Enriching rice with Zn and Fe while minimizing Cd risk

Inez H. Slamet-Loedin1*, Sarah E. Johnson-Beebout2, Somayanda Impa2 and Nikolaos Tsakirpaloglou1

1 Plant Breeding, Genetics, and Biotechnology Division, International Rice Research Institute, Manila, Philippines
2 Crop and Environmental Sciences Division, International Rice Research Institute, Manila, Philippines

INTRODUCTION

Iron (Fe) and zinc (Zn) deficiencies affect more than two billion people globally (McLean et al., 2009; Wessells and Brown, 2012). Fe-deficiency anemia can cause impaired cognitive and physical development in children and reduction of daily productivity in adults (Black et al., 2013; Stevens et al., 2013). Recently, low maternal Fe intake has been linked to autism spectrum disorder in their offspring (Schmidt et al., 2014). Adequate Zn nutrition is also important for child growth, immune function, and neurobehavioral development (Wessells and Brown, 2012). Biofortification, defined as increasing the micronutrient content in staple food (Bouis et al., 2011), has the potential to combat Fe and Zn deficiencies, but it is important to ensure low presence of undesirable toxic metals. Because cadmium (Cd) tends to accumulate in kidneys throughout a person’s life, there is concern that regular consumption of rice with even moderate Cd concentration may result in health problems, especially for people who consume rice as a staple food (Meharg et al., 2013). Here we review the genetics and nutrient management approaches to increasing Fe and Zn and minimizing possible Cd contamination.

CONVENTIONAL, MARKER ASSISTED AND TRANSGENIC BREEDING APPROACHES FOR BIOFORTIFICATION TO ENHANCE Fe AND Zn CONCENTRATIONS IN RICE

Nutritional studies suggested that 24–28 mg kg−1 Zn and 13 mg kg−1 Fe concentration in polished grain is essential to reach the 30% of human estimated average requirement (Bouis et al., 2011). Based on this, rice germplasm diversity has been exploited to breed Zn-dense varieties conventionally (Graham et al., 1999). Two Zn-enriched varieties, reaching up to 19 and 24 mg kg−1 Zn in rice grains, have been released by Bangladesh Rice Research Institute (BRRI) in collaboration with the International Rice Research Institute (IRRI) under the HarvestPlus project. Identification of quantitative trait loci (QTLs) for low to moderate Zn enhancement in the existing rice germplasm was reported (Stangoulis et al., 2006; Anuradha et al., 2012; Neelamraju et al., 2012). In addition, genome wide association mapping revealed several loci associated with Zn levels in grains (Norton et al., 2014). However, large effect Zn QTLs (≥30% phenotypic variation) have not been identified yet. Conventional breeding efforts for developing Fe-enriched polished rice have not progressed effectively due to limited variation of Fe concentration in polished rice. Evaluation of more than 20,000 rice accessions from Asia, Latin America, and the Caribbean for Fe and Zn concentration revealed a maximum of only 8 mg kg−1 in polished grains (Gregorio et al., 2000; Graham, 2003; Martinez et al., 2010). Most Fe and Zn are concentrated in the aleurone layers of rice bran. There are between 1 and 5 aleurone layers in different rice accessions (del Rosario et al., 1968); therefore, the high Fe levels in unpolished grains can be due to thickness of the bran layers. Conventional breeding has so far been unsuccessful in the development of Fe-enriched polished rice (Bashir et al., 2013a).

Transgenic approaches to enhance Fe in the starchy endosperm were first explored more than a decade ago (Goto et al., 1999). Since then, researchers have attempted to increase Fe content in rice endosperm by overexpressing genes involved in Fe uptake from the soil and translocation from roots, shoot, flag leaf to grains, and by increasing the efficiency of Fe storage proteins (Table 1; Kobayashi and Nishizawa, 2012; Lee et al., 2012; Bashir et al., 2013a;
Table 1 | Summary of transgenic approaches to improve Iron (Fe)/Zinc (Zn) concentrations in rice grains and to reduce Cadmium (Cd).

| Gene            | Promoter       | Cultivar                  | Growth conditions   | Generation of seeds | Fe concentration (ppm) | Fold increase in Fe | Zn concentration (ppm) | Fold increase in Zn | Effect on Cd concentration in the grains | Reference                   |
|-----------------|----------------|---------------------------|---------------------|---------------------|------------------------|---------------------|------------------------|---------------------|------------------------------------------|-----------------------------|
| **(A) Overexpression approaches** | | | | | | | | | | |
| **(1) Brown seeds** | | | | | | | | | | |
| SoyFeH1         | OsGlubl        | Japonica cv. Kitaake     | Greenhouse          | T1                  | ~38.0                  | 3.0                 | n.a.                   | n.a.                | n.a.                                    | Goto et al. (1999)          |
| SoyFeH1         | OsGlu,OsGtbi   | Japonica cv. Kitaake     | Greenhouse          | T3 to T6            | up to 27.0             | 3.0                 | up to 46.0             | 1.1                 | Similar to WT                           | Qu et al. (2005)            |
| PyFerritin+rgMT | OsGlubi        | Japonica cv. Taipei 309  | Greenhouse          | T1                  | ~22.0                  | 2.0                 | n.a.                   | n.a.                | n.a.                                    | Luca et al. (2002)          |
| TDM1            | CaMV 35S       | Japonica cv. Tsukinohikari | Hydroponic          | T1                  | ~18.0                  | 12                  | ~45.0                  | 1.6                 | n.a.                                    | Nozoye et al. (2011)        |
| SoyFeH1         | ZmUbil         | Indica cv. M12           | Greenhouse          | T2                  | ~18.0                  | No significant increase | n.a.              | n.a.                | n.a.                                    | Drakakaki et al. (2000)     |
| OsIRO2          | CaMV 35S       | Japonica cv. Tsukinohikari | Greenhouse          | T1                  | up to 15.5             | 2.8                 | up to 13.0             | 1.4                 | Similar to WT                           | Lee et al. (2009a)          |
| OsYSL 15        | OsAcinl        | Japonica cv. Dongjin     | Paddy field         | T1                  | ~14.0                  | 1.1                 | ~23.5                  | 1.0                 | Similar to WT                           | Lee and An (2009)          |
| OsIRT1          | ZmUbil         | Japonica cv. Dongjin     | Paddy field         | T3                  | ~12.0                  | 1.1                 | ~22                    | 1.1                 | Similar to WT                           | Lee et al. (2009b)          |
| HvNAS1, HvNAS1+ | Genomic fragments | Japonica cv. Tsukinohikari | Paddy field         | T1                  | up to 73               | 12                  | up to 15.3             | 1.4                 | n.a.                                    | Suzuki et al. (2008)        |
| HvNAS1AT, IDS3  |                | (Calcareaeous soil)      |                     |                     |                        |                     |                        |                     |                                          |                             |
| OsNAS1          | OsGlubl        | Japonica cv. Xiushui 110 | Field               | ??                  | ~5.0                   | 10                  | ~30.0                  | 1.3                 | n.a.                                    | Lee et al. (2009b)          |
| **(2) Milled seeds** | | | | | | | | | | |
| SoyFeH1         | OsGlubl        | Indica cv. IR88144       | Screenhouse         | T2                  | ~37.0                  | 3.7                 | ~55.0                  | 1.4                 | Similar to WT                           | Vasconcelos et al. (2003)   |
| SoyFeH1         | Indica cv. Swama | Basmati rice (Indica cv. | Greenhouse          | BC2F5               | up to 16.0             | 2.5                 | up to 275              | 1.5                 | n.a.                                    | Paul et al. (2014)          |
| OsFe2           | OsGlub1        | Pusa-Sugandh II          | Greenhouse          | T3                  | up to 15.9             | 2.1                 | up to 30.75             | 1.4                 | Similar to WT                           | Paul et al. (2012)          |
| OsNAS1          | OsGlub1        | Japonica cv. Dongjin     | Greenhouse          | T1                  | ~12.0                  | 2.6                 | ~35.0                  | 2.2                 | Similar to WT                           | Lee et al. (2009a)          |
| OsNAS1          |                | (Calcareaeous soil)      |                     |                     |                        |                     |                        |                     |                                          |                             |
| OsNAS1          | Activation tagging | Japonica cv. Dongjin     | Greenhouse          | T1                  | ~10.0                  | 3.0                 | ~42.0                  | 2.7                 | Similar to WT                           | Lee et al. (2011, 2012)     |
| **(3) Polished seeds** | | | | | | | | | | |
| OsNAS1, OsNAS2, | CaMV 35S       | Japonica cv. Nipponbare  | Glasshouse          | T1                  | up to 19.0             | 2.2, 2.4, 2.2        | up to 76.0             | 1.4, 2.2, 1.4         | n.a.                                    | Johnson et al. (2011)       |
| OsNAS3         |                | (Calcareaeous soil)      |                     |                     |                        |                     |                        |                     |                                          |                             |
| SoyFeH1         | Glub1          | Indica cv. BR29          | Greenhouse          | T3                  | up to 9.2              | 2.4                 | n.a.                   | n.a.                | n.a.                                    | Khalekuzzaman et al. (2006) |
| HvNAS1          | CaMV 35S       | Japonica cv. Tsukinohikari | Greenhouse          | T2                  | ~8.5                   | 2.5                 | ~28.0                  | 1.5                 | n.a.                                    | Higuchi et al. (2001), Masuda et al. (2009) |

(Continued)
Table 1 | Continued

| Gene                | Promoter                      | Cultivar                        | Growth conditions | Generation of seeds | Fe concentration (ppm) | Fold increase in Fe | Zn concentration (ppm) | Fold increase in Zn | Effect on Cd concentration in the grains | Reference        |
|---------------------|-------------------------------|---------------------------------|-------------------|---------------------|------------------------|---------------------|------------------------|---------------------|----------------------------------------|------------------|
| SoyFerH1, SoyFerH2, OsFer1C, OsFer2C | CluB1 and GluB4, CluB1 and GluB4, CluB1 and GluB4 | Indica cv. 1R64 | Greenhouse | T4                  | up to 76               | 2.3                  | n.a                    | n.a                  | n.a                                   | Oliva et al. (2014) |
| SoyFerH1, SoyFerH2 | CluB1 and GluB4, CluB1        | Indica cv. IR64                | Greenhouse        | T5                  | up to 5.9              | 18                   | n.a                    | n.a                  | n.a                                   |                  |
| HvNAS1              | OsActinl                      | Japanica cv. Tsukinohikari     | Greenhouse        | T1                  | ~75                    | 3.4                  | ~35.0                  | 2.3                  | n.a                                   | Masuda et al. (2009) |
| OsYSL2              | OsSUT1                        | Japanica cv. Tsukinohikari     | Glasshouse        | T1                  | ~75                    | 4.4                  | n.a                    | n.a                  | n.a                                   | Ishimaru et al. (2010) |
| AtNAS1+, Pdferitin+ | CaMV 35S, Glbl, Glbl          | Japanica cv. Taipei 309        | Hydroponic        | T1                  | ~70                    | 6.3                  | ~33.0                  | 1.6                  | n.a                                   | Wirth et al. (2009) |
| OsYSL2+, SoyFerH2+, HvNAS1 | OsSUT1 and Glbl, GluB1 and Glb, OsAct       | Japanica cv. Tsukinohikari     | Greenhouse        | T2 (and T3)         | up to 70               | 6 (and 4)             | ~20.0                  | 1.6                  | Similar to WT                          | Masuda et al. (2012) |
| SoyFerH2, OsNAS1+, OsYSL2 | OsGluB1 and OsGlb, OsSUT1 and OsGlb | Tropical Japanica cv. Paw and OsAct, OsGlb | Greenhouse | T1 (and T2)       | up to 5.02             | 2 (up to 3.4)         | 34.2 (up to 39.2)       | 1.1 (up to 1.3) | n.a                                    | Aung et al. (2013) |
| SoyFerH2, HvNAS1, HvNAAT, -A, -B and IDS3 genome fragments | OsGluB1, OsGlb, | Japanica cv. Tsukinohikari Greenhouse | T3                  | up to 4.0             | 2.6                  | up to 31               | 1.5                  | n.a                                    | Masuda et al. (2013b) |
| HvNAS1, HvNAS1, TyNAS1+ | Genomic fragments       | Japanica cv. Tsukinohikari     | Paddy field       | T1                  | 1.1, 1.19, 1.49        | 10, 11, 1.4          | 11.3, 11.9, 14.3       | 1.0, 1.1, 1.3 | n.a                                    | Masuda et al. (2008) |
| HvNAS1, IDS3        | Genomic fragments            | Japanica cv. Tsukinohikari     | Paddy field (Andosolsoi) | T1                  | 11.1, 1.19, 1.49       | 10, 11, 1.4          | 11.3, 11.9, 14.3       | 1.0, 1.1, 1.3 | n.a                                    | Masuda et al. (2008) |

(B) Silencing approaches

| Gene                | T-DNA mutant | Cultivar                        | Growth conditions | Generation of seeds | Fe concentration (ppm) | Fold increase in Fe | Zn concentration (ppm) | Fold increase in Zn | Effect on Cd concentration in the grains | Reference        |
|---------------------|--------------|---------------------------------|-------------------|---------------------|------------------------|---------------------|------------------------|---------------------|----------------------------------------|------------------|
| OsVIT               | T-DNA mutant | Japanica cv. Zhonghual 1        | Paddy field       | ~16                 | ~1.4                   | ~31                 | ~1.2                   | ↑                  | Zhang et al. (2012)                    |                  |
| OsVIT2              | T-DNA mutant | Japanica cv. Dongjin            | Paddy field       | ~14                 | ~1.5                   | ~30                 | ~1.3                   | ↑                  | Zhang et al. (2012)                    |                  |
| OsNRAMP5            | RNAi         | Japanica cv. Tsukinohikari     | Glasshouse        | n.a.                | n.a.                   | n.a.                | n.a.                   | ↓                  | Ishimaru et al. (2012)                 |                  |

*Silencing of OsNRAMP5 (Natural Resistance-Associated Macrophage Protein 5) has also been obtained through ion-beam irradiation (Ishikawa et al., 2012). Different approaches have been grouped based the transgenic over expression vs. down regulation (silencing) approaches, and available Fe/Zn data (polished grain or brown rice). The arrows (↑) or (↓) indicate the increase/decrease of Cd concentration in rice grains.*
Masuda et al., 2013a). Among these studies, the concomitant increase in Fe and Zn content in rice grains was obtained by the overexpression or activation of the NAS (nicotianamine synthase) genes, either in solo or in combination with other transporters or Fe storage genes (Table 1). NAS catalyzes the synthesis of the divalent metal chelator nicotianamine acid (NA) from the precursor molecule 2’-deoxymugeneic acid (MA). Constitutive expression of OsNAS2 resulted in increased Fe concentration as high as 19 mg kg\(^{-1}\) and Zn concentration to as high as 76 mg kg\(^{-1}\) within the endosperm of polished rice grains (Johnson et al., 2011). On the other hand, the baseline of O. japonica cv. Nipponbare in this study is 4 mg kg\(^{-1}\) Fe, which is higher than other studies employing japonica accessions (Table 1), possibly due to a favorable micro-environment. Combinations of genes involved in chelating, transporting or storing Fe significantly enhanced Fe concentration in polished grain (Masuda et al., 2012, 2013b; Aung et al., 2013). These studies also demonstrated the stability of the trait over multiple plant generations; nevertheless, reaching the recommended target level still remains a challenge. Furthermore, to accelerate the farmers’ adoption and consumers’ acceptance, Oliva et al. (2014) generated phytoferritin over-expressor events in popular indica variety without selectable marker genes; however, the level of Fe was not sufficient to reach the target.

The average of 2 mg kg\(^{-1}\) Fe in well-polished rice grains is the general baseline in popular varieties (Bouis et al., 2011). However, there was a marked variation in the baseline of Fe concentration between genotypes used in the studies described in Table 1. Such variation could be due to differences in the milling degree of rice grains, the respective genotypes as such, or the growth conditions, and fertilizer applications. In addition, Fe measurement is also highly prone to contamination during seed processing, milling, and analytical process.

Most Fe biofortification studies were conducted under favorable glasshouse conditions, with only limited studies performed under field conditions (Masuda et al., 2008, 2012). In the first study, moderate increases of 1.40-fold for Fe and 1.35-fold for Zn concentrations of transgenic polished rice grains were observed compared to the control (Masuda et al., 2008). In the second study, a significant decrease (up to 50%) was observed in the Fe concentration in polished grains in the subsequent generation of T\(3\) homozygous plants grown under paddy field conditions (4 mg kg\(^{-1}\)) compared to the earlier generation grown under the glasshouse condition (Masuda et al., 2012) that reached up to 7–8 mg kg\(^{-1}\) (six times the concentration of the wild type control).

Among genetic improvement options for increasing rice grain Fe and Zn, we recommend the prioritization of the sink and source strategy (Wirth et al., 2009; Masuda et al., 2013a). However, despite the fast progress, reaching the nutritionist recommended target level of 13 mg kg\(^{-1}\) for Fe under field conditions (Bouis et al., 2011) still remains a challenge (Bashir et al., 2013a). Therefore, to enhance Fe and Zn content in polished rice grains, the expression of most optimum orthologues of chelator(s), transporter genes and iron storage genes still needs to be evaluated. In addition, for product development, data on the trans gene copy number is required.

### GENETICS OF CADMIUM UPTAKE

In general, indica varieties accumulated higher Cd concentrations compared to japonica in Cd-polluted soils or in hydroponic solution with high Cd (Arao and Ishikawa, 2006). The physiological mechanisms for Cd uptake and its translocation to shoots in rice have been associated with several chemically related metal ions (Kim et al., 2002; Arao and Ishikawa, 2006; Uraguchi and Fujisawa, 2012). Absorption of Cd in hydroponically grown Fe-deficient plants was thought to be mediated through the Fe-uptake system, particularly through the OsIRT1 and OsIRT2 genes (Nakanishi et al., 2006). OsNRAMP1 (Natural Resistance-Associated Macrophage Protein 1) is another transporter protein shown to be related to the absorption of Cd in rice roots (Takahashi et al., 2011). Functional analysis of the gene confirmed its expression in roots, whilst the protein was localized in the plasma membrane, indicating its role in Cd absorbance and transport (Takahashi et al., 2011).

Recently, it has been demonstrated that the OsNRAMP5 gene in rice acts as a major transporter of Cd and Mn in the roots (Ishikawa et al., 2012; Sasaki et al., 2012). Expression analysis showed that its presence was restricted to roots, as well as in tissues around the xylem (Ishimaru et al., 2012; Sasaki et al., 2012). In addition, extensive analysis of silencing, insertion knock-out plants, and ion-beam irradiation mutants confirmed the role of OsNRAMP5 in reducing the Cd accumulation both in straw and in grains to negligible levels, even when grown in Cd-contaminated paddy fields (Ishikawa et al., 2012; Ishimaru et al., 2012; Sasaki et al., 2012). Using a different approach, hydroponic and soil culture experiments suggested root-to-shoot Cd translocation via the xylem as the major physiological process for determining grain Cd accumulation in rice (Uraguchi et al., 2009). Analysis of mapping populations for identification of QTLs related to Cd accumulation in rice grains indicated the presence of a genetic locus in chromosome 7 (qGCd7; Ishikawa et al., 2005, 2010). This QTL was shown to be specific to Cd since it was not related to the absorption/translocation of other metal cations or to any agronomic characteristics. Fine mapping of the qGCd7 resulted in the identification of OsHMA3, a gene responsible for limiting the root-to-shoot translocation of Cd by selectively sequestering it within the vacuoles (Ueno et al., 2010; Miyadate et al., 2011). OsHMA2, a close homolog of OsHMA3, has also been shown to be involved in the root-to-shoot translocation of Cd in rice plants, through the xylem network (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012).

Furthermore, Uraguchi et al. (2011) proposed a different route for reducing Cd within the rice grains. The identification of the low-affinity cation transporter (OsLCT1) reduced the Cd accumulation within rice grains by significantly decreasing its phloem-mediated transport. Suppression of OsLCT1 did not have any negative effect on the content of other metal ions in the grains, indicating its specificity for Cd (Uraguchi et al., 2011, 2014). Among genetic strategies for decreasing Cd concentration in rice, we recommend prioritization of strategies reducing the sequestration of Cd in roots, such as down-regulation of OsNRAMP5. This has been achieved recently by RNAi transgenic approach and mutation technologies (Ishikawa et al., 2012; Ishimaru et al., 2012).
HAS CADMIUM BEEN ACCUMULATED IN ENRICHED Fe/Zn RICE?

Conventional breeding lines with enriched grain Zn have not been reported to contain elevated Cd. The fact that Fe/Zn-biofortification by transgenic approaches exploited different transporter genes (Table 1) raises the possibility of Cd accumulation because Zn-associated transporters often co-transport Zn-mimic Cd (Olsen and Palmgren, 2014). The upper limit of Cd set by FAO/WHO in rice grain is 0.4 mg kg⁻¹ (Codex Alimentarius, 2010). The transgenic approaches that tended to simultaneously increase grain Zn as well as Fe were the ones involving the NAS family genes (Table 1). However, assessment of seedlings of OsNAS3 activation tag lines and its wild counterpart in plant growth medium with elevated Cd showed no difference in Cd level amongst different germplasm and tissues (Lee and An, 2009; Lee et al., 2009b, 2011), suggesting the specificity of NA to Zn over Cd (Olsen and Palmgren, 2014). In addition, a 20% reduction in the Cd accumulation was identified in T₃ polished grains compared to the non-transgenic counterparts expressing transporters and phytoferritin genes (Aung et al., 2013). Another transporter protein, OsIRT1, has been suggested to be involved in the Fe and Cd uptake pathway earlier (Nakanishi et al., 2006). However, the translocation of excess Cd from the roots to shoots was minimal. Recent studies in osvt1 and osvt2 T-DNA knock out mutants reported some increase in Cd level in rice grains (Zhang et al., 2012). To date only one report on transgenic biofortified rice shows a slight increase in the Cd levels (Zhang et al., 2012), whilst there have been no reports yet on the grain Cd level on the Zn-enriched conventional breeding lines. In all the reported approaches, the acquired Cd concentrations were significantly lower than the threshold toxic levels for the polished rice grains.

MANAGEMENT AND ENVIRONMENT EFFECTS ON Fe, Zn, and Cd UPTAKE IN RICE

The performance of biofortified genotypes is often restricted due to low available pools of Zn or Fe in soil. Under these conditions, enriching Fe or Zn concentration in grains through either fertilization or water management, called agronomic biofortification, is a short term strategy which would complement the breeding programs. Some of these management and environment effects have also been shown to change Cd uptake patterns.

WATER MANAGEMENT

Irrigation management in rice strongly influences soil redox potential, which affects the availability of Fe, Zn, and Cd. Rice was domesticated under flooded conditions, and it is still grown with continuous soil submergence in many places. However, for a variety of reasons, rice is now produced across the entire range of irrigation management options, including fields which are always aerobic, always anaerobic, and many variations along the aerobic-anaerobic spectrum (Bouman et al., 2007). Because socioeconomic drivers are so important in designing irrigation systems, it seems unlikely that farmers would choose irrigation options solely for the purpose of changing the soil availability of Fe, Zn, or Cd. Therefore, we need to understand the effect that water management has on the benefits and risks of enriching grains with metals, even though the opportunities for managing the risks this way are limited.

As a soil changes from aerobic to anaerobic conditions after flooding, Fe-oxides are dissolved when the Fe³⁺ is reduced to Fe²⁺ (Figure 1), which weakens the oxide stability and increases its water-solubility (Kirk, 2004). This releases much more Fe into the soil solution, so flooded soil nearly always has sufficient Fe for plant uptake, and rice has therefore become somewhat adapted to Fe toxicity. Most rice plants have mechanisms to prevent excessive uptake of Fe. Anti-oxidative mechanisms, including induction of ferritin gene, have been reported as one of the plant mechanisms against excessive plant endogenous Fe²⁺ (Briat et al., 2010). In contrast, in aerobic soils, Fe deficiency can occur (Zuo and Zhang, 2011), while Zn and Cd both tend to be more available in this soil. Both elements are predominantly present in the +2 oxidation state, regardless of soil redox potential, so the effect of flooding is indirect (rather than direct as with Fe). The availability of Zn decreases with flooding due to precipitation (Figure 1) as insoluble zinc sulphide (after sulfate is reduced to sulphide, Bostick et al., 2001) or as insoluble carbonate mixtures (after decomposing organic matter causes an increase in the partial pressure of carbon dioxide in soil solution, Kirk, 2004). Cadmium behaves similarly to Zn (Du Laing et al., 2009). In summary, changing a soil from aerobic to anaerobic conditions by flooding will increase Fe availability and suppress Cd, but will also decrease Zn availability (Figure 1). The possibility of managing irrigation to optimize the plant uptake of Fe, Zn, and Cd simultaneously is negligible.

FERTILIZATION OPTIONS

Most evidence has shown that applying Fe or Zn fertilizers to the soil is ineffective at increasing grain Fe or Zn in rice. Under aerobic water management, the soil-applied Fe (usually in the form of Fe²⁺, either chelated or as a sulfate salt) is rapidly converted to unavailable Fe³⁺, and hence, foliar application is a better option to overcome Fe deficiency and to increase grain Fe and its bioavailability in rice (Wei et al., 2012a). Under anaerobic water management, Fe²⁺ is readily available to rice plants (Figure 1), so no fertilization is needed. Application of Zn at 5–25 kg Zn ha⁻¹ as zinc sulfate incorporated to the soil before flooding or after transplanting is the most common Zn fertilizer recommendation for rice (Dobermann and Fairhurst, 2000). However, soil-applied zinc sulfate has often been unsuccessful in improving grain Zn concentration and yield under flooded paddy due to redox induced fixation of applied Zn (Srivastava et al., 1999; Johnson-Beebout et al., 2009). In rice, positive effects of soil Zn fertilization on grain Zn have been noticed primarily with aerobic water management (Wang et al., 2014). On the other hand, foliar Zn application has been more effective in improving grain Zn concentration in flooded rice compared to soil Zn fertilization (Wissuwa et al., 2008; Wirth et al., 2009). Zn and Fe fertilization strategies and its effects on the uptake and accumulation of Zn, Fe, and Cd in rice are illustrated in Figure 1.

Although foliar application of Fe or Zn is more promising than soil application for enhancing grain Fe or Zn, the efficiency of foliar applied Fe or Zn varies depending on the time of fertilization, source of Zn fertilization and ability of genotypes to remobilize Zn or Fe from source tissues to grain (Karak et al., 2006; Cakmak,
FIGURE 1 | Illustration of water and fertilizer managements and their effects on zinc (Zn), iron (Fe), and cadmium (Cd) uptake and accumulation in rice grain. The arrows (↓) under the compounds indicate precipitation.

2009; Wei et al., 2012b). Late season foliar application of Zn or Fe at flowering or at early grain filling stage is more effective in improving grain Zn or Fe, respectively, than early season application (Phattarakul et al., 2012; Mabesa et al., 2013). Though the levels of Zn and Fe in grains are positively related, fertilization of one element did not affect the grain concentration of the other (Cakmak et al., 2010; Wei et al., 2012a,b). However, foliar fertilization of combined Fe and Zn fertilizers enhanced both grain-Fe and -Zn content without any antagonistic effects (Wei et al., 2012a). Among fertilization strategies for flooded rice, the most likely to succeed is a combined foliar Zn and Fe spray soon after flowering or at early grain filling stage, and it is important to study how to make foliar fertilizers more effective.

Optimized management of N fertilizer could improve grain Fe and Zn, as indicated by a strong correlation of seed Fe and Zn with N in several crop species under sufficient Zn supply (Zhang et al., 2008; Cakmak et al., 2010; Kutman et al., 2010). Better N nutrition promotes protein synthesis, which is a major sink for Fe and Zn, and enhances the expression of Zn and Fe transporter proteins, such as ZIP family transporters (Cakmak et al., 2010). Better N nutrition may also enhance the production of other nitrogenous compounds such as NA and deoxymugineic acid (DMA), and YSL proteins involved in Zn transport within the plant (Haydon and Cobbett, 2007; Curie et al., 2009). Under high N supply, vegetative growth is enhanced and plants remain green for a longer time, resulting in longer grain filling periods, and delayed senescence (Kutman et al., 2010). However, under low Zn conditions, increased biomass production induced by optimal N fertilization can decrease grain Zn concentration due to biological dilution (Zhang et al., 2008; Kutman et al., 2012). In summary, it is always important to optimize N fertilization in rice production, but there is not very much scope for adjusting N management for the purpose of biofortification.

Phosphate fertilizers are major sources of Cd input in agricultural land and in cereal crops (Eriksson, 1990; He and Singh, 1993; Gao et al., 2010). They can contain significant amounts of Cd due to its presence in the rock phosphate used for production (Williams and David, 1973). However, once recognized, these relatively high-Cd phosphate rock sources have been avoided in the production of fertilizer, so there is very little evidence of actual P-fertilizer-related Cd uptake in rice. The effect of Zn fertilization on Cd uptake by plants is highly dependent on the soil Cd and Zn concentrations. Higher biomass accumulation under high NPK fertilization, results in enhanced Cd uptake but may either increase or decrease concentration, depending on the balance of fertilizer effects on crop growth, root distribution, and Cd availability. This could be a useful strategy for phytoremediation but not for cereal production. Increase in Cd uptake under higher rate of fertilization than lower rate of fertilization (Singh, 1990), suggests that efficient management of fertilizers is necessary to keep a control on Cd accumulation in agricultural crops.

IMPROVING IRON AND ZINC NUTRITION, AND MITIGATING CADMIUM TOXICITY RISK THROUGH GENETICS AND MANAGEMENT APPROACHES

Biofortified rice has a potential to reach areas that currently could not be reached by other interventions since rice consumption is
high in affected regions. In flooded rice fields, Cd uptake risk is low (Uraguchi and Fujisawa, 2012), but the trend is for more rice fields to become aerobic due to erratic rain or scarce water resources. Therefore, the risk of Cd accumulation will increase with more aerobic water management, particularly in Cd contaminated areas. To mitigate this, it is essential to develop a low Cd accumulating cultivar by down-regulating the expression of endogenous genes involved in Cd uptake and/or translocation by identifying a genetic marker and subsequently introgressing the trait into the popular varieties through marker assisted breeding. The latter approach has been validated in the field using the dysfunctional OsNRAMP5 mutant (Ishikawa et al., 2012). It significantly decreases root Cd uptake and Cd content in the straw and grain, apparently without decreasing Fe uptake in root, shoot, and straw (Ishimaru et al., 2012; Sasaki et al., 2012). As we continue to identify new pathways to biofortification of rice with Fe and Zn, it is critical to examine the potential for each biofortification mechanism to affect Cd uptake.

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