	27.8	32.1	24.3	19.3	7
	Sep(6)	28.5	33.8	23.9	19.9	0
	Growing season	25.0	29.2	21.6	18.9	188
2011	May (25)	17.3	20.0	14.7	13.6	67
	Jun	21.7	25.2	18.8	16.0	126
	Jul	25.8	29.5	22.5	19.0	104
	Aug	25.8	29.8	22.7	17.0	120
	Sep(13)	25.7	30.0	22.3	16.7	38
	Growing season	24.1	27.9	21.0	17.0	456

The numbers in the brackets for May and September are the day of month for transplant and harvest, respectively. Summary weather data are only for that period.

Table 2.2 Yields information of rice cultivar Koshihikari in 2010 and 2011 Tsukuba FACE experiments (n, duplications; $\bar{X}_A$ $\bar{X}_E$, mean values under ambient and elevated $[CO_2]$).

N applied (g m^{-2})	Year	Variables	n	$\bar{X}_A$	SD$_A$	$\bar{X}_E$	SD$_E$
0	2010	Brown rice yield[1] (g m^{-2})	4	400	24	455	54
0	2011	Brown rice yield[1] (g m^{-2})	4	430	55	459	49
8	2010	Brown rice yield[1] (g m^{-2})	4	552	23	642	55
8	2011	Brown rice yield[1] (g m^{-2})	4	604	18	698	22
0	2010	Above ground biomass[2] (g m^{-2})	4	980	66	1122	130
0	2011	Above ground biomass[2] (g m^{-2})	4	1035	104	1192	121
8	2010	Above ground biomass[2] (g m^{-2})	4	1293	57	1472	100
8	2011	Above ground biomass[2] (g m^{-2})	4	1403	28	1640	71
0	2010	Harvest index[3] (%)	4	44.1	0.5	43.5	1.2
0	2011	Harvest index[3] (%)	4	44.2	2.1	41.4	0.3
8	2010	Harvest index[3] (%)	4	46.1	0.3	46.7	0.7
8	2011	Harvest index[3] (%)	4	45.7	1.1	45.5	0.7
0	2010	Panicle number (m^{-2})	4	257	6	272	18
0	2011	Panicle number (m^{-2})	4	278	17	297	40
8	2010	Panicle number (m^{-2})	4	321	18	351	23
8	2011	Panicle number (m^{-2})	4	347	10	403	37
0	2010	Spikelet number (m^{-2})	4	21108	1159	23321	2539
0	2011	Spikelet number (m^{-2})	4	21790	2705	23843	2652
8	2010	Spikelet number (m^{-2})	4	28764	1169	32372	2875
8	2011	Spikelet number (m^{-2})	4	31990	1471	37951	4016
0	2010	% of ripened spikelets[4]	4	89.1	1.7	89.0	2.0
0	2011	% of ripened spikelets[4]	4	89.2	3.3	88.6	1.2
8	2010	% of ripened spikelets[4]	4	85.7	1.0	88.6	1.0
8	2011	% of ripened spikelets[4]	4	82.9	2.7	82.4	8.3
0	2010	Thousand grain weight[1] (g)	4	21.3	0.2	21.9	0.6
0	2011	Thousand grain weight[1] (g)	4	23.9	0.4	23.6	0.7
8	2010	Thousand grain weight[1] (g)	4	22.4	0.2	22.4	0.2
8	2011	Thousand grain weight[1] (g)	4	23.7	0.7	23.5	0.7
0	2010	Panicle C concentration (mg g^{-1})	4	429	2	426	4
0	2011	Panicle C concentration (mg g^{-1})	4	430	1	430	1
8	2010	Panicle C concentration (mg g^{-1})	4	427	2	426	2
8	2011	Panicle C concentration (mg g^{-1})	4	433	2	433	2
0	2010	Panicle N concentration (mg g^{-1})	4	9.00	0.36	8.57	0.24
0	2011	Panicle N concentration (mg g^{-1})	4	9.34	0.18	8.81	0.33
8	2010	Panicle N concentration (mg g^{-1})	4	10.10	0.23	8.96	0.30
8	2011	Panicle N concentration (mg g^{-1})	4	10.61	0.51	9.95	0.38

1) Expressed on the 15% moisture content basis.
2) 0% moisture, dry mass basis.
3) Paddy rice yield divided by the aboveground mass expressed on the 0% moisture.
4) Estimated by sorting unhulled seeds in an ammonium sulfate solution with a specific gravity of 1.06 PRS is % of sunken spikelets.

Table 2.3 Effects of [CO_2] (A, ambient; E, elevated) and nitrogen fertilization levels (0 g m^{-2} and 8 g m^{-2}) on the panicle number, grain yield of rice cultivar Koshihikari in 2010 and 2011 and the results of an analysis of variance (ANOVA).

N applied (g m^{-2})	Year	Brown rice yield[1] (g m^{-2})			Harvest index[2] (%)			Above ground biomass (g m^{-2})		
		A	E	E/A	A	E	E/A	A	E	E/A
0	2010	400	455	1.14	44.1	43.5	0.99	980	1122	1.14
	2011	430	459	1.07	44.2	41.4	0.94	1035	1192	1.15
8	2010	552	642	1.16	46.1	46.7	1.01	1293	1472	1.14
	2011	604	698	1.16	45.7	45.5	0.99	1403	1640	1.17
ANOVA[3]										
Year			ns			†			†	
CO_2			**			†			**	
Year*CO_2			ns			†			ns	
N			***			**			***	
Year*N			†			ns			†	
CO_2*N			*			*			ns	
Year*CO_2*N			ns			ns			ns	

1) Expressed on the 15 % moisture content basis.
2) Paddy rice yield divided by the aboveground mass expressed on the 0 % moisture (dry mass basis).
3) †, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Table 2.4 Effects of $[CO_2]$ (A, ambient; E, elevated) and nitrogen fertilization levels (0 g m^{-2} and 8 g m^{-2}) on the yield component of rice cultivar Koshihikari in 2010 and 2011 and the results of an analysis of variance (ANOVA).

N applied (g m^{-2})	Year	Spikelet number (m^{-2})			Ripened spikelet[1] (%)			Single grain mass (mg)			Spikelet number (panicle^{-1})			Panicle number (m^{-2})		
		A	E	E/A	A	E	E/A	A	E	E/A	A	E	E/A	A	E	E/A
0	2010	21108	23321	1.10	89.1	89.0	1.00	21.3	21.4	1.00	82	86	1.04	257	272	1.06
	2011	21790	23843	1.09	89.2	88.6	0.99	23.9	23.6	0.99	78	80	1.03	278	297	1.07
8	2010	28764	32372	1.13	85.7	88.6	1.03	21.4	21.4	1.00	90	92	1.03	321	351	1.09
	2011	31990	37951	1.19	82.9	82.4	0.99	23.7	23.5	0.99	92	94	1.02	347	403	1.16
ANOVA[2]																
Year			†			ns			***			ns			*	
CO$_2$			*			ns			ns			†			*	
Year*CO$_2$			ns			ns			ns			ns			ns	
N			***			**			ns			***			***	
Year*N			*			ns			ns			*			ns	
CO$_2$*N			†			ns			ns			ns			†	
Year*CO$_2$*N			ns			ns			ns			ns			ns	

1) Estimated by sorting unhulled seeds in an ammonium sulfate solution with a specific gravity of 1.06 PRS is % of sunken spikelets.

2) †, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Table 2.5 Effects of [CO_2] (A, ambient; E, elevated) and nitrogen fertilization levels (0 g m^{-2} and 8 g m^{-2}) on the panicle structure of rice 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[1]																
CO_2	ns		ns		ns		ns		ns		ns		ns		ns	
N	ns		**		*		**		**		**		**		**	
CO_2×N	ns		ns		*		ns		ns		*		ns		ns	

1) *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

Table 2.6 Efficiency of nitrogen fertilization on the brown yield enhancement of rice cultivar Koshihikari [(Yield enhancement under 8 g m^{-2} nitrogen fertilizer level- Yield enhancement under 0 g m^{-2} nitrogen fertilizer level) / 8, g/g] under different

	2010	2011	2 years
Ambient	19.0	21.7	20.4
FACE	23.4	29.9	26.6
FACE/Ambient	1.23	1.38	1.31
ANOVA		P value	
CO_2		0.0208	0.0346
Year		0.0648	
CO_2*Year		0.3820	

Table 2.7 Effects of $[CO_2]$s (A, ambient; E, elevated) and nitrogen fertilization levels (0 g m^{-2} and 8 g m^{-2}) on the occurrence of various types of chalky kernels in Koshihikari in the 2010 FACE experiment and the results of an analysis of variance (ANOVA)

N levels	CO$_2$	Chalky sum (%)[1]	Milky white (%)	Basal white (%)	White back (%)	White belly (%)	Opaque (%)
0	A	67.1 a	9.13 b	36.3 a	21.0 b	0.75 a	1.88 a
	E	63.8 a	15.5 a	12.5 c	35.5 a	0.13 a	1.50 a
8	A	44.2 b	8.00 b	22.4 b	12.0 c	1.38 a	3.50 a
	E	68.4 a	11.9 ab	29.3 ab	24.8 b	1.50 a	1.00 a
ANOVA				P value			
CO$_2$		0.0145	0.0007	0.0055	0.0002	0.5334	0.0506
N levels		0.0243	0.0252	0.4902	0.0013	0.0384	0.3769
CO$_2$*N levels		0.0041	0.1701	0.0003	0.6352	0.3600	0.1216

1) Chalky sum is the sum of milky white, basal white, white back, white belly and mix type white kernels divided by total grains that analyzed in each sample (200 grains)

CHAPATER 3

A CLOSER LOOK INTO PANICLES
Grain growth of rice under elevated [CO$_2$] at different nitrogen application rates

This material is an edited version of materials presented in

The effects of free-air CO$_2$ enrichment (FACE) on carbon and nitrogen accumulation in grains of rice (*Oryza sativa* L.)

GY Zhang, H Sakai, T Tokida *et al.* 2013, Journal of Experimental Botany, 64 (11), 3179-3188

The effects of free-air CO$_2$ enrichment (FACE) and nitrogen levels on the grain mass, grain carbon and nitrogen accumulation in the rice plants

GY Zhang, H Sakai, T Tokida *et al.* 2012, Japanese Journal of Crop Sciences, 81 (Extra issue 1), 348-349 (poster)

Chapter 3

3.1 Introduction

Although higher $[CO_2]$ increases the grain yield of rice (Long *et al.*, 2006; Kobayashi *et al.*, 2006; Hasegawa *et al.*, 2007), it reduces grain quality, especially in terms of grain chalkiness and protein content (Seneweera *et al.*, 1996; Lieffering *et al.*, 2004; Terao *et al.*, 2005; Yang *et al.*, 2007; Taub *et al.*, 2008; Seneweera, 2011). The grain protein content may also affect the occurrence of grain chalkiness, as Terao *et al.* (2005) found a closely negative relation between grain N concentration and grain whiteness. To maximize the yield enhancement and maintain grain quality in the future, a better understanding is needed on rice grain growth, yield stimulation, and grain quality degradation, especially the reduction in grain protein, under future elevated atmospheric $[CO_2]$.

Grain yield stimulation by elevated $[CO_2]$ 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 $[CO_2]$ 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; Yang and Zhang, 2010; Zhang *et al.*, 2012).

While elevated [CO_2] 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 [CO_2] may be different. There are several possible ways in which elevated [CO_2] might affect the grain filling of superior and inferior spikelets. First, both the superior and inferior spikelets are better filled, producing heavier overall grains; second, the superior spikelets might be better filled and the inferior ones are unaffected, producing heavier superior grains; and third, the inferior spikelets are better filled and the superior ones are not affected, resulting in heavier inferior grains. It is yet to be known which change is the case.

The distinction between the superior and inferior spikelets is important for understanding the mechanisms of reduction in grain nitrogen concentrations due to elevated [CO_2], which are not fully understood (Taub *et al.*, 2008). The nitrogen and protein concentrations may be diluted by increased carbohydrates under elevated [CO_2] (Gifford *et al.*, 2000). Superior spikelets usually accumulate nitrogen earlier and faster than inferior spikelets (Iwasaki *et al.*, 1992; Iwasaki *et al.*, 1993). Therefore, under elevated [CO_2], a limited quantity of available nitrogen 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 nitrogen is supplied by fertilization, there may be less available nitrogen in the whole plant, which would allow reductions in nitrogen and protein to be more easily detected.

Grain growth is mainly accomplished by the accumulation of grain carbon (GC), grain nitrogen (GN), and grain mass (GM) during grain filling. Because elevated [CO_2] 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 protein reduction may not be detected. The inferior spikelets, however, begin to fill rapidly around 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 nitrogen (N) has already been taken up. As a result, they may be more likely to show reduced protein.

In order to test the hypothesis stated above, we conducted a FACE experiment with two nitrogen fertilization levels, trying to find answers for the following questions:

1) Do grains located at different panicle positions respond differently to an elevated $[CO_2]$ in their growth and weight?

2) Under an elevated $[CO_2]$, if the average grain N concentrationis decreased, is the reduction restricted to superior or inferior grains or is it observed in all grains?

3) Will the N concentration in rice grains be reduced by an elevated $[CO_2]$ more at a lower nitrogen fertilization level?

4) When does the elevated $[CO_2]$ start to decrease grain N 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 the grain filling and growth responses to an increased source supply, and of the mechanisms of rice yield enhancement, under elevated $[CO_2]$. In addition, the results may suggest how grain yield might be increased and grain quality might be maintained in future.

3.2 Materials and methods

3.2.1 [CO$_2$] 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 [CO$_2$]. Briefly, four blocks were established in paddy fields, with each block consisting of two octagonal plots (240 m^2, 17 m across): an ambient CO$_2$ (A-[CO$_2$]) plot and an elevated CO$_2$ (E-[CO$_2$]) treatment plot. The E-[CO$_2$] plots were equipped with emission tubes on the perimeter, where CO$_2$ was released from the windward side to keep the [CO$_2$] measured at the central point at 200 µmol mol^{-1} above the ambient control level. The season-long daytime average [CO$_2$] in the ambient plots and in the E-[CO$_2$] plots was 386 and 584 µmol mol^{-1} in 2010; and 386 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 the average year of about 23°C (Table 2.1). The 2010 season was also sunnier and drier than was the 2011 season.

3.2.2 Crop cultivation

Crop cultivation practices in 2010 were reported by Hasegawa *et al.* (2013), and the 2011 growing season followed essentially the same practices, which were described in Chapter 2.

3.2.3 Sampling and analysis

In 2011, we monitored 20–30 hills in each plot every day and recorded the date of panicle emergence; the date was written with a marker on adhesive tape, which was wrapped around each panicle below the flag leaf sheath. We sampled five

panicles from each plot at 5, 10, 15, 20, 30, and 40 DAH, and oven-dried them at 80 ºC. We then separated the superior and inferior spikelets (Figure 3.3) 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).

3.2.4 Statistical analysis

A blocked split-plot design was used for the experiment, with the CO_2 treatment as the main plot and the N treatment as the sub-plot, with four replicates. The statistical significance of each factor (E-[CO_2] versus A-[CO_2], 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 grain mass data, we fitted the Richard's function to the time course of grain mass accumulation in the superior and inferior spikelets and then estimated the active grain filling duration and average grain-filling rate according to Zhang *et al*. (2012).

3.3 Results

3.3.1 Panicle structure

Elevated [CO_2] had no significant effect on the number of primary and secondary branches and the number of spikelets within a panicle (Table 2.4). 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 $[CO_2]$ and the N levels, which is that at 0N the number of primary spikelets was increased by E-$[CO_2]$, while at SN it was decreased by E-$[CO_2]$.

Elevated $[CO_2]$ 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 3.1). The effect of E-$[CO_2]$ on the number of fertile spikelets were N level and the grain position dependent: at 0N, the number of fertile superior spikelets was higher in E-$[CO_2]$ than in the ambient plots; at SN, the number of fertile superior spikelets in E-$[CO_2]$ was lower and the number of fertile inferior spikelets was higher than in ambient.

3.3.2 Effects of elevated [CO$_2$] and N levels on grain mass, grain N, and grain C contents in superior and inferior spikelets at maturity

Elevated $[CO_2]$ affected grain mass (GM) of superior and inferior spikelets differently depending on the N fertilization level (Table 3.2). At 0N, the grain mass of superior spikelets in E-$[CO_2]$ was higher than that in ambient, whereas the grain mass of inferior spikelets in E-$[CO_2]$ was lower than that in ambient. At SN, the grain mass of superior spikelets in E-$[CO_2]$ was lower than that in ambient, whereas the GM of inferior spikelets in E-$[CO_2]$ was higher than that in ambient. The grain carbon (GC) content of superior spikelets was not affected by $[CO_2]$, while grain carbon of inferior spikelets was similar to the grain mass of inferior spikelets: at 0N, grain carbon of inferior spikelets in E-$[CO_2]$ was lower than that in ambient, whereas the grain carbon content of inferior spikelets in E-$[CO_2]$ was higher than that in ambient at SN. The grain nitrogen (GN) content of superior spikelets was significantly decreased (by 24%) by FACE (P <0. 01), whereas GN in inferior spikelets was not affected by E-$[CO_2]$. 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 of the superior and inferior spikelets.

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

Grain-filling patterns differed between superior and inferior spikelets (Figure 3.2). In the superior spikelets, GM accumulation started to increase immediately after panicle emergence (Figure 3.2A), whereas in the inferior spikelets, rapid GM accumulation began at about 20 DAH (Figure 3.2B). The final GM of inferior spikelets was lower than that of superior spikelets: at 0N, the GM of inferior spikelets was 52% lower in E-$[CO_2]$ and 32% lower in ambient compared with superior spikelets; and at SN, the GM of inferior spikelets was 28% lower in E-$[CO_2]$ and 50% lower in ambient compared with superior spikelets.

The effects of elevated $[CO_2]$ on GM accumulation were more apparent in the inferior spikelets at SN (Figure 3.2B). 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 E-$[CO_2]$ 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 E-$[CO_2]$ 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 around 20 DAH (Figure 3.1A). In inferior spikelets, a difference became apparent at around 30 DAH, and the inferior spikelets apparently continued to accumulate GM even after 40 DAH (Figure 3.1B).

E-[CO_2] and N levels modified the relationship between GN and GM accumulation (Figure 3.1, lower panels). E-[CO_2] 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 (Figure 3.1C, D). The GN concentration in the superior spikelets was apparently ($P < 0.10$) decreased by E-[CO_2] at 5 DAH, 30 DAH ($P < 0.01$), and 40 DAH ($P < 0.05$) (Figure 3.1C). 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 E-[CO_2] at 30 DAH ($P < 0.05$) and increased by SN at 10 DAH ($P < 0.10$) (Figure 3.1D).

With the increase of GM from inferior spikelets to superior spikelets, the GN concentration was decreased (Figure 3.2), the slope of the relationship between the GN concentration and GM differed significantly between E-[CO_2] and ambient ($P < 0.05$). For superior spikelets, although the GM was not affected by E-[CO_2], the GN concentration was decreased by FACE ($P < 0.05$).

3.4 Discussion

3.4.1 Is a reduction of GN by elevated [CO_2] a result of dilution?

It has been commonly reported that elevated [CO_2] decreases GN in rice (Seneweera *et al.*, 1996; Lieffering *et al.*, 2004; Terao *et al.*, 2005; Yang *et al.*, 2007; Seneweera, 2011). We also observed a significant reduction in N concentration of the bulk panicle samples ($P < 0.01$, Table 2.3). Further analysis showed, however, that this decrease is apparent only in superior spikelets (Table 3.2).

The mechanisms by which elevated [CO_2] decreases plant nitrogen and protein concentrations are not yet well understood, but one plausible mechanism is that the stimulated production of carbohydrates by elevated [CO_2] dilutes plant

nitrogen (Gifford *et al.*, 2000; Taub *et al.*, 2008). We also found a negative correlation between grain mass and grain nitrogen concentration when we pooled superior and inferior spikelets (Figure 3.3). When we examined the effect of E-[CO_2] separately in superior and inferior spikelets, however, we found that the E-[CO_2] treatment decreased the GN of the superior spikelets, but that their GM was not increased by elevated [CO_2].

Moreover, whereas the GM of inferior spikelets was significantly increased by elevated [CO_2], their GN was not reduced. These results suggest that the reduced GN 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 [CO_2] must be a part of the mechanism that accounts for the reduced GN.

In previous experiments under current [CO_2], 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 [CO_2] greatly affects rice growth and N partitioning among different organs and the present study demonstrated that elevated [CO_2] affects the partitioning of N between superior and inferior spikelets.

3.4.2 Reduction of grain N under elevated [CO_2] occurred at early grain filling

GN accumulated differently in superior and inferior spikelets during the grain filling period in response to elevated [CO_2], and the reduction in GN occurred at the beginning of the grain-filling period (Figure 3.2C, D). The superior spikelets started the rapid growth immediately after heading, whereas growth and N accumulation in inferior spikelets started only around 20 DAH (Iwasaki *et al.*, 1992), when superior spikelets had already completed the rapid growth.

In this study, under ambient [CO_2], 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 reports under elevated [CO_2]

(Figure 3.2C, D), which reduced the 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 $[CO_2]$, with a greater need for N. In contrast, the early drop in GN concentration may reflect dilution by stimulated carbohydrate accumulation in elevated $[CO_2]$ against lagging N accumulation.

At 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 $[CO_2]$ 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 $[CO_2]$, 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 $[CO_2]$, whereas the GM of inferior spikelets was significantly increased at SN (Table 3.2). Inferior spikelets are usually limited by the C supply (Murty and Murty, 1982; Fu *et al.*, 2011); thus, the increased C supply by elevated $[CO_2]$ stimulated the growth of inferior grains.

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

The different responses of superior and inferior spikelets to elevated $[CO_2]$ were N level dependent (Table 3.2, Figure 3.2A, B). The GM of superior spikelets was little affected by elevated $[CO_2]$. On the other hand, the GM of inferior spikelets was lower at 0N and higher at SN under elevated than ambient $[CO_2]$. Poor grain filling of inferior spikelets is reported to be attributed to a sink limitation due to poor activity of enzymes in relate with starch synthesis (Patel *et al.*, 1996; Liang *et al.*, 2001; Ishimaru *et al.*, 2005), whereas our results showed that when the source supply was increased by elevated $[CO_2]$, the grain filling of inferior spikelets was improved. Manderscheid *et al.* (2009) reported that grain growth of winter barley under elevated $[CO_2]$ is strongly sink-limited, whereas our results showed that the GM of superior spikelets, which have a larger sink capacity than inferior spikelets, responded negatively to elevated $[CO_2]$. 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 $[CO_2]$ (Table 3.2). The stimulation of biomass and yield by elevated $[CO_2]$ is usually small at low nitrogen levels (Amthor, 2001; Kim *et al.*, 2003; Manderscheid *et al.*, 2009). In this study, the grain yield enhancement and panicle N reduction by elevated $[CO_2]$ were 11% and 5.5% at low nitrogen, and 16% and 8.5% at ample nitrogen (Table 2.3). At low nitrogen, the carbohydrate stimulation by elevated $[CO_2]$ may not be enough to significantly dilute the GN concentration 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 a 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 $[CO_2]$ may be an indication of how rice adapts to

environmental change. The present study suggests that under elevated $[CO_2]$, 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.

3.4.4 Relevance of the grains' position-dependent response to elevated $[CO_2]$ 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 $[CO_2]$. The ratio of the number of superior to inferior grains was not affected (Tables 2.4, 3.1), but the number of panicles was increased by elevated $[CO_2]$. Therefore, the numbers of inferior and superior grains increased simultaneously.

  Compared with superior grains, the cooking quality and appearance quality 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 $[CO_2]$, 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 (the range of inferior grain mass under $e[CO_2]$ were 11.4~16.6 mg, almost reaching to that of superior grains under ambient $[CO_2]$, which were 17.4~20.6 mg) might not be discarded during sieving, but the small grain size would have a negative impact on milling quality (Jongkaewwattana and Geng, 2001). Head rice loss under elevated $[CO_2]$ 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 $[CO_2]$ might affect the overall grain quality of the yield.

3.4.5 Implications for future experiments

There is an urgent need to understand how future elevated CO_2 concentrations will affect yield and grain quality so that rice yields and grain quality can both be maximized. Elevated [CO_2] 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 [CO_2]. 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 [CO_2]. 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 [CO_2].

3.5 Conclusion

Future elevated [CO_2] 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 [CO_2], 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.

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Table 3.1 Effects of [CO_2] (A, ambient; E, elevated) and nitrogen fertilization levels (0 g m^{-2} and 8 g 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	Spikelet number				% of sterile spikelets (%)				Fertile spikelet number				% in total fertile spikelets			
(g m^{-2})	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[1]																
CO_2	ns		ns		ns		ns		ns		ns		ns		ns	
N	ns		***		ns		ns		ns		**		*		*	
$CO_2 \times N$	ns		ns		ns		ns		†		ns		ns		ns	

†, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Table 3.2 Effects of $[CO_2]$ (A, ambient; E, elevated) and nitrogen fertilization levels (0 g m^{-2} and 8 g 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

N applied (g m^{-2})	Grain mass (mg·kernel^{-1}) Superior		Inferior		C concentration (mg g^{-1}) Superior		Inferior		C content (mg·kernel^{-1}) Superior		Inferior		N concentration (mg g^{-1}) Superior		Inferior		N content (mg·kernel^{-1}) 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[1]																				
CO_2	ns		ns		ns		ns		ns		ns		*		ns		**		ns	
N	***		*		ns		*		***		†		ns		ns		*		†	
$CO_2 \times N$	†		*		*		ns		ns		*		ns		ns		*		ns	

1) †, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.
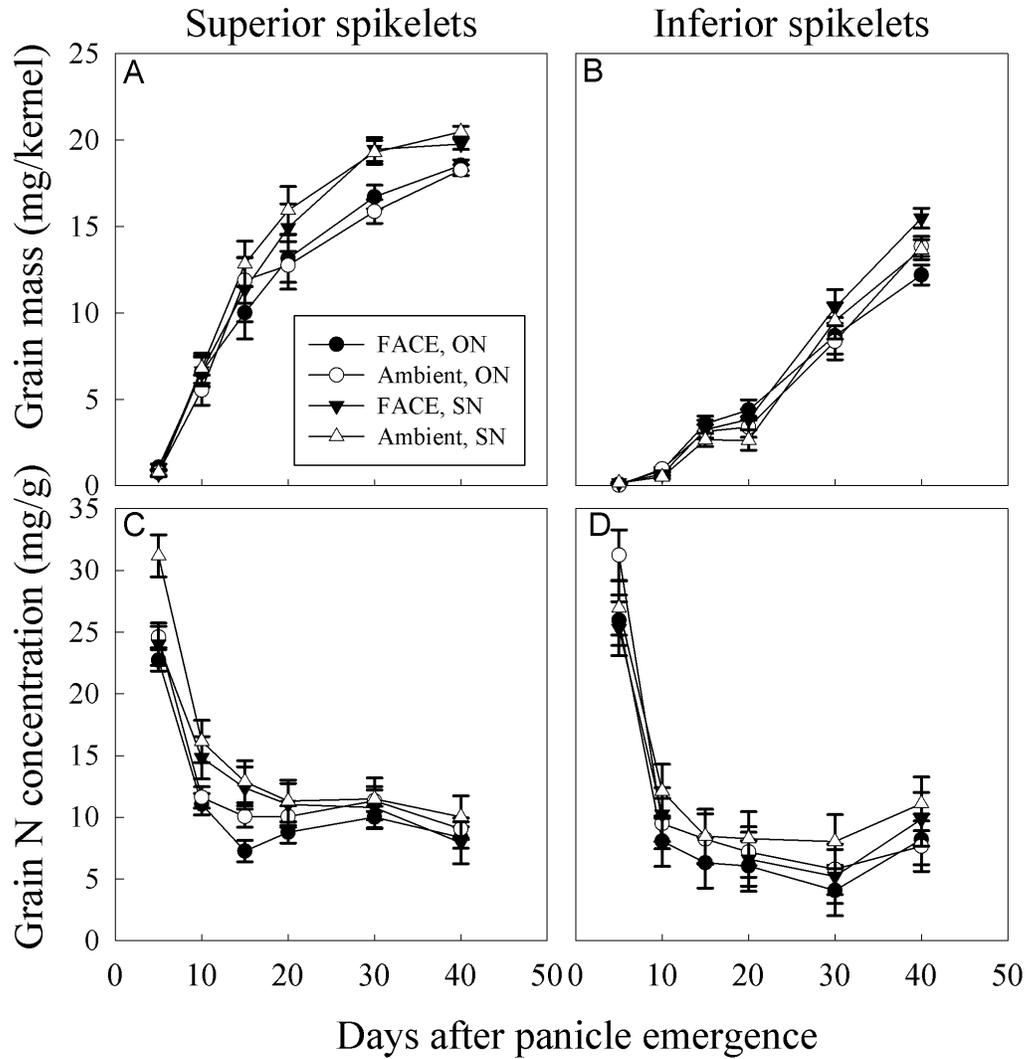
Figure 3.1 Changes in grain mass (A, B) and N concentration (C, D) of superior and inferior spikelets grown under two CO_2 and N regimes in the 2011 FACE experiment. Bars indicate standard error for the mean (n=4).
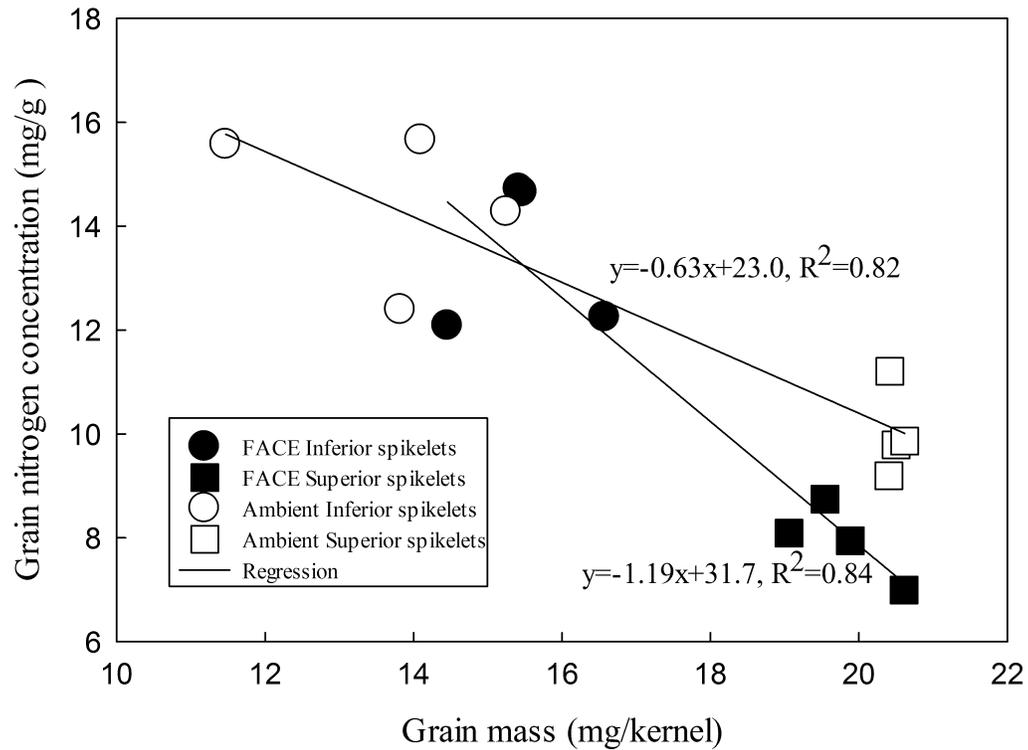
Figure 3.2 Relationships between grain mass and N concentration under different atmospheric CO_2 concentrations (FACE and ambient) for grains at maturity under two CO_2 regimes (SN) in 2011 FACE experiment. The equations were linear regressions with x stands for grain mass, and y stands for N concentration, R^2 is the percentage of variation in y explained by the variable x. The slopes between FACE and ambient were significantly different ($P < 0.05$).
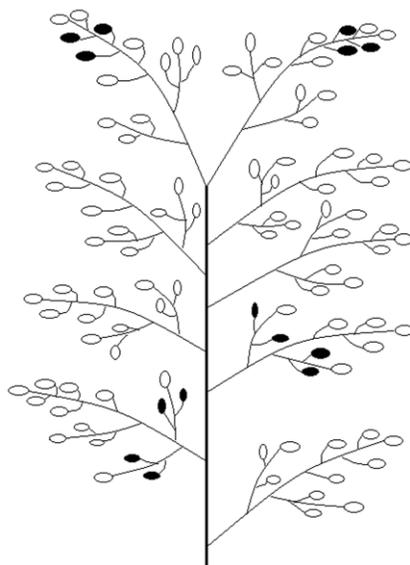
Figure 3.3 Superior and inferior spikelets within a rice panicle, the upper black spikelets located on primary branches are referred to as superior spikelets, the lower black spikelets located on secondary branches are referred to as inferior spikelet.

CHAPTER 4

CULTIVAR DIFFERENCE

Grain growth of rice cultivars with different panicle structures under elevated [CO$_2$]

This material is an edited version of materials presented in

The effects of free-air CO$_2$ enrichment (FACE) on the grain growth and appearance quality of rice cultivars
GY Zhang, H Sakai, T Tokida *et al*. 2013, Japanese Journal of Crop Sciences, 82 (Extra issue 1): 142-143 (oral presentation)
Effects of free-air CO$_2$ enrichment (FACE) on the grain growth and quality of rice cultivars differing in panicle structure
GY Zhang, H Sakai, Y Usui *et al*. *Journal of Experimental Botany* submitted
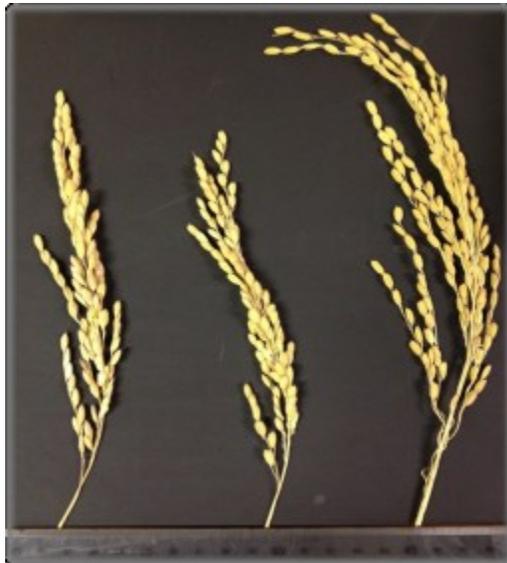
4.1Introduction

In general, the projected increases in $[CO_2]$ will promote photosynthesis, biomass production, and grain yield, and the expected yield stimulation is promising with regard to improving crop productivity in the future (Ziska *et al.*, 2012; Tausz *et al.*, 2013). On the other hand, elevated $[CO_2]$ (E-$[CO_2]$) is known to have some negative effects on grain quality (Terao *et al.*, 2005; Yang *et al.*, 2007; Taub *et al.*, 2008), which will affect the rice market price as well as the nutrient intake of hundreds of millions of people (Borrell *et al.*, 1999). E-$[CO_2]$ reduces the protein content in rice grains (Lieffering *et al.*, 2004; Terao *et al.*, 2005; Taub *et al.*, 2008), and the quality of grain appearance is also degraded by E-$[CO_2]$, which induces chalky grains (Yang *et al.*, 2007). The mechanisms underlying this quality degradation, however, are not well understood.

Grain growth is the key process that determines both grain yield and quality, and is sensitive to the changes in climatic factors, such as temperature, elevated concentrations of ozone and CO_2 (Mulholland *et al.*, 1998; Li *et al.*, 2000; Kobata & Uemuki, 2004; Hu *et al.*, 2007; Manderscheid *et al.*, 2009; Asseng *et al.*, 2011; Liu *et al.*, 2012). In rice, however, the responses of grain growth to E-$[CO_2]$ remain unclear. Grain growth is the result of series of processes including floret initiation and development, pollination, and the accumulation of carbon (C) and nitrogen (N). E-$[CO_2]$ could potentially affect all these processes. In E-$[CO_2]$, the stimulated photosynthesis followed by the changes in N nutrition (Leakey *et al.*, 2009) would alter C and N accumulation in grains, which could, in turn, influence the quality of rice grains.

Growth rates are not uniform among grains located at different positions within a panicle of rice. The early-flowering spikelets located on primary branches at the top of the panicle are referred to as superior spikelets (SS), whereas the late-flowering spikelets located on secondary branches of the lower primary branches are referred to as inferior spikelets (IS) (Yang & Zhang, 2010; Peng *et al.*, 2011).

The time interval for flowering of an entire panicle is normally 4 to 7 days. The different timing of flowering and grain filling leads to great variation in the weight and quality of grains borne on SS and IS, referred to as superior and inferior grains, respectively (Matsue *et al.*, 1994; Liu *et al.*, 2005; Cheng *et al.*, 2007; Wang *et al.*, 2007).

In experiments using the standard *japonica*-type rice cultivar Koshihikari, Zhang *et al*. (2013) indicated that E-$[CO_2]$ stimulates the growth of inferior grains at the expense of reductions in the protein content of superior grains. The results suggested that, rather than a dilution effect caused by the stimulated accumulation of carbohydrates by E-$[CO_2]$, the differential allocation of N and assimilates between superior and inferior grains is responsible for the reduction in grain protein content and the resultant change in grain quality due to E-$[CO_2]$. These changes in rice grain growth, however, could vary greatly among cultivars that differ in panicle structures and sink capacity.

Hasegawa *et al.* (2013) reported that yield responses to E-$[CO_2]$ differ among rice cultivars, and a large sink capacity is the trait that confers a greater yield advantage under E-$[CO_2]$. This finding suggests that future rice cultivars will need to have a larger sink capacity to take greater advantages of higher productivity in E-$[CO_2]$. The increased sink capacity can be achieved through changes in panicle structures for either greater grain size or a larger number of grains. The both traits have been effective for achieving higher productivity in cultivars Akita 63, a large-grained *japonica* cultivar (Mae *et al.*, 2006), and Takanari, an *indica* cultivar bearing numerous spikelets within a panicle (Nagata *et al.*, 2001; Taylaran *et al.*, 2011). Both Akita 63 and Takanari have indeed shown greater yield enhancements under E-$[CO_2]$ than other cultivars (Hasegawa *et al.*, 2013).

It must be noted that the changes in panicle structure for the greater sink capacity would alter the responses of grain growth to E-$[CO_2]$. If E-$[CO_2]$ preferentially supports grain mass and N accumulation of IS, reduction in grain

protein could be more pronounced in SS than in IS, as shown in Koshihikari (Zhang et al., 2013). If the proportion of IS is larger in a cultivar with a larger number of secondary rachis branches, as typically seen in Takanari, the increment of grain yield maybe larger due to the stimulated grain growth of IS and the grain N reduction in SS may be more than that seen in Koshihikari under E-[CO_2]. The increment of grain yield in Akita 63 may be smaller and the grain N reduction in SS of Akita 63 may be not as great as that in Koshihikari, because Akita 63 bears fewer secondary spikelets. Thus, our hypothesis was that E-[CO_2] would stimulate the grain growth of IS and decrease the grain protein in SS and thereby increase the grain yield and degrade the grain quality of rice, and that the grain yield stimulation would be largest in Takanari followed by Koshihikari and Akita 63, and that the grain quality degradation would be largest in Takanari followed by Koshihikari and Akita 63.

Among the various quality traits in rice, grain appearance often plays a major role, since rice is predominantly sold in markets as grains rather than flours. Chalkiness, for example, is a major appearance trait that affects milling quality and rice price (IRRI, 2006). In Japan, chalky grains have conventionally been classified into categories based on the position of opaque spots in the endosperm: milky white, white back, basal white, white core, and white belly kernels (Yoshioka et al., 2007), and generally ranked lower than the translucent ones. Grain chalkiness is a result of anomalous grain development and loosely packed starch granules, and is frequently found under high temperatures during the grain-filling stage (Tashiro & Wardlaw, 1991; Wang et al., 2008). Various studies have been conducted to better understand the genetic and physiological mechanisms that underlie the chalkiness (Cheng et al., 2003; Patindol & Wang, 2003; Tabata et al., 2005; Ishimaru et al., 2009). In addition to the temperature effects during the grain-filling stage, the occurrence of white back and basal white kernels are more likely to be related to plant N conditions, whereas the milky white kernels are more likely to be related to source–sink interactions (Kondo, 2011). E-[CO_2]

has been reported to increase rice grain chalkiness (Yang *et al.*, 2007), but the mechanisms are not fully understood.

In order to better understand the effects of E-[CO_2] on rice grain yield and quality, we conducted a free-air CO_2 enrichment (FACE) experiment using three cultivars with contrasting panicle structures and sink sizes to answer the following questions. Firstly, does E-[CO_2] affect the grain growth in SS and IS differently in the various rice cultivars? Secondly, is the degree of N reduction in rice grains caused by E-[CO_2] cultivar dependent? Finally, is grain chalkiness under E-[CO_2] associated with grain growth responses to E-[CO_2] in the three cultivars?

4.2 Materials and methods

4.2.1 Site conditions

Site conditions were described in Chapter 2 and Chapter 3.

4.2.2 Crop cultivation

Crop cultivation practices in 2010 and 2011 were reported by Zhang *et al.* (2013). Briefly, equal amounts of phosphorus (P) and potassium (K) were given to all the plots in early April, before plowing; compound P-K fertilizer was applied at a rate of 4.36 g P m^{-2} and 8.30 g K m^{-2}. Fields were kept submerged after late April. Three kinds of N fertilizer were applied just prior to puddling: 2 g N m^{-2} as urea, 4 g N m^{-2} as coated urea controlled-release fertilizer (type LP100, JCAM Agri. Co. Ltd., Tokyo, Japan), and 2 g N m^{-2} as another type of controlled-release fertilizer (type LP140, JCAM Agri. Co. Ltd.).

Seeds of the rice cultivars (Akita 63, Koshihikari, and Takanari) were first soaked in water to germinate and then 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). Cells of the seedling trays were filled with sterilized soil amended with fertilizer at a rate of 0.4 g N, 0.35 g P, and 0.5 g K

per 1 kg of soil, and 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 2010 and 25 May 2011, at a density of 22.2 hills m^{-2} and three seedlings per hill. After transplanting, the fields were kept flooded until 20 August, about 3 weeks after heading, when the ponding water was drained in preparation for the harvest. In order to keep the soil moist for the period after the water drainage, flush irrigation was applied as needed.

4.2.3 Harvesting, sampling, and analysis

In each plot, 21 hills of each cultivar were harvested at physiological maturity, equivalent to an area of 0.945 m^2 in both years. Harvested plants were air-dried under a rain shelter, and the total weight and panicle number were measured. The spikelets were weighed after threshing and were then dehulled to determine the brown rice weight. A grain moisture tester (Riceter f, Kett Electric Laboratory, Tokyo, Japan) was used to measure the moisture content of the grains, and the moisture content of rice straw was determined by the gravimetric method after oven-drying at 80 °C for 72 hours. Brown rice yield and single-grain weight were expressed on the basis of 15% moisture content, whereas all other mass-based variables were for dry mass. The sink capacity and sink-filling ratio were calculated using the following equations:

sink capacity (g m^{-2}) = spikelet number (m^{-2}) × single-grain weight (mg) /1000

sink-filling rate (%) = [brown rice yield (g m^{-2})/sink capacity (g m^{-2})] × 100.

In 2010, the grain chalkiness of fully matured grains was evaluated visually, and the grains were classified into milky white, basal white, white back, white belly, white core, cracked, and opaque kernels according to the criteria of Tashiro and Wardlaw (1991).

In 2011, 20 to 30 hills of each cultivar were monitored in each plot every day. The dates of panicle emergence were recorded with a marker on adhesive tape,

which was wrapped around each panicle below the flag leaf sheath. We collected 10 panicles from each plot at 5, 10, 15, 20, 30, and 40 days after heading (DAH) for all three cultivars, as well as at 50 DAH for Takanari. After oven-drying at 80 °C for 72 hours, SS and IS were separated according to the method of Peng *et al.* (2011). Grains were dehusked, weighed, ground into powder, and then subjected to N and C analysis with an NC analyzer (Sumigraph NC-22F, Sumika Chemical Analysis Service, Ltd, Tokyo, Japan).

At maturity, 15 panicles were collected from each plot and stored in an 80% ethanol solution. On each panicle, we counted the numbers of primary and secondary branches, which included the few tertiary branches only in Takanari, spikelets on primary and secondary branches, sterile spikelets on primary and secondary branches, and SS and IS.

4.2.4 Statistics

A blocked split-plot design was used for the experiment, with the CO_2 treatment as the main plot and the cultivar as the subplot, with four replicates. The statistical significance of each factor was tested by using the mixed model procedure of the SAS package (SAS Add-In 4.3 for Microsoft Office, SAS Institute, Tokyo, Japan). Comparison of means was conducted with Tukey's honestly significant difference (HSD) criterion. Percentage occurrence of anomalous grains, e.g. milky white, was converted to a rank on the ordinal scale and analyzed with logistic regression to avoid the problem of heterogeneity in the error variance. We fitted the Richard's function to the time course of grain mass accumulation in the SS and IS and then estimated the active grain-filling duration and average grain-filling rate according to the method of Zhang *et al.* (2012).

4.3 Results

4.3.1 Grain yield

E-[CO_2] significantly increased the brown rice yield, by 15% (93 g m^{-2}) averaged across the 2 years and three cultivars ($P<0.001$, Table 4.2). The grain yield in 2011 was larger than that in 2010 for all three cultivars, with a significant effect of year ($P<0.05$). The difference among cultivars was also highly significant ($P<0.001$): Takanari had the largest brown rice yield, followed by Akita 63 and Koshihikari. There was no significant interaction between CO_2 and cultivars for grain yield in this study.

E-[CO_2] significantly increased the panicle number ($P<0.001$) and spikelet number ($P<0.01$). The percentage of ripened spikelets was modestly improved ($P<0.1$), but single-grain weight of the bulk sample was not affected by E-[CO_2]. As was expected, single-grain weight differed greatly among cultivars, being largest in Akita 63, followed by Koshihikari and Takanari. On the other hand, the harvest index was largest in Takanari, followed by Akita 63 and Koshihikari, with a significant cultivar effect ($P<0.001$) but no significant effect of E-[CO_2].

4.3.2 Panicle survey

E-[CO_2] had no effect on either the number of primary and secondary branches or the number of spikelets within a panicle for any of the cultivars (Table 4.3). The interaction between E-[CO_2] and cultivar had significant effects on the primary spikelet number ($P<0.1$) and fertile primary spikelet number ($P<0.05$); the counts were increased by E-[CO_2] in Takanari, but decreased or unchanged in Akita 63 and Koshihikari. Cultivar differences of the panicle structure were significant for all the characteristics surveyed. The number of secondary spikelets and fertile secondary spikelets were highest in Takanari followed by Koshihikari and Akita 63 ($P<0.001$), and the number of primary spikelets and primary fertile spikelets were highest in Takanari followed by Akita 63 and Koshihikari ($P<0.001$).

E-[CO_2] or its interaction with cultivar had no effect on the numbers of SS and IS in any of the cultivars, but cultivar differences were significant (Table 4.4).

The number of SS was highest in Koshihikari followed by Akita 63 and Takanari ($P<0.001$), whereas the number of IS was highest in Takanari followed by Koshihikari and Akita 63 ($P<0.001$). The percentage of sterile SS in Takanari was highest among the three cultivars, and the percentage of sterile IS was highest in Akita 63. The number of fertile SS was lowest in Takanari. The number of fertile IS was similar in Takanari and Koshihikari, and Akita 63 had the fewest fertile IS.

4.3.3 Effects of E-[CO_2] on grain mass (GM), grain nitrogen (N), and grain carbon (C) at maturity

E-[CO_2] had no effect on GM in SS, but increased it in IS ($P<0.05$, Table 4.5). The effect of E-[CO_2] on GM of IS differed among cultivars ($P<0.01$): Takanari was increased by 21.8% and Koshihikari by 13.1%, whereas Akita 63 was unchanged. Grain C concentration in IS was increased by E-[CO_2] ($P<0.05$), and the grain C content (C weight per kernel) in IS responded to E-[CO_2] in the same manner as GM. Grain N concentration in SS was decreased by E-[CO_2] in Koshihikari (20.7%, $P<0.01$). Grain N content (N weight per kernel) was decreased by E-[CO_2] in SS ($P<0.05$), but was slightly increased in IS ($P<0.1$).

4.3.4 Temporal effects of E-[CO_2] on GM accumulation in SS and IS

E-[CO_2] stimulated the accumulation of GM in IS of Koshihikari and Takanari over time (Figure 4.1a, b). The accumulation of GM in SS was not significantly affected by E-[CO_2], but there were decreasing trends in general; the effects were marginally significant at 20 and 40 DAH for Koshihikari ($P<0.1$) and significant at 15 and 20DAH for Takanari ($P<0.05$). There was also a marginally significant stimulation of GM at 10 DAH by E-[CO_2] in SS of Takanari ($P<0.1$). There was no effect of E-[CO_2] on the GM accumulation in either SS or IS of Akita 63 (Figure 4.1c).

E-[CO_2] increased the average grain-filling rate of IS ($P<0.01$). The effect was most pronounced in Takanari followed by Koshihikari, whereas there was almost no effect on Akita 63(Figure 4.1, Table 4.6). E-[CO_2] shortened the active grain-

filling duration ($P<0.05$). The average grain-filling rate of SS was highest in Takanari ($P<0.001$), and that of IS was highest in Akita 63 ($P<0.1$). The active grain-filling duration of SS was shortest in Takanari followed by Koshihikari and Akita 63 ($P<0.001$).

4.3.5 Effects of E-[CO₂] on grain C and grain N accumulation in SS and IS of Koshihikari and Takanari

Grain C accumulation in IS was slower than that in SS (Figure 4.2a, b). The greater accumulation in IS started at 20 DAH, when the accumulation in SS was almost complete. The response of grain C accumulation to E-[CO_2] was similar to that of GM. Under E-[CO_2], the grain C content in IS of Takanari came close to that in SS at the end of grain filling.

Grain N accumulation in SS was depressed by E-[CO_2], but was improved by E-[CO_2] in IS particularly in Takanari (Figure 4.2d), for which the effect was significant at 15 and 20 DAH in both IS and SS, and the grain N in IS exceeded that in SS at 50 DAH (Figure 4.2d). For Koshihikari, the effect of E-[CO_2] on SS was marginally significant at 15 DAH ($P<0.1$) and significant at 40 DAH ($P<0.01$; Figure 4.2c).

4.3.6 Occurrence of various types of chalky kernels in Akita 63, Koshihikari, and Takanari

E-[CO_2] significantly increased the occurrence of milky white, basal white, and white back kernels in the three cultivars ($P<0.05$, Table 4.7). Akita 63 had the highest percentage of milky white kernels, followed by Takanari and Koshihikari, and the difference between cultivars was significant ($P<0.05$). We found significant interactions between cultivar and [CO_2] with regard to the percentage of basal white ($P<0.05$) and white back ($P<0.01$) kernels, and the cultivar ranks were Koshihikari>Akita 63>Takanari. The percentages of cracked, white core, and white belly kernels were not affected by E-[CO_2], although the differences among cultivars were significant ($P<0.001$, $P<0.05$, and $P<0.001$, respectively);

all were highest in Takanari. The percentage of opaque kernels was significantly decreased by E-[CO_2] in all the cultivars ($P<0.01$).

4.4 Discussion

We had found with a rice cultivar Koshihikari that E-[CO_2] stimulated the grain growth of IS at the expense of grain N reduction in SS (Zhang *et al*., 2013), and hypothesized that a cultivar with more secondary spikelets should exhibit a greater decline in grain N of SS as stimulated grain growth in IS in response to E-[CO_2]. The results of this study showed the expected changes of grain growth in IS and grain N in SS in other cultivars than Koshihikari. The stimulation in the grain growth of IS was indeed largest in Takanari, as we had hypothesized. However, the grain N degradation in Takanari was less than that in Koshihikari and Akita 63 under E-[CO_2]. Mechanisms underlying the differential changes between SS and IS and among the cultivars are discussed in the following sections.

4.4.1 The response of grain growth to E-[CO_2] could increase the grain yield enhancement

Grain growth responses to E-[CO_2] were dependent on the spikelet position and cultivar. For IS, E-[CO_2] significantly stimulated the grain-filling rate while shortening the grain-filling duration (Table 4.6), but had no effects on SS during the whole grain-filling period.

The stimulation of GM in IS by E-[CO_2] appeared to differ among the cultivars (Table 4.5) with a significant increase in Takanari followed by Koshihikari but a slight decline in Akita 63, conforming to the cultivar rank in the responsiveness of IS grain growth to E-[CO_2] (Figure 4.1). In Takanari, the GM of IS under E-[CO_2] came close to that of SS in ambient [CO_2] (Figure 4.1b) due partly to the smaller disparity between SS and IS in GM for this cultivar than the others and the continuously supply of nitrogen from leave maybe also a reason (Charles P. Chen, unpublished data). The opposite was true in Akita 63 and Koshihikari was located between the two cultivars. The greater response in GM to E-[CO_2] in Takanari

could contribute to larger sink strength (Marcelis LFM, 1996) and a greater yield response in this than other cultivars. This has indeed been observed when Hasegawa *et al*. (2013) reported varietal difference in grain yield response to E-[CO_2] (their Table 3), although, in this study, the effect of CO_2 x cultivar interaction on single grain weight did not reach the significance level (Table 4.2).

4.4.2 Grain protein reduction by E-[CO₂] varies among superior and inferior grains

E-[CO_2] is well known to decrease protein concentration in rice grains (Lieffering *et al.*, 2004; Taub *et al.*, 2008), and the reduced grain protein or N concentration has been explained by the dilution effect; that is, stimulated carbohydrate accumulation dilutes the N and protein concentrations in the plant (Gifford *et al.*, 2000; Lam *et al.*, 2012). When the superior and inferior grains were separately observed, however, the stimulation in carbohydrate accumulation was found only in IS whereas the reduction in N concentration was observed in SS only for cultivar Koshihikari (Table 4.5). This finding suggests a need to consider mechanisms besides the dilution to account for the E-[CO_2] induced grain N reduction.

Previous studies showed that grain N accumulation in IS was usually slow and suppressed by the accumulation in SS (Iwasaki *et al*., 1992, 1993). In this study, however, we found that E-[CO_2] stimulated grain N accumulation in IS at the expense of less accumulation in SS, and the effect was apparent at 20 DAH, especially in Takanari (Figure 4.2d). The simultaneous and opposite changes in grain N accumulation in superior and inferior grains under E-[CO_2] may indicate the allocation of N away from superior grains toward inferior grains (Figure 4.2c, d), i.e. distribution rather than dilution as a mechanism of reduced N concentration in E-[CO_2].

Alteration by E-[CO_2] to the distribution and partitioning of N and assimilates among rice organs has been reported (Weerakoon *et al.*, 2005; Seneweera, 2011;

Zhang *et al.*, 2013). The changes in distribution of N among superior and inferior grains and different rice organs may enhance the N-use efficiency, which will change the physiological requirements of plants for the nutrient under E-[CO_2] (Ainsworth & Long, 2005; Leakey *et al.*, 2009; McGrath & Lobell, 2013).

4.4.3 E-[CO_2] increased grain chalkiness of rice

In addition to the effect on grain nutritional quality, E-[CO_2] also affected grain appearance quality with the increased percentages of milky white, basal white, and white back kernels (Table 4.7), and the decreased percentage of opaque kernels. Chalky grains, especially basal white, white back, and milky white kernels, are frequently found when rice plants are subjected to heat stress or N limitation (Borrell *et al.*, 1999; Tsukaguchi & Iida, 2008; Ishimaru *et al.*, 2009).

In this study, the average air temperatures during the 20 days after heading were similar between ambient and E-[CO_2] treatments for all three cultivars (Table 4.1). Nevertheless, the temperature in the rice panicles could be higher under E-[CO_2] due to stomatal closure (Yoshimoto *et al.*, 2005), and the rice spikeletes under E-[CO_2] may have experienced greater heat stress than those in ambient [CO_2]. The increased chalkiness in E-[CO_2] could thus have been caused by elevated panicle temperature.

E-[CO_2] could have also increased the grain chalkiness via disturbed source–sink interactions. With the grain growth in IS of Koshihikari and Takanari being significantly stimulated by E-[CO_2] (Figure 4.1a, b), the assimilate supply per SS may be subjected to a greater competition for carbohydrates especially in Takanari, which had more IS than Koshihikari (Table 4.3). Previous studies have indeed shown greater percentages of milky white kernels due to manipulative reduction of the sink size relative to the source supply (Tsukaguchi & Iida, 2008; Kondo, 2011).

E-[CO_2] could have also increased the grain chalkiness via increased N limitations, particularly in Koshihikari which showed a significant grain N reduction in SS. N limitation has been reported to increase ratio of basal white kernels (Borrell *et al.*, 1999; Kondo, 2011).

Opaque kernels are more likely to be observed among small grains (Tsukaguchi & Iida, 2008). Because inferior grains are usually smaller and have poorer appearance quality than superior grains (Iwasaki *et al.*, 1992; Jongkaewwattana *et al.*, 1993; Wang *et al.*, 2008; Mohapatra *et al.*, 2011), opaque kernels are more likely to occur in IS. E-[CO_2] stimulated the growth of inferior grains which may have reduced the percentage of opaque kernels.

Previous studies have found large variations among cultivars in the occurrence of different types of chalky kernels (Nagahata & Yamamoto, 2005; Tsukaguchi & Iida, 2008). We also found significant effects of cultivar and, furthermore, the interaction between [CO_2] and cultivar on the occurrences of basal white and white back kernels (Table 4.7): the percentages of basal white and white back kernels were increased by E-[CO_2] in Koshihikari, but unchanged in Akita 63 and Takanari. This result could be related with plant N limitation, and Koshihikari migh have experienced greater N limitation as shown by the significant reduction in grain N concentration under E-[CO_2] (Table 4.5).

4.5 Implications for future research

As larger sink capacity confers greater yield advantage under E-[CO_2] (Hasegawa *et al*., 2013), Takanari and Akita 63 have larger sink capacities by having more spikelets per panicle and larger grain size, respectively, both of them gained larger yield stimulation by E-[CO_2] compared with Koshihikari. Although, in this study, the effect of CO_2 x cultivar interaction on brown rice yield did not reach the significance level, the net yield enhancement was most pronounced in Takanari, which have largest sink strength as shown in the stimulated grain growth. The negative relationship between sink capacity and sink-filling rate in

Koshihikari suggests the importance of sink strength in maximizing the grain yield stimulation by E-[CO_2] (Figure 4.3).

Less grain quality degradation occurred in Akita 63 and Takanari compared with Koshihikari under E-[CO_2]. Whether this can be explained by increased N uptake or N translocation from other organs of rice remains to be clarified. Our results also highlight the importance of higher N-use efficiency in selecting and breeding rice cultivars for the projected higher [CO_2].

Grain growth varies by panicle position and cultivar. The panicle structure was not affected by E-[CO_2] (Tables 4.2, 4.3), but the panicle number was significantly increased by E-[CO_2]. The simultaneous increase in the number of SS and that of IS (Table 4.4) indicates that the variation in grain weight and quality between superior and inferior grains will have substantial effects on the grain yield and quality responses of rice to E-[CO_2]. Further studies on the grain quality responses of superior and inferior grains to E-[CO_2], as well as the underlying mechanisms, will help rice breeding programs to deal more effectively with the future environmental changes.

4.6 Conclusion

In conclusion, future E-[CO_2] is likely to stimulate grain yield of rice but cause declines in grain nutritional and appearance quality. The responses of grain yield and quality to E-[CO_2] depend on spikelet position and cultivars, and increasing the number of spikeletes per panicle can take better advantages of E-[CO_2]. High N-use efficiency or greater N-uptake capability will be a key mechanism for maintaining rice grain quality under E-[CO_2].

4.7 References

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Table 4.1 Heading dates of Akita 63, Koshihikari and Takanari and air temperatures at different [CO_2] (A, ambient; E, elevated) at 5, 20, and 20–40 days after heading (DAH) in 2010 and 2011 growing seasons at Tsukuba FACE site, Japan.

Year	Cultivar	Heading date		Air temperature (ºC)						
		A	E	5 DAH		20 DAH		20–40 DAH		Growing season
				A	E	A	E	A	E	
	Akita 63	27 July	27 July	27.0	27.0	27.6	27.6	28.0	28.0	
2010	Koshihikari	31 July	30 July	28.4	27.9	27.7	27.7	27.6	27.8	25.0
	Takanari	5 Aug.	4 Aug.	27.5	27.8	27.7	27.7	27.0	27.2	
	Akita 63	2 Aug.	1 Aug.	25.9	25.1	26.4	26.5	24.9	24.6	
2011	Koshihikari	4 Aug.	3 Aug.	27.2	26.5	26.6	26.5	25.3	25.2	24.1
	Takanari	10 Aug.	8 Aug.	28.6	28.4	25.8	26.1	25.8	25.6	

The daily averaged air temperatures were calculated basing on the different heading dates.

Table 4.2 Effects of [CO_2] (A, ambient; E, elevated) on the grain yield, panicle traits, single-grain weight and harvest index of Akita 63, Koshihikari and Takanari in the 2010 and 2011 FACE experiments and the results of an analysis of variance (ANOVA)

Year	Cultivar	CO_2	Brown rice yield[1] (g m^{-2})	Panicle number (m^{-2})	Spikelet number (m^{-2})	Ripened spikelets (m^{-2})	Single-grain weight (mg)	Harvest index[2] (%)
2010	Akita 63	A	577	290	23872	75.2	32.2	49
		E	691	302	26631	82.7	31.4	51
	Koshihikari	A	552	321	28764	85.7	22.4	46
		E	642	351	32372	88.6	22.4	47
	Takanari	A	697	250	40124	86.0	20.2	54
		E	845	269	46311	88.4	20.6	56
2011	Akita 63	A	726	339	28620	79.9	29.5	51
		E	783	372	30885	81.2	29.6	50
	Koshihikari	A	604	347	31990	82.9	23.7	46
		E	698	403	37951	82.4	23.5	45
	Takanari	A	811	259	44193	81.1	21.4	56
		E	865	285	48950	79.4	21.5	56
ANOVA[3]			P-value					
CO_2			0.0003	0.0021	0.0023	0.2231	0.5344	0.1060
Year			0.0230	0.0061	0.0333	0.0590	0.9225	0.6180
CO_2 × Year			0.0950	0.1702	0.7947	0.2147	0.6957	0.0111
Cultivar			<0.0001	<0.0001	<0.0001	0.0005	<0.0001	<0.0001
CO_2 × Cultivar			0.8399	0.1366	0.3131	0.1853	0.1780	0.4307
Year × Cultivar			0.0397	0.0016	0.8110	0.0096	<0.0001	0.0861
CO_2 × Year × Cultivar			0.1859	0.6921	0.5186	0.9249	0.1450	0.2755

[1] Expressed on the basis of 15% moisture content.

[2] Estimated by sorting unhulled seeds in an ammonium sulfate solution with a specific gravity of 1.06 PRS; value is the percentage of spikelets that sank.

[3] Brown rice yield divided by the aboveground mass expressed on the 0% moisture (dry mass) basis.

Table 4.3 Effects of $[CO_2]$ (A, ambient; E, elevated) on the panicle structures of Akita 63, Koshihikari and Takanari in the 2011 FACE experiment and the results of an analysis of variance (ANOVA).

Cultivar	CO_2	Branch number [a]		Spikelet number [a]			Fertile spikelet number [a]		
		Primary	Secondary	Primary	Secondary	Total	Primary	Secondary	Total
Akita 63	A	11 b	16 b	60 abc	40 b	99 b	56 ab	33 b	89 b
	E	11 b	16 b	59 abc	40 b	99 b	56 ab	33 b	89 b
Koshihikari	A	10 b	20 b	58 bc	56 b	113 b	55 b	51 b	106 b
	E	10 b	19 b	55 c	54 b	109 b	53 b	49 b	102 b
Takanari	A	12 a	39 a	61 ab	128 a	189 a	56 ab	110 a	166 a
	E	13 a	37 a	64 a	119 a	184 a	59 a	100 a	159 a
ANOVA		P value							
CO_2		1.000	0.3523	0.9317	0.4399	0.5163	0.8254	0.2900	0.3343
Cultivar		<0.0001	<0.0001	0.0005	<0.0001	<0.0001	0.0019	<0.0001	<0.0001
$CO_2 \times$ Cultivar		0.6845	0.8401	0.0938	0.6681	0.9066	0.0462	0.4902	0.7700

a. Difference between the means across the cultivar x CO_2 combinations is indicated by the alphabets after the mean values. Means sharing a same character are not significantly different (p=0.05) from each other with Tukey-Kramer HSD test.

Table 4.4 Effects of $[CO_2]$ (A, ambient; E, elevated) on the number of superior and inferior spikelets within a panicle of Akita 63, Koshihikari and Takanari in the 2011 FACE experiment and the results of an analysis of variance (ANOVA).

Cultivar	CO_2	Spikelet number [a]		Sterile spikelet (%)[a]		Fertile spikelet number [a]	
		Superior	Inferior	Superior	Inferior	Superior	Inferior
Akita 63	A	6.5a	4.5b	4.3b	36.5a	6.5a	2.8b
	E	7.0a	3.8b	6.5ab	39.0a	6.8a	2.3b
Koshihikari	A	7.5a	8.3a	7.0ab	19.8b	7.0a	7.0a
	E	7.5a	8.5a	5.8b	18.8b	6.8a	7.3a
Takanari	A	4.0b	10.5a	25.8a	31.5ab	3.5b	7.0a
	E	4.5b	9.5a	19.8ab	36a	3.5b	6.3a
ANOVA		P value					
CO_2		0.1081	0.4167	0.6274	0.4233	0.9750	0.5428
Cultivar		<0.0001	<0.0001	<0.0001	0.0002	<0.0001	<0.0001
$CO_2 \times$ Cultivar		0.5098	0.6988	0.5986	0.7257	0.7613	0.7919

a. Difference between the means across the cultivar x CO_2 combinations is indicated by the alphabets after the mean values. Means sharing a same character are not significantly different (p=0.05) from each other with Tukey-Kramer HSD test.

Table 4.5 Effects of $[CO_2]$ (A, ambient; E, elevated) on grain mass (GM), grain carbon (C), and grain nitrogen (N) in superior and inferior spikelets of Akita 63, Koshihikari and Takanari in the 2011 FACE experiment and the results of an analysis of variance

Cultivar	CO_2	Grain mass [a] (mg kernel^{-1})		Grain C concentration [a] (mg g^{-1})		Grain C content [a] (mg kernel^{-1})		Grain N concentration [a] (mg g^{-1})		Grain N content [a] (mg kernel^{-1})	
		Superior	Inferior	Superior	Inferior	Superior	Inferior	Superior	Inferior	Superior	Inferior
Akita 63	A	27.8a	15.3ab	435b	439a	12.1a	6.72ab	11.8a	10.8c	0.33a	0.16b
	E	25.7a	14.0b	441ab	446a	11.4a	6.25b	11.4a	11.8bc	0.29a	0.16b
Koshihikari	A	20.5b	13.7b	447ab	442a	9.17b	6.04b	10.0ab	14.5a	0.21b	0.20ab
	E	19.8b	15.5ab	457a	447a	9.02b	6.92ab	7.93b	13.4ab	0.16b	0.21ab
Takanari	A	19.6b	14.7b	440ab	435a	8.61b	6.38b	10.9a	13.9ab	0.21b	0.21ab
	E	19.6b	17.9a	435b	444a	8.49b	7.95a	10.9a	13.5ab	0.21b	0.24a
ANOVA		P value									
CO_2		0.1354	0.0213	0.3279	0.0355	0.2511	0.0087	0.0674	0.7186	0.0456	0.0858
Cultivar		<0.0001	0.0195	0.0048	0.4159	<0.0001	0.0323	0.0009	0.0005	<0.0001	0.0002
$CO_2 \times$ Cultivar		0.3713	0.0059	0.1824	0.8775	0.6004	0.0059	0.1560	0.1661	0.3592	0.2141

a. Difference between the means across the cultivar x CO_2 combinations is indicated by the alphabets after the mean values. Means sharing a same character are not significantly different (p=0.05) from each other with Tukey-Kramer HSD test.

Table 4.6 Effects of $[CO_2]$ (A, ambient; E, elevated) on the grain filling in superior and inferior spikelets of Akita 63, Koshihikari and Takanari in the 2011 FACE experiment and the results of an analysis of variance (ANOVA).

Cultivar	CO_2	Averaged grain filling rate [a] (mg kernel^{-1} day^{-1})		Active grain filling duration [a] (days)	
		Superior	Inferior	Superior	Inferior
Akita 63	A	0.96bc	0.52ab	29a	37a
	E	0.99bc	0.59a	27a	33a
Koshihikari	A	0.89bc	0.40ab	22a	43a
	E	0.77c	0.53ab	26a	32a
Takanari	A	1.31ab	0.39b	14b	42a
	E	1.51b	0.54ab	13b	32a
ANOVA		P value			
CO_2		0.6641	0.0040	0.9515	0.0448
Cultivar		0.0003	0.0644	<0.0001	0.8439
CO_2 × Cultivar		0.3378	0.5659	0.1955	0.7429

a. Difference between the means across the cultivar x CO_2 combinations is indicated by the alphabets after the mean values. Means sharing a same character are not significantly different (p=0.05) from each other with Tukey-Kramer HSD test.

Table 4.7 Effects of $[CO_2]$ (A, ambient; E, elevated) on the occurrence of various types of chalky kernels in Akita 63, Koshihikari and Takanari in the 2010 FACE experiment and the results of ordinal logistic regression.

Cultivar	CO_2	Milky white (%)	Basal white (%)	White back (%)	White belly (%)	Opaque (%)
Akita 63	A	13.5	6.8	3.5	1.4	4.0
	E	18.4	7.1	2.9	0.1	1.4
Koshihikari	A	8.0	22.4	12.0	1.4	3.5
	E	11.9	29.3	24.8	1.5	1.0
Takanari	A	10.5	3.5	1.0	32.4	3.0
	E	16.0	3.7	0.4	36.3	0.6
ANOVA				P value [a]		
CO_2		0.0007	0.1207	0.4725	0.0909	0.0004
Cultivar		0.0017	<0.0001	<0.0001	<0.0001	0.1905
$CO_2 \times$ Cultivar		0.7717	0.0236	0.0063	0.0132	0.6513

a. P values for each treatment effect denote the statistical significance of the coefficients for each term in the ordinal logistic regression.
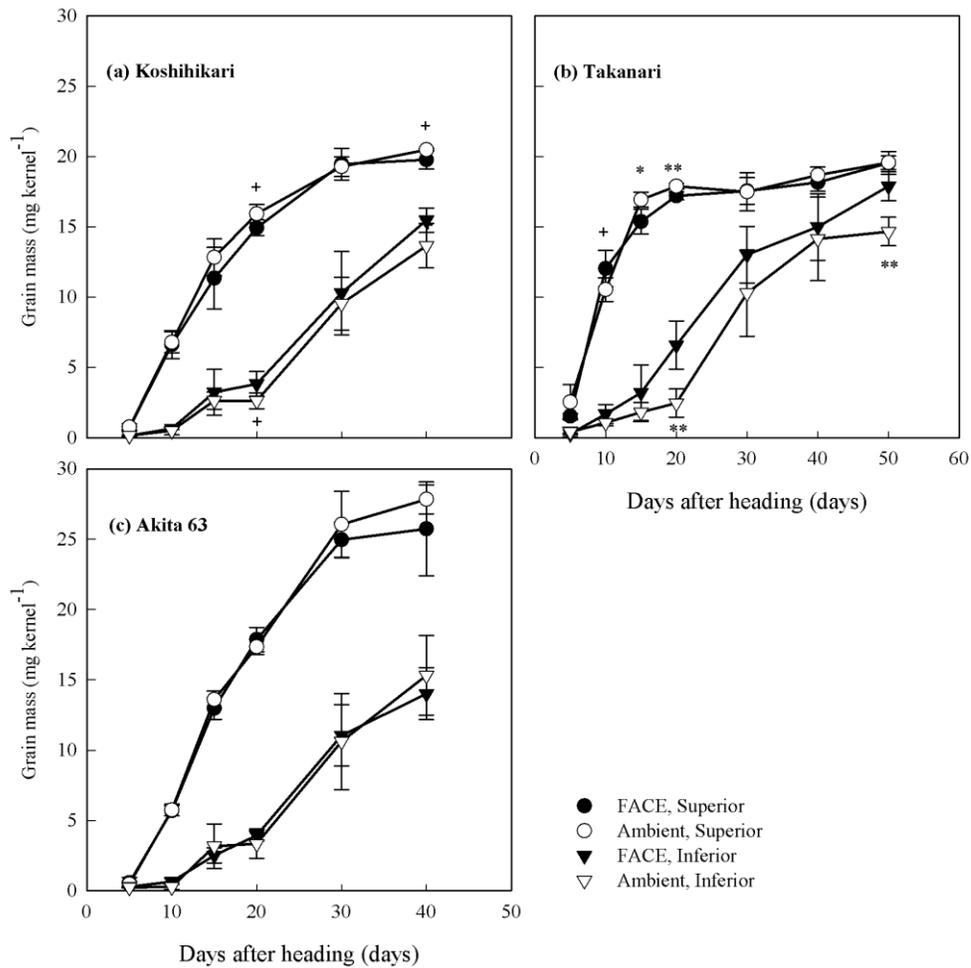
Figure 4.1 Accumulation of grain mass in superior and inferior spikelets in Koshihikari (a), Takanari (b), and Akita 63 (c) under different $[CO_2]$ (Ambient, ambient; FACE, elevated) in the 2011 FACE experiment. Bars indicate standard error of the mean (n=4). Significant differences between FACE and ambient in the superior and inferior spikelets, respectively, are marked as +, $P<0.1$; *, $P<0.05$; **, $P<0.01$.
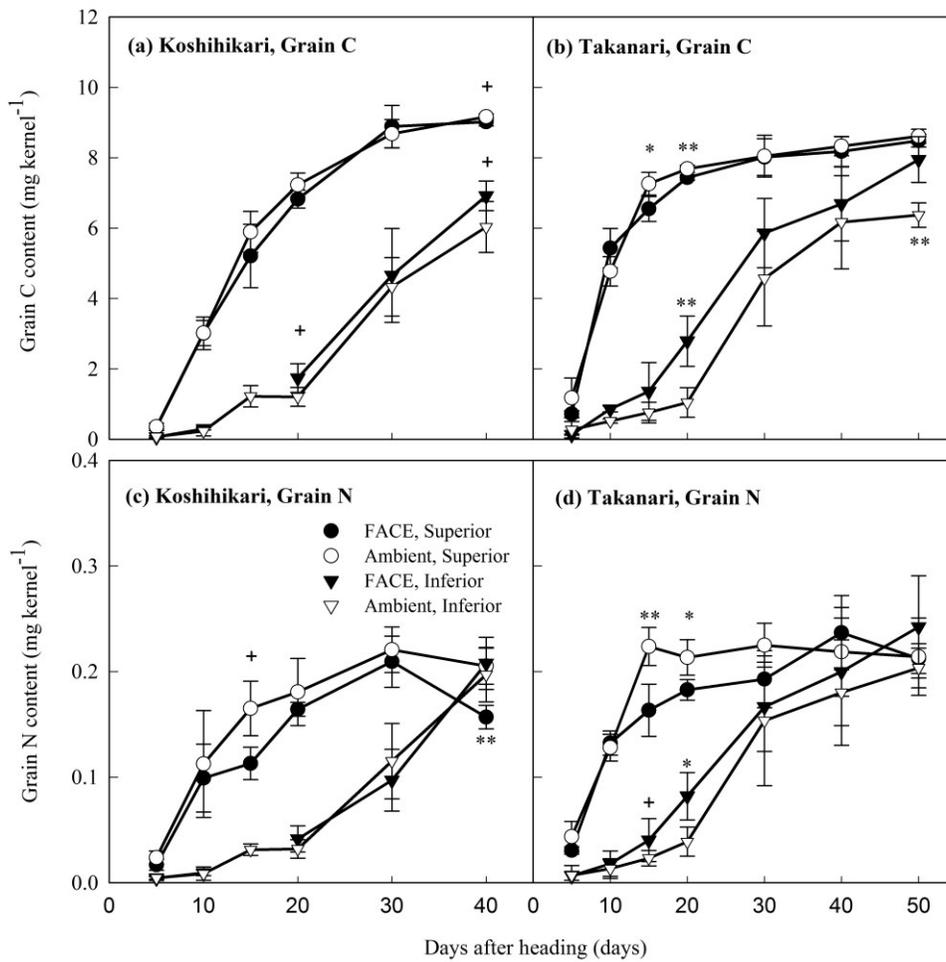
Figure 4.2 Accumulation of grain carbon and grain nitrogen content in superior and inferior spikelets in Koshihikari and Takanari under different [CO_2] (Ambient, ambient; FACE, elevated) in the 2011 FACE experiment. Bars indicate standard error of the mean (n=4). Significant differences between FACE and ambient in the superior and inferior spikelets, respectively, are marked as +, $P<0.1$; *, $P<0.05$; **, $P<0.01$.
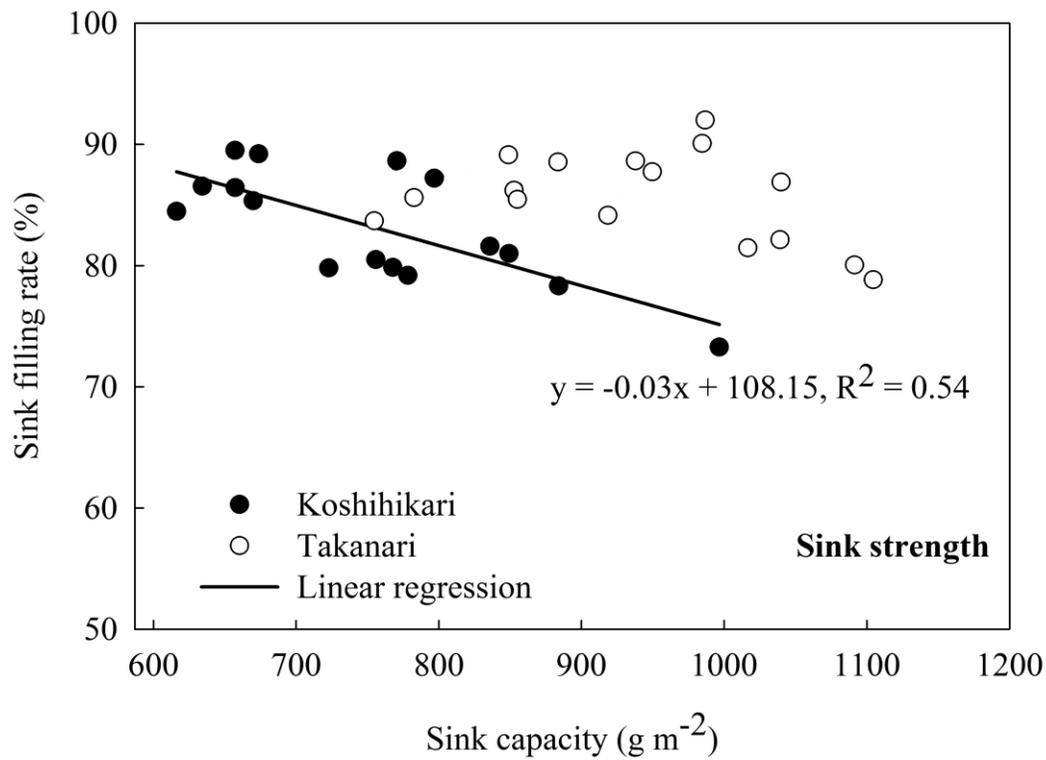
Figure 4.3 Relationships between sink capacity and sink filling rate of Koshihikari and Takanari in the 2010 and 2011 FACE experiments. The regression lines were drawn for Koshihikari and Takanari respectively. And the regression for Koshihikari is significant ($P< 0.01$).
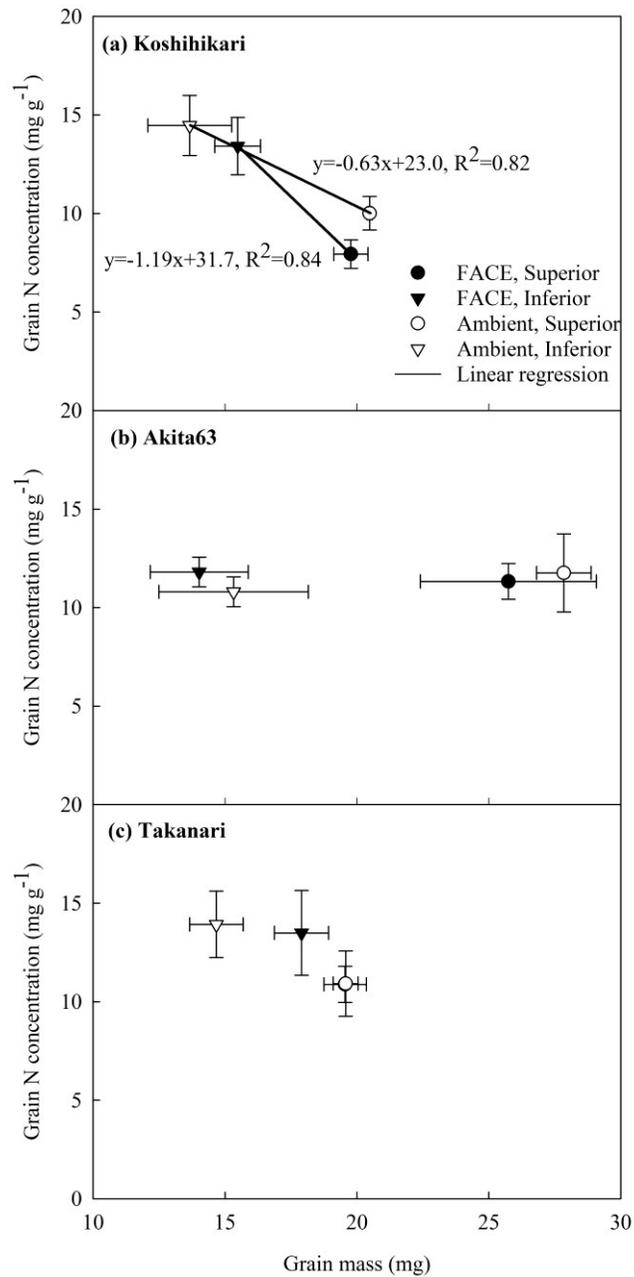
Figure 4.4 Relationships between grain mass and grain nitrogen concentration in superior and inferior spikelets of Koshihikari (a), Akita 63 (b), and Takanari (c) under different $[CO_2]$ (FACE, elevated; Ambient, ambient) in the 2011 FACE experiment. Bars indicate standard error of the mean (n=4). The regression lines were drawn for FACE and ambient, respectively in Koshihikari (a). The slopes between FACE and ambient were significantly different (P<0.05).

CHAPTER 5

FUTURE RICE

Synthesis of findings and implications for future studies

Chapter 5

In Chapter 1 of this dissertation, I presented three research topics, namely

1. The effects of nitrogen fertilizer application on the responses of rice yield and quality to elevated $[CO_2]$,

2. The differences of grains located at different panicle positions in their responses to elevated $[CO_2]$, and

3. The cultivar difference in the grain growth responses to elevated $[CO_2]$.

Synthesizing the findings described in the preceding chapters, I would try to give answers to the above research questions in this chapter. Admittedly, however, my answers are bound by various limitations, which are also discussed below.

5.1 Nitrogen fertilizer applications under elevated CO_2 concentrations

5.1.1 Nitrogen fertilizer applications enhance the yield stimulation of rice by elevated $[CO_2]$

The study on the interactive effects of nitrogen fertilizer application and rising CO_2 on rice started at last century. Ziska *et al.* (1996) found that the growth and photosynthetic response of rice to elevated $[CO_2]$ were highly dependent on the supply of nitrogen. If the nitrogen is not enough, the additional CO_2 may not be fully used by rice in their photosynthesis and growth as a lack of sinks. This was tested by my study (Chapter 2), the yield enhancement by elevated $[CO_2]$ was 5% lower at low nitrogen condition than ample nitrogen condition, and the difference was mainly caused by the limited number of spikelet.

Kim *et al.* (2001) suggested that sufficient nitrogen at panicle initiation stage can increase the sink capacity through the increment of spikelet number, which can make use of the additional CO_2 to gain larger grain yield. While (Figure 5.1), redundant nitrogen fertilizer application failed to stimulate larger sink capacities and the grain yield stimulations under elevated $[CO_2]$ were not proportionally related with nitrogen fertilizer applications (Kim *et al.*, 2003a; Yang *et al.*, 2006).

Moreover, nitrogen fertilizer application exacerbated the nitrogen reduction under elevated $[CO_2]$ (Zhang *et al.*, 2013).

Although the redundant nitrogen fertilizer application cannot gain proportional yield stimulation under elevated $[CO_2]$, but how will the extreme low nitrogen fertilizer application affect the effects of elevated $[CO_2]$ on grain yield and quality is yet unclear. The low nitrogen levels stated above are not uniform, for (Kim *et al.*, 2003a; Kim *et al.*, 2003b), the low nitrogen fertilizer applications was 4 g m^{-2}; for (Yang *et al.*, 2006), that was 15 g m^{-2},   neither of them tried the extreme low nitrogen conditions, 0 g m^{-2} of nitrogen fertilizer application.

In my study, grain yield of rice cultivar Koshihikari was increased by 11% under elevated $[CO_2]$ at 0 g m^{-2} of nitrogen fertilizer application, the yield enhancement was higher than that of Akitakomachi at a low nitrogen fertilizer application of 4 g m^{-2}, which was 7% (Kim *et al.*, 2003a). This can be explained by the larger sink capacities of Koshihikari than Akitakomachi (Hasegawa *et al.*, 2013). The differences of these studies should be also related with soil heterogeneity, temperature difference, cultivar variation, and mainly the efficiency of nitrogen fertilizer application, which was closely related with the nitrogen uptake.

Besides the nitrogen fertilizer application, nitrogen uptake affects the response of rice growth and yield to elevated $[CO_2]$ (Kim *et al.*, 2003b). The nitrogen uptake response to elevated $[CO_2]$ dependent on growth stages (Shimono and Bunce, 2009), and the effect of elevated $[CO_2]$ on the nitrogen uptake at reproductive stages was negative (Kim *et al.*, 2003b). Yamakawa *et al.* (2004) found that the nitrogen uptake was higher under elevated $[CO_2]$ compared with ambient $[CO_2]$ at panicle initiation, while after panicle initiation, the nitrogen uptake was only related with the soil nitrogen availability. Thus additional nitrogen fertilizer applications at reproductive stages may help rice to enhance nitrogen uptake under elevated $[CO_2]$.

Future studies should focus on how to improve the efficiency of nitrogen fertilizer applications in ensuring the sufficient plant nitrogen at both vegetative

and reproductive stages under elevated [CO_2], for example, top-dressing of nitrogen fertilizer from 35 to 30 days before heading can efficiently generate a larger spikelet number (Kamiji *et al.*, 2011), adjusting the methods of nitrogen fertilizer application even without increasing the total nitrogen fertilizer application rate can also increase grain yield (Zhang *et al*., 2012), whether these are common under elevated [CO_2] is unknown.

5.1.2 Nitrogen fertilizer application cannot counteract the negative effects of elevated CO_2 on grain quality of rice

 Elevated [CO_2] reduce grain quality of rice, especially in terms of protein content and grain chalkiness (Taub *et al.*, 2008; Yang *et al.*, 2007). Increasing the nitrogen fertilizer application can minimize the effects of elevated [CO_2] on crop protein concentration (Idso and Idso, 2001; Taub *et al.*, 2008), and nitrogen fertilizer application suppressed the occurrence of chalky kernels in rice (Qiao *et al.*, 2011). Thus nitrogen fertilizer application seems to be able to counteract the negative effects of elevated [CO_2] on grain quality of rice.

 While, in this study (Chapter 2), the nitrogen fertilizer application suppressed the occurrence of grain chalkiness at ambient [CO_2], at elevated [CO_2], the occurrence of grain chalkiness was less dependent on nitrogen fertilizer applications. These should be better explained by the efficiency of nitrogen fertilizer applications in improving the plant nitrogen concentration under ambient and elevated [CO_2]. Yang *et al.* (2007) found that reduction in grain protein and increment in grain chalkiness under elevated [CO_2] were independent of nitrogen fertilizer applications. And Zhang *et al.* (2013) found that the nitrogen reduction by elevated [CO_2] under ample nitrogen fertilizer applications was larger than low nitrogen fertilizer applications, which can be explained by the dilution effects (Gifford *et al.*, 2000), as the growth and yield stimulations were usually larger in ample nitrogen fertilizer applications than low nitrogen fertilizer applications (Amthor, 2001; Manderscheid *et al.*, 2009; Zhang *et al.*, 2013). Thus even with reductant nitrogen fertilizer supplied by the traditional applications, the plant

nitrogen concentration may not be maintained but still reduced under elevated $[CO_2]$, inducing the increase of the occurrence of grain chalkiness.

Mechanisms for the occurrence of grain chalkiness under elevated $[CO_2]$ are not fully understood, further studies are needed. For example, as the grain chalkiness is a result of abnormally developed and loosely packed starch granules in the endosperm (Fitzgerald *et al.*, 2009), through the grain section image analysis (Matsushima *et al.*, 2010; Morita *et al.*, 2005), we can check on the accumulation of starch granules and endosperm cells to understand whether the grain chalkiness was induced by disturbed nitrogen limitations or source-sink interactions under elevated $[CO_2]$.

Grain protein is an important trait for rice nutritious quality. Elevated $[CO_2]$ is well known to reduce grain protein in rice and higher nitrogen fertilizer application rate cannot counteract the negative effect (Yang *et al.*, 2007; Zhang *et al.*, 2013). In this study, the grain nitrogen reduction by elevated $[CO_2]$ was larger at ample nitrogen fertilizer application than low nitrogen fertilizer application. And indeed, the grain nitrogen reduction by elevated $[CO_2]$ was not significant at zero nitrogen fertilizer application. But the yield stimulation was small too, further studies are needed to better balance the grain yield (quantity) and grain quality. Not only protein, the concentrations of several essential micronutrients such as iron and zinc are also low in rice grain (Johnson, 2013). As a result, billions of people suffer the protein and micronutrient malnutrition with symptoms including iron-deficiency anaemia, growth retardation and blindness. Rising atmospheric $[CO_2]$ imbalances plant stoichiometry, decreases grain protein in rice (Taub *et al.*, 2008), but little is known about the effects of elevated $[CO_2]$ on grain micronutrient mineral concentrations (Johnson, 2013). Further studies in revealing the grain elementary concentration response to elevated $[CO_2]$ may help us better evaluate the effects of elevated $[CO_2]$ on rice.

Moreover, rice, wheat, corn and soybean are the major crops in the world, in order to evaluate an overall effects of future higher $[CO_2]$ on the grain quality of global crops. The nutritious quality (protein and mineral) of these grains (rice,

wheat, corn and soybean) under elevated $[CO_2]$ should be studied in the near future.

5.2 Effects of elevated $[CO_2]$ on grain growth of rice at different positions in a panicle

The response of individual grain weight to elevated $[CO_2]$ has been inconsistent (Baker and Allen, 1993; Kim *et al.*, 2003a; Yang *et al.*, 2006; Ziska *et al.*, 1997). As 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 this study (Chapter 3 and Chapter 4), the grain weight and nitrogen content responded to elevated $[CO_2]$ differently according to their panicle positions. We had described with a rice cultivar Koshihikari that elevated $[CO_2]$ stimulated the grain growth of inferior spikelet at the expense of grain nitrogen reduction in superior spikelet (Zhang *et al.*, 2013). Understanding the variations in the responses of superior and inferior spikelets may help us better reveal the adaptation of rice to the environment change. With an increased carbon supply by elevated $[CO_2]$, rice would like to produce more seed even if they are inferior grains. The increased quantity of seed was at an expense of degraded grain quality. Whether rice was not wise enough to balance the quantity and quality of their offspring remains unknown. Whether these quantity-stimulated inferior grains and quality-degraded superior grains could be well qualified seeds for rice under elevated $[CO_2]$ remains unknown. Actually, the superior and inferior grains are two extreme groups, there are still normal grains located at the panicle, which are nether superior or inferior. Further studies on the single grain response may give us more information on rice physiology.

5.3 Effects of elevated [CO_2] on grain growth of rice in different cultivars

I hypothesized that a cultivar with more secondary spikelet should exhibit a greater increase in grain growth in inferior spikelet and a larger decline in grain nitrogen of superior spikelet in response to elevated [CO_2]. The results of the cultivar x [CO_2] study showed the expected changes of grain growth in inferior spikelet and grain nitrogen in superior spikelet in other cultivars also. The stimulation in the grain growth of inferior spikelet was indeed largest in Takanari, as we had hypothesized. However, the grain nitrogen degradation in Takanari was less than that in Koshihikari and Akita 63 under elevated [CO_2] (Chapter 4).

Larger sink capacity confers greater yield advantage under elevated [CO_2] (Hasegawa *et al*., 2013), although, in this study, the effect of [CO_2] x cultivar interaction on brown rice yield did not reach statistical significance level. The varietal difference was found in grain quality degradation due to elevated [CO_2]. Whether this can be explained by increased nitrogen uptake or nitrogen translocation from other organs of rice warrants further studies. The grain growth variation within a panicle in relation to the distribution of carbon and nitrogen will be a focus of the future studies. The better understandings of the mechanisms of rice grain yield and quality responses to elevated [CO_2] will help the breeding efforts for cultivars without degraded grain quality under elevated [CO_2] in the future.

5.4. Implications for rice production better adapted to elevated CO_2 concentrations.

In order to make sure of the food security in future, besides the grain yield, it is urgent to understand the underlying changes in grain quality and grain growth that occur when crops are grown under what is predicted to be future atmospheric and climate conditions, discover and reveal the underlying mechanisms, develop applicable resolutions to help farmers adapt and inspire a new greener revolution.

Chapter 5

This definitely need the efforts from all of us human beings, especially, scientists, politicians and farmers.

With this dissertation study, I focused on the effects of elevated $[CO_2]$ on the grain yield and quality of rice. The study covers: regulations of nitrogen fertilizer applications on the effects of elevated $[CO_2]$ on rice yield and quality, differences of grains located at different panicle positions in response to elevated $[CO_2]$, response of grain growth to elevated $[CO_2]$, cultivar differences of rice in response to elevated $[CO_2]$. There are some new findings as well as limitations, while from this study advices on future nitrogen application and breeding were given as follows:

In synthesis of this dissertation, advices for nitrogen fertilizer applications under elevated $[CO_2]$ is that on base of the ample nitrogen applications, extra nitrogen fertilizer should be applied to the plant at panicle initiation stage, and the type of the fertilizer and the way of applications should be studied more in details in future.

Insight for cultivar selection and breeding for future high $[CO_2]$ is that cultivars that have more secondary branches and spikelet number within a panicle may benefit more from the elevated $[CO_2]$ on grain yield when accompanied by higher capacity to supply sufficient nitrogen to the growing grain, a higher nitrogen use efficiency is important to ensure the grain quality.

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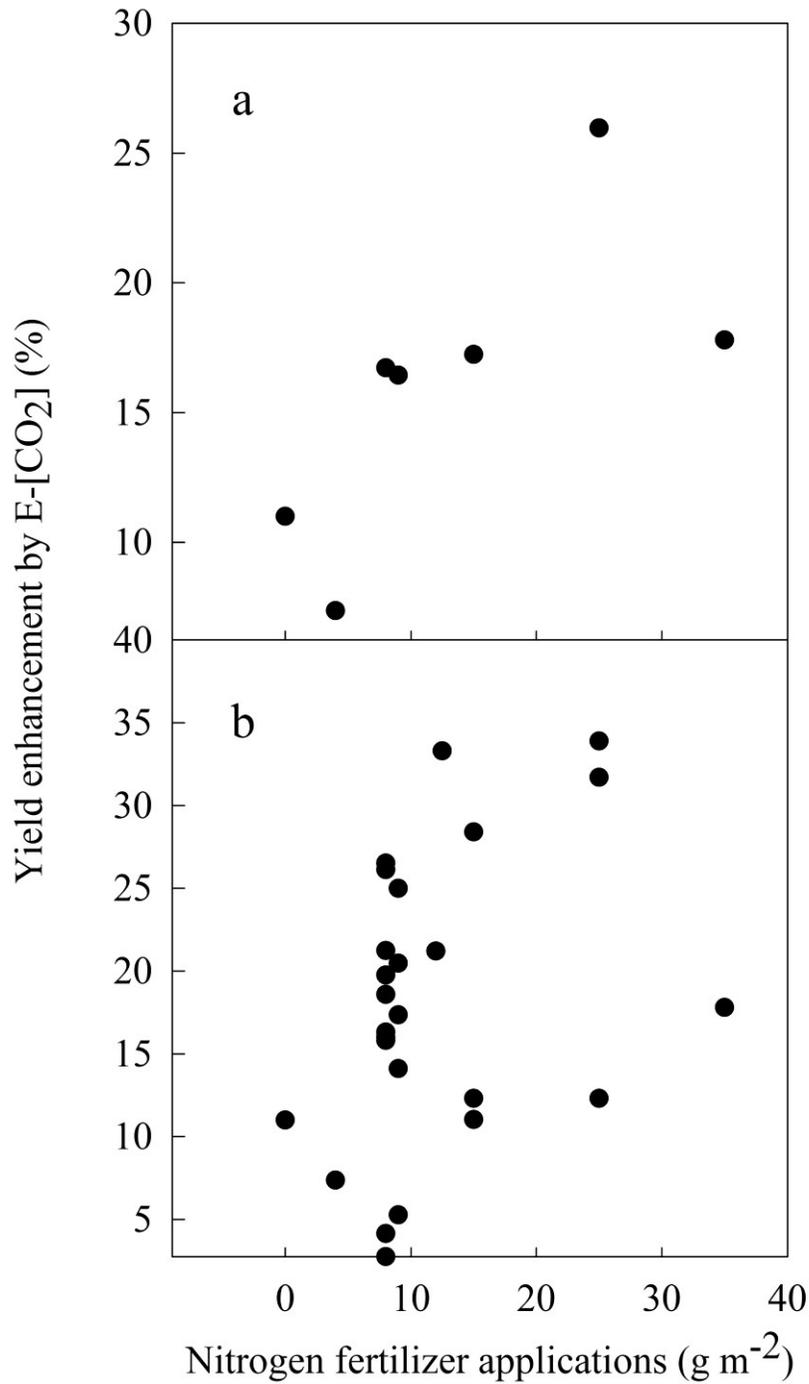
Figure 5.1 Yield enhancement by elevated [CO_2] and nitrogen fertilizer applications, a is the average data, b is the original data, datas were collected from 6 published papers in three rice FACE experiments (Shizukuishi and Tsukuba, Japan; Jiangsu, China) with different rice cultivars and growing seasons.

Effects of elevated CO_2 concentration on grain yield and quality of rice (*Oryza sativa* L.)

A thesis submitted in fulfilment of the requirements for admission to the degree of Doctor of Philosophy

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