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Enhancing the chelation capacity of rice to maximise iron and zinc concentrations under elevated atmospheric carbon dioxide

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Abstract. Roughly half of the Earth's seven billion people rely on rice as their primary source of food. The milled grain of rice, often referred to as polished or white rice, serves as a rich source of energy but is low in protein and several essential micronutrients such as iron and zinc. As a result, billions of people in rice-based countries suffer the debilitating effects of protein-energy and micronutrient malnutrition with symptoms including iron-deficiency anaemia, growth retardation and blindness. By 2050, the Earth's atmospheric carbon dioxide concentration ([CO_2]) is expected to reach 550 μmol mol^{-1}, representing a 70% increase from today's concentration of 392 μmol mol^{-1}. The impacts of elevated [CO_2] on plant growth will likely include agronomically useful traits such as increased biomass, yield and water-use efficiency. However, increased plant productivity is likely to be accompanied by decreased protein and micronutrient mineral concentrations of cereal grain. This review focuses on the effects of carbon dioxide-enrichment on rice physiology and nutritional composition and proposes increased activity of the Strategy II iron uptake pathway as a promising method to maintain or increase iron and zinc concentrations in rice grain, and perhaps cereal grain in general, under elevated [CO_2].

Additional keywords: biofortification, FACE, hidden hunger, nicotianamine, phytate, Strategy II.

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Rice as a staple food today – areas of consumption and nutritional composition of the grain

Rice (*Oryza sativa* L.) is the staple food for approximately half of the world's population and provides more than 70% of total food energy in many developing countries such as Bangladesh, Cambodia and Myanmar (Juliano 1993; FAO 2006). Rice is also the staple food for three of the four most populated countries on Earth: China, India and Indonesia. Accordingly, Asia produces most of the world's rice with six Asian countries in particular – China, India, Indonesia, Bangladesh, Vietnam and Japan – producing and consuming ~80% of the world's rice supply each year. Outside of Asia, rice is an important staple food for many countries in Latin America, the Caribbean and Africa. Despite its widespread use as a food staple, the nutritional composition of rice grain is lacking in several areas and malnutrition disorders frequently occur in rice-based diets.

Most of the world's rice is milled to produce polished or white rice. The polished grain is suitable for long-term storage and because of the removal of the outer aleurone, bran (pericarp, seed-coat, nucellus) and germ (embryo) through milling, it is composed primarily of starch-containing endosperm cells that provide dietary energy. In total, starch constitutes 90% of the dry weight of polished rice. The protein content of polished rice is 7% and, although rich in the limiting essential amino acid lysine, represents the lowest protein content among the cereal crops (Juliano 1993). As a result, diets high in rice can result in protein-energy malnutrition (PEM), a disorder frequently seen in young children as failure to thrive. Alarmingly, more than a third of children around the world are affected by PEM, with ~80% of those children living in Asia where rice is the major staple food (de Onis *et al*. 1993).

With regards to micronutrient composition, the polished rice grain contains no provitamin A and only low concentrations of iron (Fe) and zinc (Zn). Rice-based diets often lead to deficiencies in these essential micronutrients and international research consortiums such as the HarvestPlus Challenge Program have emerged to produce 'biofortified' rice and other food staples with nutritionally adequate concentrations of these three micronutrients (Ramakrishnan 2002; Mayer *et al*. 2008). Golden rice is a genetically-engineered variety of rice containing transgenes that direct β-carotene accumulation to the polished grain (Ye *et al*. 2000). It is hoped that Golden rice will provide the daily recommended allowance of provitamin A in 100–200 g of polished rice, this corresponding to the daily intake of rice for the estimated 100–200 million children currently affected by vitamin A deficiency and at risk of blindness or death. Iron is critical for proper mental development, immune function and oxygen transport and iron deficiency is the most common nutritional deficiency in the world, affecting more than two billion people, many of them in developing countries. Conventional breeding has yielded only modest increases in Fe concentrations of rice grain and, as a result, numerous genetic engineering approaches have been employed to boost levels to those sufficient for iron biofortification (Lucca *et al*. 2002; Lee *et al*. 2009; Wirth *et al.* 2009). Zinc deficiency is estimated to affect ~50% of people in rice-based countries such as Bangladesh and causes a variety of disorders such as impaired immune function and child stunting (Arsenault *et al*. 2010). Conventional breeding for high Zn rice has made considerable progress and, unlike the situation with provitamin A and Fe, appears to be an effective approach for producing Zn biofortified rice.

Increased protein and micronutrient composition of rice grain is urgently needed and could radically improve the nutritional health of billions of people in developing countries around the world. In 2008, the Copenhagen Consensus ranked biofortification as the fifth most important solution in a list of 30 solutions to global challenges, recognising it as one of the most effective and economical methods (along with micronutrient fortification and supplements) to deal with the enormous burden of global malnutrition (Bouis *et al*. 2011).

Rice as a staple food in 2050 and beyond – assessing the impacts of population increase and an increasingly carbon dioxide-enriched atmosphere

The world's population reached 7 billion in 2011 and is expected to rise to ~9.3 billion by 2050. Most of this population increase will occur in developing countries, where populations are projected to rise sharply from roughly 5.6 billion today to 7.9 billion by 2050 (United Nations Department of Economic and Social Affairs Population Division 2009). China and India will remain, by far, the most populated countries in 2050 with roughly 3 billion people in these two countries alone. Increasingly large populations in China and India and other rice-consuming countries such as Indonesia will have major impacts on the production of rice, with many estimating that world demand for rice will increase by more than 50% by 2050 (von Braun and Bos 2005).

Due largely to the burning of fossil fuels and deforestation, the Earth's atmospheric carbon dioxide concentration ([CO_2]) is rising rapidly and is expected to reach a concentration of 550 μmol mol^{-1} by 2050. This represents a 70% increase from today's concentration of ~392 μmol mol^{-1} CO_2 and is predicted to have major effects on the growth of rice and all terrestrial plants. Through a combination of growth chamber, open-top chamber (OTC) and, most recently, free-air CO_2 enrichment (FACE) experiments, we know that rice will almost certainly be a larger, higher-yielding and more water-efficient crop at 550 μmol mol^{-1} CO_2. These agronomic changes are due to the fact that rice, like all C_3 plants, responds to elevated [CO_2] with increased photosynthetic activity and decreased stomatal conductance of CO_2 and water (Ainsworth and McGrath 2010). Initial growth chamber studies at 700 μmol mol^{-1} CO_2 reported aboveground biomass gains of 26% and yield gains of 58% for rice (Seneweera *et al*. 1996; Seneweera and Conroy 1997). However, subsequent field studies using FACE technology have found that enclosed CO_2-enrichment studies tend to overestimate biomass and yield gains and it is more likely that 550 μmol mol^{-1} CO_2 will result in yield increases of ~12% for rice, with most of the yield increase due to increased numbers of panicles per area (Long *et al*. 2006; Yang *et al*. 2006; Ainsworth 2008; Leakey *et al*. 2009). Other environmental changes predicted to occur as a result of increased [CO_2], such as warmer night temperatures, however, could largely negate the yield gains that have been observed in CO_2-enrichment studies (Cheng *et al*. 2009).

A near universal finding of CO_2-enrichment studies, both enclosed and FACE, is that nitrogen (N) concentrations of plant tissues decrease significantly under elevated [CO_2]. Although many hypotheses have been put forward to explain this result, it is widely recognised that decreased concentrations of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) in photosynthetic tissues are responsible for a significant portion of the N decrease observed under elevated [CO_2]. Additional mechanisms thought to contribute to decreased N concentrations in plant tissues include 'dilution' as more biomass accumulates under elevated [CO_2] as well as reduced N uptake due to reduced transpiration, all of which have been reviewed extensively (Pang *et al*. 2006; Taub and Wang 2008; Taub *et al*. 2008). Although decreased N concentrations in leaf tissues translate into higher photosynthetic nitrogen-use efficiencies for plants under elevated [CO_2], these decreased concentrations are also thought to reduce the overall pool of N that is available for translocation from leaf to cereal grain during seed filling. Numerous studies have shown that the already low protein content of rice grain decreases a further 10–14% under elevated [CO_2] (Seneweera and Conroy 1997; Lieffering *et al*. 2004; Terao *et al*. 2005). With the exception of cystine and arginine, elevated [CO_2] decreases the concentration of all amino acids in unpolished and polished rice grain by 30–40% (Wang *et al*. 2011). The closely related cereal species, wheat, displays similar drops in protein content of grain to the extent that the required concentration for bread making (11.5%) may not be attainable under growth at 550 μmol mol^{-1} CO_2 (Conroy *et al*. 1994; Kimball *et al*. 2001; Högy and Fangmeier 2008; Ainsworth and McGrath 2010). Without significant increases to the protein content of rice grain, it appears certain that the severity of PEM as a global nutritional disorder will increase significantly under future, CO_2-enriched atmospheres. Grain protein content does, however, show considerable variability across rice varieties and conventional breeding may be a viable strategy for producing new cultivars with increased grain protein content (Kennedy and Burlingame 2003; Mahmoud *et al*. 2008).

In addition to the well documented physiological effects of elevated [CO_2] on rice growth (increased biomass, yield and water-use efficiency) and protein content of rice grain, there is accumulating evidence that points towards a significant decrease in the concentrations of Fe and Zn in rice grain under elevated [CO_2]. Early growth room experiments at 700 μmol mol^{-1} CO_2 found 17 and 28% decreases in Fe and Zn concentrations, respectively, of unpolished rice grain (Seneweera *et al*. 1996; Seneweera and Conroy 1997). Similar studies with wheat using open-top chambers at 718 μmol mol^{-1} CO_2 found ~25% reductions in grain Fe and Zn concentrations in two cultivars (Manderscheid *et al*. 1995). Decreased Fe and Zn concentrations have been found not only in grain but also in some vegetative tissues of wheat and rice under elevated [CO_2] (Seneweera and

Conroy 1997; Pal *et al*. 2003). Results of these and other studies, many of them enclosed, led to predictions that elevated [CO_2] would lower the elemental composition of major food staples and further exasperate malnutrition disorders such as Fe and Zn deficiency that already affect billions today (Loladze 2002).

Rice FACE experiments have reported widely on the yield and N effects of CO_2-enrichment, as well as the interaction of these two factors, although comparatively little has been published regarding the effects of CO_2 increase on mineral concentrations in rice grain (Pang *et al*. 2006; Ainsworth 2008). In one of the few FACE studies focussed on CO_2-enrichment effects on mineral concentrations of rice, Lieffering *et al*. (2004) examined the nutritional composition of archived temperate rice grain that had been produced under FACE in Japan. Although grain N concentrations were significantly decreased, as expected, they found no changes in the concentrations of other elements and proposed that 'dilution' of nutrient supplies may have occurred in enclosed, potted studies examining the effects of elevated [CO_2]. In their study, they found that the root biomass increase brought about by elevated [CO_2] was more than double that of the shoot biomass increase and therefore elemental uptake may have increased to support the aboveground biomass. FACE studies of wheat, in contrast, have shown that the concentrations of all microelements decrease in wheat grain, with 18.3% decreases reported for Fe and 13–23% decreases reported for Zn (Högy and Fangmeier 2008; Erbs *et al*. 2010). Low N supply, in conjunction with elevated CO_2, appears to increase the magnitude of grain micronutrient decreases that are observed in wheat FACE studies. All of these results, from both enclosed and FACE experiments, indicate that grain mineral concentrations are affected by a multitude of potentially confounding factors in CO_2 enrichment studies, such as fertiliser application and nutrient availability of the soil medium, yet there is an overall trend towards reduced grain concentrations of most macroelements and all microelements under elevated [CO_2] (Ainsworth and McGrath 2010).

An examination of grain P concentrations under elevated [CO_2] warrants special review because of the significant role that P plays in determining the nutritional quality of cereals for humans. Additionally, P is one of a small number of macroelements (calcium and potassium included) that has not presented consistently reduced concentrations under elevated [CO_2]. Nearly all P in cereal grain is present in the outer embryo and aleurone layer in the form of phytic acid (PA), or phytate, a molecule that has traditionally been viewed as an 'antinutrition' factor because of its ability to form stable complexes, through phosphate group binding, with cations such as potassium, magnesium, calcium, Fe and Zn. Although PA provides a rich source of P and minerals to the germinating embryo, the PA-mineral complex is insoluble and largely unavailable for human intestinal absorption (Hurrell 2003). Phosphorus concentrations in plant tissues have not shown a clear trend under elevated [CO_2], however, some rice studies have found increased total P content per grain under elevated [CO_2] (Seneweera *et al*. 1996; Seneweera and Conroy 1997). It is likely that the increased P contents in grain were in the form of PA, due to the fact that Mg concentrations correlated with P concentrations in brown grain, suggestive of PA-Mg complex formation (Seneweera *et al*. 1996). Increased grain P concentrations have also been reported in CO_2-enrichment studies of wheat and barley (Manderscheid *et al*. 1995; Högy and Fangmeier 2008). It is conceivable that increased PA content in rice grain, accompanied by decreased Fe and Zn concentrations, could significantly reduce the bioavailability of these key micronutrient minerals under elevated [CO_2] (Manoj-Kumar 2011). Additionally, increased accumulation of PA in endosperm tissues that lie below the typically PA-rich aleurone layer, as a result of elevated [CO_2], could lower the bioavailability of the small amount of Fe and Zn that is found in polished rice. Detailed analysis of the deposition of PA, as well as Fe and Zn, within cereal grain under elevated [CO_2] is required to shed light on these issues.

The mechanisms behind decreased mineral concentrations in cereal grain are poorly understood but are likely to be caused, at least in part, by dilution from increased biomass production under elevated [CO_2], as described previously for decreased grain N concentrations. Altered translocation of minerals to grain, both temporally and spatially, may also play a significant role and could explain why the concentrations of some macroelements, such as potassium and P, have increased in several CO_2-enrichment studies. Regardless of the biological mechanisms behind micronutrient decreases in grain, it is crucial from a nutritional point of view that any decrease in the already low Fe and Zn concentrations of rice grain under elevated [CO_2] be avoided. The production of biofortified rice with increased Fe and Zn grain concentrations, already an ambitious undertaking today, may very well present even greater challenges in a CO_2-enriched future where, despite higher yields, the uptake of nutritionally important micronutrients from soil and translocation of those micronutrients to grain, is compromised. In the proceeding section I review how stimulation of the metal chelating-capacity of rice significantly increases grain micronutrient concentrations under ambient [CO_2] and discuss the applicability of this approach for improving rice nutritional quality under elevated [CO_2].

Enhancing the chelation-based Fe uptake system of rice – nutritional benefits under ambient [CO_2] and prospects for future climates

Graminaceous plants such rice, wheat and maize employ a chelation-based strategy to absorb Fe from the rhizosphere – an approach commonly referred to as Strategy II Fe uptake (Fig. 1). In rice, this uptake system involves the synthesis of a small molecular weight mugineic acid phytosiderophore called deoxymugineic acid (DMA) that is subsequently secreted from the root, in a diurnal pattern, via the plasma membrane-localised phytosiderophore transporter TOM1 (Nozoye *et al*. 2010). The released DMA phytosiderophore solubilises ferric Fe (Fe^{3+}) in the rhizosphere and the Fe^{3+}-DMA complex is then absorbed into root cells via yellow stripe 1-like (YSL) transporters such as OsYSL15 (Inoue *et al*. 2008). Once inside the rice plant, Fe can remain complexed to DMA or transfer to several additional chelating molecules, such as citrate, depending on pH of the surrounding plant tissue.

Central to the chelation-based Fe uptake strategy of graminaceous plants is the DMA precursor nicotianamine

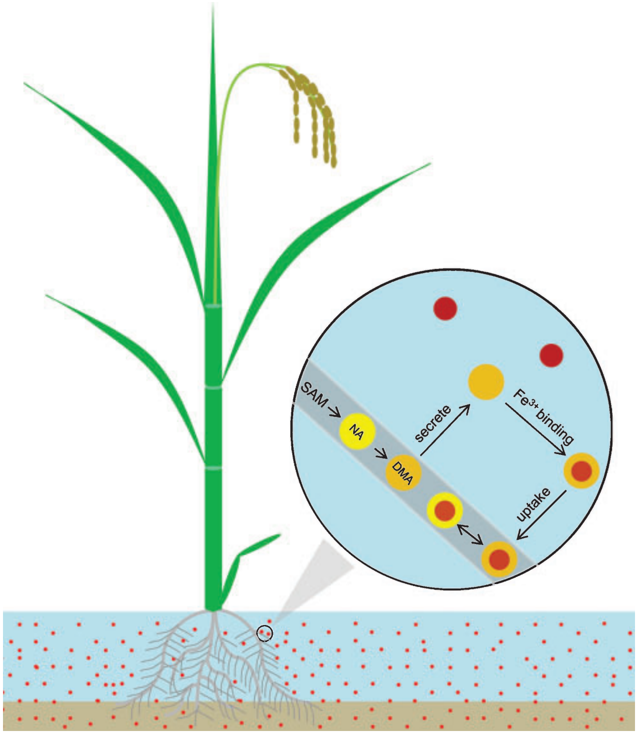

Fig. 1. An overview of the chelation-based strategy II iron uptake system in rice. Iron readily oxidises to ferric form (Fe^{3+}, red circles) under aerobic conditions and is generally insoluble in water and soil at neutral to high pH. Rice and other graminaceous plants take up Fe^{3+} by secreting phytosiderophores from their roots into the local environment (water or soil) and then absorbing the soluble, ferric-phytosiderophore complex into root tissues. As indicated in the magnified view (circled), strategy II uptake begins with trimerisation of S-adenosyl methionine (SAM) to form nicotianamine (NA), a process catalysed by the nicotianamine synthase enzymes. An additional enzyme, nicotianamine aminotransferase, subsequently converts NA to a 3′-keto DMA intermediate that is reduced to form the phytosiderophore, deoxymugineic acid (DMA). DMA is secreted from rice roots, via the plasma membrane-localised transporter TOM1, into the environment where it binds Fe^{3+} to form a soluble Fe^{3+}-DMA complex. The Fe^{3+}-DMA complex is absorbed into root cells via uptake through plasma membrane-localised yellow stripe 1-like (YSL) transporters. Once inside the plant, Fe is likely to remain complexed to DMA or NA in phloem tissues whereas the more acidic pH of xylem tissues favours complexes with citrate.

(NA), a non-protein amino acid that is a strong chelator of transition metals in all higher plants (von Wirén *et al*. 1999). Nicotianamine is formed by trimerisation of three molecules of S-adenosyl methionine in a process catalysed by nicotianamine synthase (NAS) enzymes. The rice genome has three genes encoding NAS enzymes that are all differentially regulated by Fe status of the plant (Inoue *et al*. 2003). NA plays important roles in transporting and remobilising metal cations such Fe, Zn, nickel (Ni), manganese (Mn) and copper (Cu) within plant tissues, including those of Strategy I species that do not release phytosiderophores (Takahashi *et al*. 2003; Cassin *et al.* 2009; Klatte *et al*. 2009). In Strategy II plants that release phytosiderophores, nicotianamine aminotransferase (NAAT) converts NA to a 3′-keto DMA intermediate that is subsequently reduced to form the DMA phytosiderophore.

For more than a decade, numerous biotechnological approaches have been employed to increase the activity of the chelation-based Strategy II uptake system in rice. The driving forces for these studies have been to extend cultivation of rice onto the estimated 30% of arable land containing alkaline soils, where metals such as Fe, Cu and Zn are highly limiting for plant growth, as well as to increase Fe concentrations inside rice grain. The genetic strategies employed to increase activity of the Strategy II system have involved manipulation of NAAT gene activity, to increase DMA production, or NAS gene activity to increase synthesis of both NA and (as a downstream product) DMA (see highlighted circle in Fig. 1). In an early effort to increase DMA production, two barley NAAT genes under the control of their native promoter were introduced into rice (Takahashi *et al*. 2001). The resulting transgenic rice plants secreted more phytosiderophore under Fe deficiency and demonstrated an impressive 4.1-fold increase in grain yield when cultivated on alkaline soil of pH 8.5. Concentrations of Fe within rice vegetative tissues and grain, however, were not reported to have changed in the transgenic plants.

Rice plants engineered to have increased NAS activity display not only enhanced growth on alkaline or calcareous soils, as seen with NAAT overexpression, but in several cases also contain more than 1.5-fold increases in Fe and Zn concentrations of root, shoot and grain tissues that are correlated with NA and DMA concentrations (Suzuki *et al*. 2008*a*; Lee *et al*. 2009, 2011; Masuda *et al*. 2009). The fact that enhanced NAS activity leads to increased concentrations of Fe and Zn is not unexpected considering that both NA and DMA, whose levels are increased by upregulated NAS activity, play important roles in the transport of Fe and Zn through plant tissues (Suzuki *et al*. 2008*b*). Large increases in concentrations of Fe and Zn in polished grain were observed when activity of two of the three endogenous rice NAS genes – *OsNAS2* and *OsNAS3* – were upregulated using CaMV 35S enhancer elements (Lee *et al*. 2009, 2011). These studies found up to 2.6-fold more Fe and 2.7-fold more Zn, in polished grain when the genes were activated, individually, in transgenic rice. Furthermore, seed from both *OsNAS2* and *OsNAS3* activation tagged lines reversed signs of Fe-deficiency when fed to anemic mice, indicating strong potential for NAS overexpression lines to improve iron stores in Fe-deficient humans (Lee *et al*. 2009, 2012).

Johnson *et al*. (2011) recently described constitutive overexpression of all three of the rice NAS genes, individually, using the full length CaMV 35S promoter in cv. Nipponbare rice. Concentrations of NA, Fe and Zn were significantly increased in polished grain of all three populations, relative to wild-type rice, and two of the *OsNAS2* overexpression lines contained Fe concentrations in polished grain that approached or surpassed the 14 $\mu g\, g^{-1}$ Fe biofortification target for rice. The *OsNAS* overexpression lines demonstrate greatly enhanced growth on alkaline soil (Fig. 2), indicating that overexpression of single *OsNAS* genes increases not only the Fe and Zn concentrations of grain but also, and as seen in previous studies, improves the mineral uptake ability of rice under nutrient limiting conditions.

Although there are currently no reports of rice lines with enhanced NAS activity grown under elevated [CO_2], it would be interesting to assess the nutritional composition of such grain

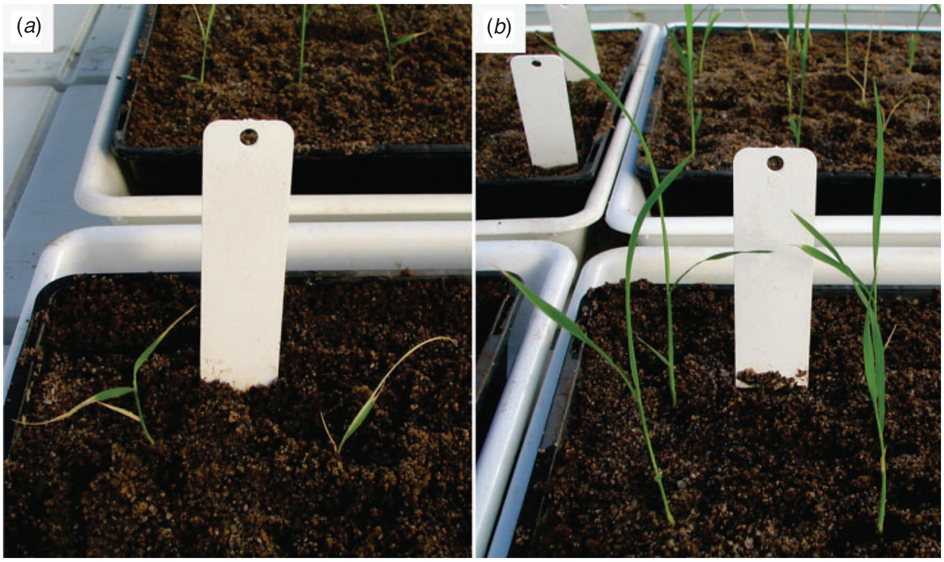

Fig. 2. Constitutive overexpression of *OsNAS* genes leads to enhanced growth of rice seedlings under nutrient-limiting conditions. Ten seedlings of either wild-type cv. Nipponbare rice (*a*) or an *OsNAS3* overexpression line in cv. Nipponbare background (*b*) were germinated on moist filter paper for 1 week before transplantation to alkaline UC Davis soil mix, pH 8.5, to produce conditions where metals such as Fe, Zn, Cu and Mn are highly limiting. After two weeks in alkaline soil, wild-type seedlings failed to establish properly, developed necrotic leaf tips and did not elongate beyond an average height of 6 cm. The *OsNAS3* overexpressing rice seedlings, by contrast, grew vigorously throughout the trial and reached an average height of 10.5 cm after two weeks on the alkaline soil. Both types of seedlings grew vigorously on control UC Davis soil mix at pH 6.0 (data not shown).

produced under CO_2-enriched conditions. The grain of *OsNAS* overexpression lines contains 10–20-fold more NA compared with wild-type rice (Lee *et al*. 2009, 2011; Johnson *et al*. 2011) and it is likely that increased concentrations of NA could reverse the trend towards reduced micronutrient grain concentrations under elevated [CO_2], by allowing more transport of Fe and Zn to the grain or creating a stronger sink for these micronutrients within the grain. Furthermore, the fact that increased NAS activity leads to plants with higher concentrations of Fe and Zn in vegetative tissues, as demonstrated through elemental analyses and enhanced growth on alkaline soil, suggests that greater amounts of these metals would be available for translocation to the grain, a feature that could minimise the 'dilution' of micronutrients that may occur as a result of increased biomass production under elevated [CO_2].

The increased Fe and Zn concentrations brought about by enhanced NAS gene activity appear to have negligible effects on the concentration of phytic acid (PA); a molecule which, as discussed earlier, has high binding affinity for both of these metal cations within rice grain. As seen in Table 1, overexpression of the three *OsNAS* rice genes causes significant increases in the concentrations of NA, Fe and Zn in unpolished rice grain whereas P concentrations (indicative of PA in the grain) remain unchanged. Grain NA concentrations are highly correlated with Fe and Zn concentrations ($r = 0.98$ for Fe and 0.93 for Zn, $P < 0.01$), suggesting that NA-Fe and NA-Zn complexes are present within the grain. Synchrotron X-ray fluorescence spectroscopy has also been used to demonstrate that overexpression of *OsNAS2* leads to significant enrichment of Fe and Zn in regions of the rice endosperm that contain only trace amounts of P (Johnson *et al*. 2011). These results are in agreement with another study showing that *OsNAS3* overexpression does not lead to an increase in the amount of Fe bound to PA (Lee *et al*. 2009) and are important in light of the previously described studies documenting a rise in P content of rice grain under elevated [CO_2]. Any biotechnological approach that is used to increase Fe and Zn concentrations under elevated [CO_2] should ideally leave levels of PA, a factor associated with decreased bioavailability of micronutrients, unchanged. Data from Caco-2 cell studies suggests that NA may actually increase the bioavailability of Fe in rice grain; however, this

Table 1. Concentrations of nicotianamine (NA), iron (Fe), zinc (Zn) and phosphorus (P) in 25 unpolished grain of wild-type cv. Nipponbare rice and three independent transgenic lines overexpressing rice *OsNAS1*, *OsNAS2* or *OsNAS3*, respectively, in cv. Nipponbare background

The results demonstrate that increases in NA concentration of the grain are accompanied by significant increases in Fe and Zn, but not P, concentrations. Nicotianamine concentrations were determined using liquid chromatography-mass spectrometry (LC-MS) and elemental concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES)

Genotype	NA ($\mu g\ g^{-1}$ DW)	Fe ($\mu g\ g^{-1}$ DW)	Zn ($\mu g\ g^{-1}$ DW)	P ($\mu g\ g^{-1}$ DW)
Wild-type	20	23	36	4900
OsNAS1 overexpression	115	40	54	5000
OsNAS2 overexpression	168	48	56	4800
OsNAS3 overexpression	209	53	63	4900

remains to be demonstrated in an animal model (Zheng *et al*. 2010*a*).

Conclusions

To provide food security for the additional 2.3 billion people expected to inhabit the Earth by 2050, most of them in developing countries of Asia, Latin America and Africa, the productivity of rice will need to increase enormously. The 550 µmol mol^{-1} CO_2 concentration predicted for the Earth's atmosphere in 2050 is likely to boost the productivity of rice through increased photosynthetic efficiency, resulting in increased biomass and ~12% gains in grain yield. Water-use efficiency may also be increased, perhaps enabling rice cultivation in more drought-prone regions. However, it will be important to increase not only the quantity of rice grain that is produced, but also its quality. A chronic lack of vitamins, minerals and quality protein in diets, often referred to as 'hidden hunger', has devastating effects on human health and productivity and affects billions of people across the world today. Alarmingly, elevated [CO_2] will almost certainly decrease the protein content of rice by 10–14%. Similarly, the protein quality of rice will likely decrease as elevated [CO_2] is associated with decreases in several essential amino acids such as lysine, methionine and leucine (Wang *et al*. 2011). Also of major concern are the decreases in Fe and Zn concentrations of rice and other cereal grain that have been documented under elevated [CO_2]. Fe deficiency is the most common nutritional deficiency in the world, affecting more than 2 billion people and can overlap with Zn and provitamin A deficiency in rice-consuming countries. A further decrease in the Fe and Zn concentrations of rice grain would have devastating consequences for the billions of people who currently rely on rice as a food staple and the billions more who will do so in the near future.

I have presented evidence that enhanced activity of the chelation-based Strategy II Fe uptake pathway affords several nutritional benefits to rice under ambient [CO_2]. Through upregulation of single NAS genes, Fe and Zn concentrations in vegetative and grain tissues are significantly increased to levels sufficient for biofortification of rice. Additional benefits include greatly increased growth on alkaline soil, which could extend the cultivation of rice to alkaline and calcareous soils, as well as an apparent lack of effect on levels of the antinutrient phytic acid in grain. These results suggest that increased activity of the Strategy II Fe uptake pathway could reverse the worrying trend towards decreased grain Fe and Zn concentrations, and potentially their bioavailability, under elevated [CO_2]. It is worth noting that rice is just one of several agriculturally important species that uses Strategy II uptake to absorb Fe from the environment. Maize, wheat, barley, rye and oats are additional examples of major cereal crops that secrete mugineic acid phytosiderophores into the rhizosphere to solubilise ferric iron. In fact, the first mugineic acid was isolated from barley (Takemoto *et al*. 1978). Since then eight additional phytosiderophore analogues have been isolated from various graminaceous species, all of them sharing the same biosynthetic pathway from S-adenosyl methionine to DMA via nicotianamine as highlighted in Fig. 1 (Ma 2005). Furthermore, the genetics of Strategy II uptake appear highly conserved among cereals. For example, maize, like rice, has three NAS genes that are differentially regulated by iron status of the plant (Mizuno *et al*. 2003). In light of these similarities, it is reasonable to assume that upregulation of Strategy II uptake in these cereal species through, for instance, overexpression of NAS genes, could yield the same increases in grain Fe and Zn concentrations that have been observed in rice.

Although genetic modification has proven extremely effective for increasing the activity of Strategy II Fe uptake, it is possible that our growing knowledge of master regulators of iron deficiency responses in rice, such as the transcription factors IRO2, IRO3 and IDEF1, could eventually facilitate a targeted, conventional breeding approach towards enhancing this uptake pathway (Kobayashi *et al*. 2007; Ogo *et al*. 2007; Zheng *et al*. 2010*b*). Without question, rapidly rising levels of atmospheric CO_2 will present a host of challenges and questions for rice breeders in the near future. Balancing grain yields with grain quality is likely to be of major concern. Increased activity of the Strategy II Fe uptake pathway represents an effective and genetically simple method for increasing rice Fe and Zn concentrations under ambient [CO_2] that may hold significant benefits for rice production in future climates.

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