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Running title: Cadmium transport in cereal crops

Transport of cadmium from soil to grain in cereal crops: A review

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ABSTRACT

Due to rapid urbanization and industrialization, many soils for crop production are contaminated by cadmium (Cd), a highly toxic heavy metal for all organisms. Cereal crops including rice, wheat, maize, and barley are the major dietary source of Cd intake for humans, therefore, reducing Cd transfer from soil to their grains is a very important issue for food safety. During last decade, great progresses have been made in understating molecular mechanism of Cd transport, especially in rice. Inter- and intra-specific variations in Cd accumulation have been observed in cereal crops. Transporters for Cd have been identified in rice and other cereal crops by using genotypic difference in Cd accumulation and mutant approaches. They belong to different transporter family and are involved in uptake, vacuolar sequestration, root-to-shoot translocation, and distribution of Cd. In addition, attempts have been made to reduce Cd accumulation in the grains by manipulating these transporters through overexpression or knockout of the transporter genes and marker-assisted selection breeding based on genotypic difference in Cd accumulation of the grains. In this review paper, we described recent progresses on molecular mechanisms of Cd accumulation in cereal crops. We also compared different molecular strategies for minimizing Cd accumulation in grains.

Key Words: barley, cadmium, genotypic difference, maize, rice, transporter, wheat

INTRODUCTION

Cadmium (Cd) and Cd-bearing minerals have been widely used in modern industries and agriculture (Clemens and Ma, 2016). However, when there are released to the environment, Cd exerts high toxicity to both plants and animals including humans (Zhao F J, Wang P. 2019). High Cd concentration in soil will inhibit plant growth through the strong interaction with sulfhydryl groups required for protein structure and function, interference with the homeostasis of essential elements such as Ca and Zn and so on (Clemens and Ma, 2016). On the other hand, excessive intake of Cd causes kidney failure, osteoporosis and osteomalacia (Bertin and Averbeck, 2006; Nawrot *et al*., 2006). Due to the regulation of the law, nowadays soils heavily contaminated with Cd are decreasing, but soils contaminated with low and moderate level are increasing due to metal mining or smelting activities, application of phosphate fertilizer with Cd impurities, contaminated sewage sludge and

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waste water, sewage effluents, and agricultural run-offs (Williams *et al*., 2009; Meharg *et al*., 2013). For example, in China, it was reported that 7% of farmland is Cd-contaminated according to the last nationwide survey (Wang *et al*., 2019a). Since the level of Cd toxicity between plants and humans are quite different, even Cd level in soil solution is not high enough to inhibit plant growth, its accumulation in edible parts can threaten human health through plant-derived foods (Clemens *et al*., 2013). Therefore, more concern has been paid on the toxicity of Cd to humans rather than Cd phytotoxicity.

Cereals including rice, wheat, maize, and barley are major dietary sources of our Cd intake because over 50% of all calories consumed in the human diet comes from these crops. For example, Cd intake from rice accounts for about 40-50% of total Cd intake for populations consuming rice as the staple food (Shimbo *et al*., 2001; Grant *et al*., 2008). The '*Itai-Itai* disease' caused by weakening of bones and renal tubular dysfunction, which occurred in the mid-1950s and mid-1960s in Japan, is the result of consuming rice grown in Cd-contaminated soils (Horiguchi *et al*., 1994; Kobayashi *et al*., 2009). Therefore, for food safety, the threshold value of the Cd concentration in cereal grains has been set to 0.4 mg kg^{-1} for rice (0.2 mg kg^{-1} in China), 0.2 mg kg^{-1} for wheat, 0.1 mg kg^{-1} for maize and barley by The Codex Alimentarius Commission/World Health Organization with responsibility for the safety of food and human health (Codex Alimentarius Commission of Food and Agriculture Organization, 2006). However, cereals produced on Cd-contaminated soil often exceeds these limits. Therefore, it is very important to restrict the transfer of Cd from soil to cereal grains for human health. During last decade, some progresses have been made in understanding Cd transport mechanisms in plants, especially in rice (Clemens and Ma, 2016; Wang *et al*., 2018; Zhao and Wang, 2019). In this review, we focus on the transport system of Cd in cereal crops and discuss the ways to reduce Cd accumulation in cereal grains.

CD AVAILABILITY IN SOIL

The average content of Cd in soil ranges from 0.07 to 1.1 mg kg^{-1} (Kabata-Pendias and Pendias, 1985). However, it could be higher in some soil contaminated with Cd. Cd concentration in the soil solution lies between 0.2 and 6 g L^{-1} in non-Cd –contaminated soil and could be as high as 400 μg L^{-1} in Cd-contaminated soil (Kabata-Pendias and Gondek, 1978). However, soluble Cd concentration in soil solution rarely exceeds 1 μmol L^{-1} range (Smolders and Mertens, 2013). The chemical forms of Cd in soil are CdS, $CdSO_4$, and Cd complex with organic chelates. These forms change with soil conditions. For example, under flooded condition, Cd is precipitated with HS and forms CdS, which has a low solubility. By contrast, under upland soil, Cd is present in the form of $CdSO_4$, which has a high solubility. The availability of Cd in soil is determined by several factors including soil pH, organic matter, cation exchange capacity, and Eh (Brown *et al*., 2004; Antoniadis *et al*., 2008; Romkens *et al*., 2011; Pietrzykowski *et al*., 2014). Amon them, the important factors are soil pH and Eh; Cd solubility in soil decreases with increasing soil pH (Arao *et al*., 2009; Hu *et al*., 2013; Hu *et al*., 2015). As shown in Fig. 1A, liming was very effective to reduce Cd accumulation in rice grain. The exchangeable Cd content in soil increases with higher Eh values under an oxidative condition. When the Eh increased from 0 to 300 mV, Cd concentration in soil solution increased from 0.2 μg L^{-1} to 0.8 μg L^{-1} (Honma *et al*., 2016). That's why flooding and liming are effective ways to reduce Cd availability in soil, thereby Cd accumulation in grains (Fig. 1A, B).

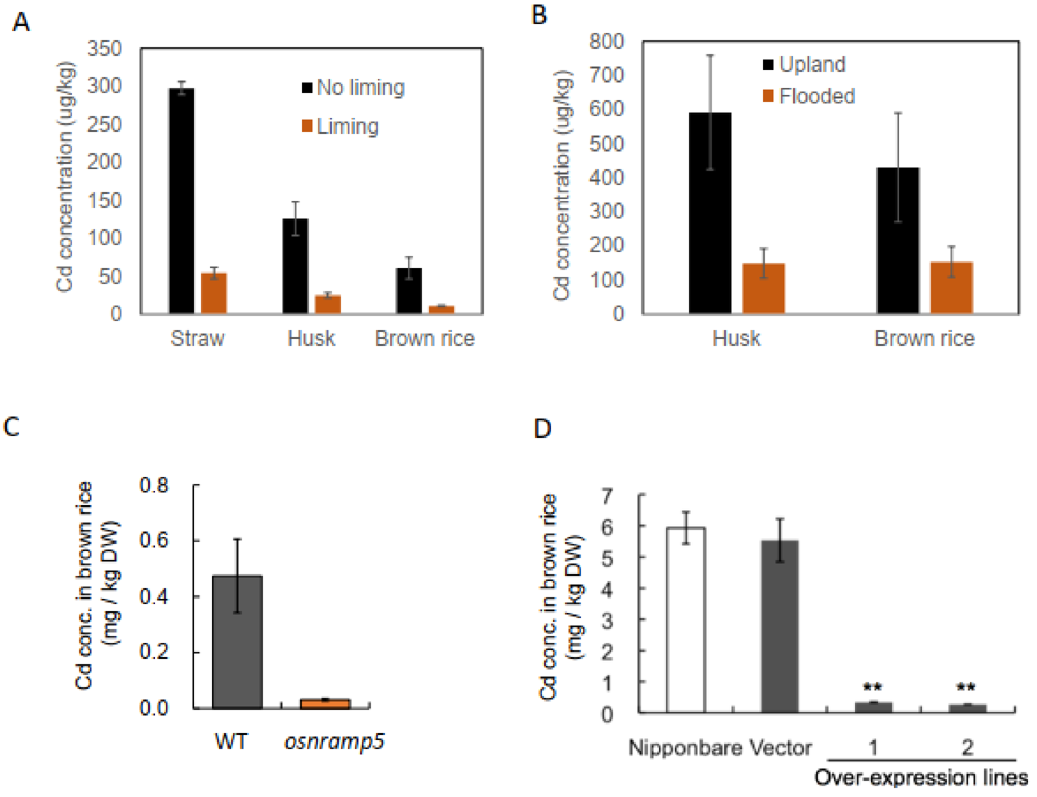

Figure 1 Approaches to migrate Cd accumualtion in rice. A-B, Effect of liming (A) and flooding (B) on Cd accumulation in rice. A, Rice plants were grown in a non Cd-contaminated soil with pH 6.28 (without liming) and 7.24 (with liming) until ripening. B, Rice plants were grown under flooded and upland condition after flowering. Date are means±SD (n=4). Data are based on unpblished data from authors' lab. C-D, Effect of knockout of *OsNramp5* (C) and over-expression of *OsHMA3* (D) on Cd accumualtion in rice grain. C and D are cited from Ueno et al. (2010) and Sasaki et al. (2012) from authors' lab.

INTRA- AND INTER-SPECIFIC VARIATION IN CD ACCUMULATION IN CEREAL CROPS

There is a wide variation in Cd accumulation between different species. In cereal crops, usually rice accumulates more Cd than wheat, barley, and maize. For example, based on a survey in Japan, Cd concentration in polished rice was 2.5-fold higher than that in wheat flour (Shimbo *et al.*, 2001). Similar trend was reported in China, the average of Cd concentrations in rice grains was 3-8 times those in wheat and other cereal grains based on an investigation of tens of thousands of samples collected (Song *et al.*, 2017). These differences in Cd accumulation between different cereal crops have been partially attributed to different expression level, transport activity and tissue-specific localization of transporters for Cd uptake as described in next section.

There is also a great variation in Cd accumulation between different cultivars within a species. For example, a 13-fold difference in shoot Cd concentration was found in 146 accessions from a rice core collection (Ueno *et al.*, 2009). In barley, the grain Cd concentration ranged from 1.6 to 102.9 μg kg^{-1} dry weight when 100 accessions of the barley core collection from different regions were grown under the same field conditions (Wu *et al.*, 2015). The leaf Cd concentration varied from 16.6 to 128.4 mg kg^{-1} in 269 maize accessions grown in nutrient solution containing high Cd (Zhao *et al.*, 2018). Genotypic variation in Cd accumulation of wheat was also reported (for review, see Zaid *et al*., 2018). These genotypic differences have been used for QTL analysis, gene isolation and breeding program as described below.

TRANSPORT SYSTEM OF CD IN CEREAL CROPS

There are several steps for the transfer of Cd from soil to the grains including uptake by the roots, vacuolar sequestration in the roots, translocation from the roots to the shoots, and distribution to the grains (Clemens and Ma, 2016; Wang *et al*., 2018). All these steps require various different transporters.

Transporters for Cd uptake in roots

The first step is the uptake of Cd by the roots from soil solution. OsNramp5 in rice and HvNramp5 in barley are responsible for the transport of Cd from soil solution to the root cells (Sasaki *et al*., 2012; Wu *et al*., 2016). Homologs of OsNramp5 and HvNramp5 are also present in maize (ZmNramp5) and wheat (TaNramp5A and TaNramp5D) (Sui *et al*., 2018). Nramp5 belongs to natural resistance associated macrophage protein transporter family and transports Cd as well as Mn (Sasaki *et al*., 2012). In rice, OsNramp5 is polarly localized at the distal side of both exodermis and endodermis of mature root region (Fig. 2A and C; Sasaki *et al*., 2012), while HvNramp5 is localized in the epidermal cells of root tips without polarity (Fig. 2B; Wu *et al*., 2016). This difference in tissue-localization could be one of reasons for high Cd accumulation in rice. Rice basal roots are characterized by having two Casparian strips: one at the exodermis and one at the endodermis and by formation of aerenchym (Ma *et al*., 2006; Yamaji and Ma, 2007). A recent study showed that both Casparian strips and polar localization of influx and efflux transporters are required for efficient Si uptake in rice (Sakurai *et al*., 2015). By contrast, in barley root tips, Casparian strip is usually weakly developed at the endodermis and there is no formation of aerenchym. Therefore, Cd taken up through HvNramp5 at the epidermal cells is radially transported to the stele symplastically with less efficiency compared with rice uptake system (Fig. 2).

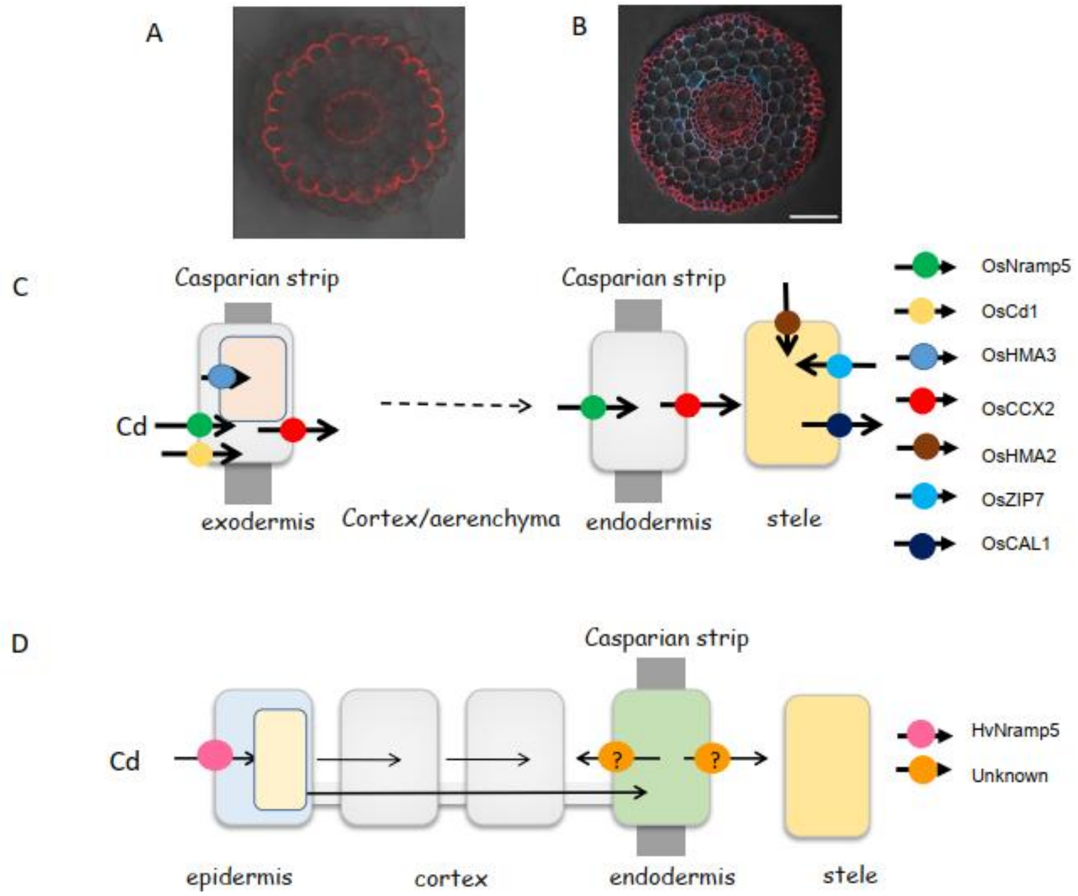

Figure 2 Difference in Cd uptake and tranlocation between rice and barley. A-B, Localization of Nramp5 in the roots of rice (A) and barley (B). C-D, Scheme of uptake and root-to-shoot trasnlocation of Cd in rice (C) and barley (D). Different transporters are shown in different color. For detail, refer to text.

In addition, there are also differences in the expression level and transport activity of *Nramp5* homologs between different cereal crops. The expression level of *OsNramp5* in rice was 4-5 times that of *TaNramp5A* and *TaNramp5D* in wheat or *ZmNramp5* in maize (Sui *et al*., 2018). The expression level of *Nramp5* was 4-7 times higher in rice than in barley (Wu *et al*., 2016). OsNramp5 from rice also showed a greater Cd transport activity than that from wheat or maize Nramp5 in yeast (Sui *et al*., 2018). These differences in expression level and transport activity could be other reasons for higher Cd accumulation in rice.

Other transporters such as OsIRT1 (Iron-regulated transporter1) and OsIRT2, OsNramp1, and OsCd1 have also been implicated in Cd uptake in rice (Nakanishi *et al*., 2006; Lee and An, 2009; Takahashi *et al*., 2011; Yan *et al*., 2019). However, knockout of *OsNramp5* resulted in complete loss of Cd uptake (Sasaki *et al*., 2012), indicating that OsNramp5 is a major transporter for Cd in rice and contribution of other transporters is relatively small.

In rice, after Cd is taken up by Nramp5 to the root cells, Cd must be transported out of the exodermis and endodermis cells toward the xylem vessel for subsequent translocation to the shoots. Therefore, an efflux transporter localized at the exodermis and endodermis must be required. Recently, it was reported that a putative cation/Ca exchanger OsCCX2 functions as an efflux transporter of Cd in rice (Hao *et al*., 2018). It may cooperatively work with OsNramp5 to release Cd from the root cells towards the root xylem vessel although further evidence is required for this role. It is unknown whether similar transporter is required for Cd uptake in other cereal crops.

Transporters for vacuolar sequestration of Cd in roots

After Cd is taken up into the root cells by influx transporter, Nramp5, part of Cd will be sequestered into the vacuoles and the remaining part will be released to the xylem for subsequent translocation to the shoots. In rice roots, the vacuolar sequestration of Cd is mediated by OsHMA3, a member of heavy metal-transporting ATPase family (Fig. 2C; Ueno *et al*., 2010). OsHMA3 was isolated based on a large QTL for Cd accumulation on the short arm of chromosome 7 using a mapping population derived from a cross between an indica variety with higher grain Cd (Anjana Dhan) and the japonica cultivar Nipponbare with lower grain Cd (Ueno *et al*., 2010). OsHMA3 is localized at the tonoplast and shows transport activity for Cd and Zn (Ueno *et al*., 2010; Miyadate *et al*., 2011; Cai *et al*., 2019). In barley, wheat, and maize, there are homologs of OsHMA3 (Wu *et al*. 2015; Zhao *et al*., 2018; Zhou *et al*., 2019), however, their role in Cd accumulation remains to be investigated.

Transporters involved in the root-to-shoot translocation

The root-to-shoot translocation of Cd is mediated by OsHMA2, a homolog of OsHMA3 in rice (Satoh-Nagasawa *et al*., 2012; Takahashi *et al*., 2012; Yamaji *et al*., 2013). Different from OsHMA3, OsHMA2 is localized to the plasma membrane. Furthermore, OsHMA2 was localized at the pericycle of the roots (Fig. 2C). OsHMA2 showed an influx transport activity for both Cd and Zn in yeast (Yamaji *et al*., 2013). In maize, a gene responsible for QTL of leaf Cd accumulation on chromosome 2 may be related to homologs of OsHMA2 and OsHMA3 (Zhao *et al*., 2018), but their roles need to be further investigated. Recently, OsZIP7 (*Oryza sativa* zinc-regulated transporter and iron-regulated transporter-like proteins) and OsCAL1 (*Oryza sativa* cadmium accumulation in leaf 1) were also reported to be implicated in the root-to-shoot translocation (Fig. 2C; Luo *et al*., 2018; Tan *et al*., 2019). OsZIP7 was localized to the plasma membrane and showed transport activity for Cd and Zn (Tan *et al*., 2019). Furthermore, it is localized at the parenchyma cells of the roots. OsCAL1 encodes a defensing-like protein and is expressed in the root exodermis and the xylem parenchyma cells (Luo *et al*., 2018). This protein can bind Cd and may facilitate its efflux to the apoplastic spaces (Fig. 2C).

Transporters for distribution of Cd to the grains

Cd will finally be distributed to the grains at the reproductive growth stage. Recently, node I has been demonstrated to play a crucible role in distribution of mineral elements including Cd in rice (Yamaji *et al*., 2013; Yamaji and Ma, 2014). In rice, several transporters including OsHMA2, OsLCT1 (*Oryza sativa* low-affinity cation transporter1), OsZIP7 and OsCCX2 have been implicated in the distribution of Cd to the grain (Fig. 3; Uraguchi *et al*., 2011; Yamaji *et al*., 2013; Hao *et al*., 2018; Tan *et al*., 2019). In rice node, OsHMA2 is localized at the phloem of enlarged and diffuse vascular bundles (E/DVB) (Yamaji *et al*. 2013). OsHMA2 is involved in reloading Zn and Cd from the intervening parenchyma tissues into the phloem of diffuse vascular bundles (Fig. 3). OsZIP7 seems to play a similar role in the inter-vascular transfer of Zn and Cd in the nodes (Tan *et al*., 2019). On the other hand, OsLCT1 seems to be also involved in the inter-vascular transfer of Cd (Uraguchi *et al*., 2011). OsLCT1 shows an efflux transport activity for Cd, K, Mg, Ca and Mn, but not for Fe, Zn, Cu and Na (Uraguchi *et al*., 2011; 2014). OsLCT1 is expressed around the EVBs and DVBs in the node I at the reproductive stage. However, in contrast to *OsHMA2* expression, which is highly expressed in the nodes throughout the reproductive growth, expression of *OsLCT1* in the nodes was only observed at the ripening stage (Uraguchi *et al*., 2011). Higher expression of *OsLCT1* was observed in the leaf blade rather than the nodes at each reproductive growth period (Uraguchi *et al*., 2011). These differences indicate that the Cd translocation pathway mediated by OsLCT1 is different from that of OsHMA2. OsLCT1 might be involved in efflux of Cd from the phloem within the nodes. On the other hand, OsCCX2 is also highly expressed in the xylem region of the enlarged vascular bundles in the nodes. It may function in releasing Cd from these cells (Hao *et al*., 2018), although further functional characterization is required. Unfortunately, no similar transporters involved in the distribution of Cd have not been identified in other cereal crops.

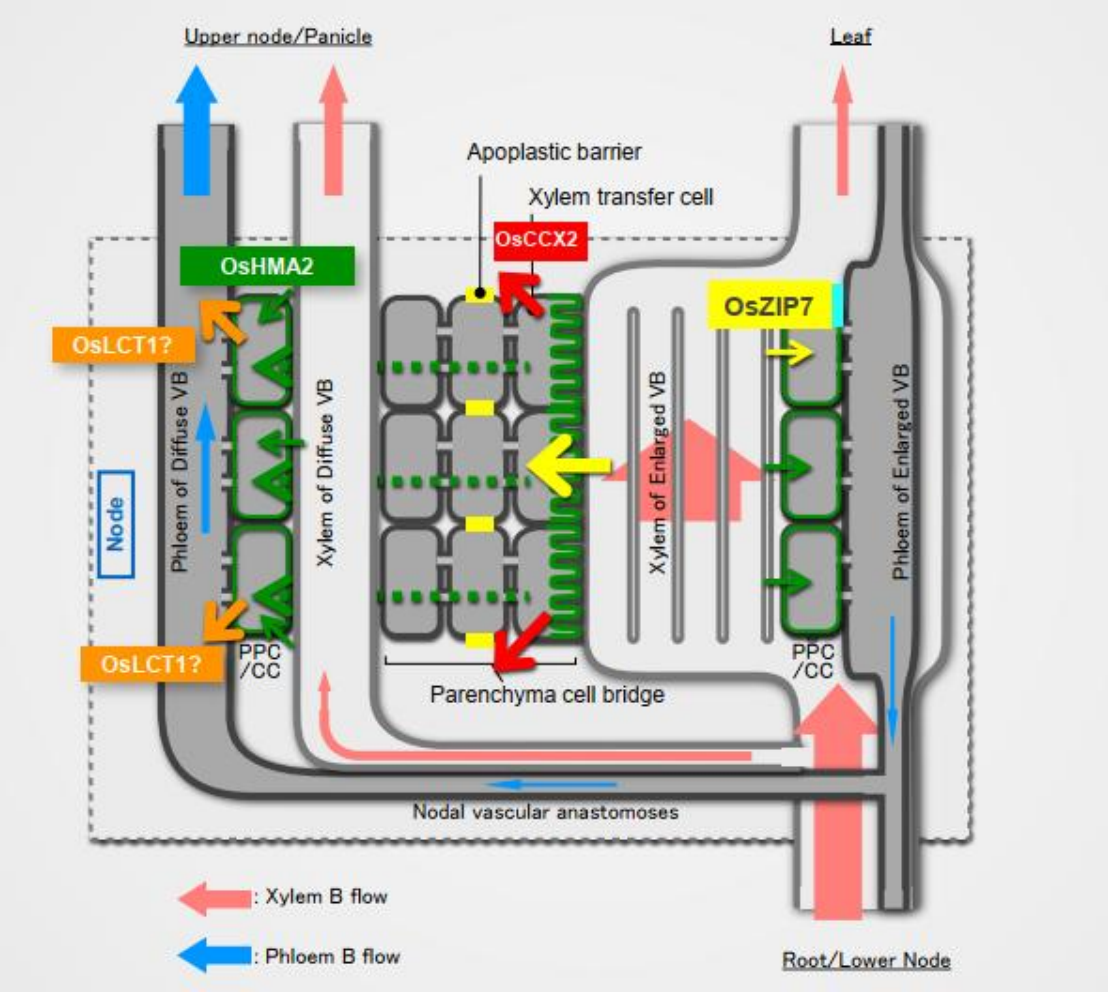

Figure 3 Transporters involved in Cd distribution in rice node. Different transporters are shown in different color. For detail, refer to text.

TRANSGENIC APPROACHES TO REDUCE CD ACCUMULATION IN CEREAL GRAINS

To reduce the transfer of Cd from soil to the cereal grain, several approaches have been tested including soil dressing, application of amendments such as biochar, liming, water management, and phytoremediation (Murakami *et al*., 2007; Arao *et al*., 2009; Ibaraki *et al*., 2009; Yu *et al*., 2014; Chen *et al*., 2018; Liu *et al*., 2018). Both liming and application of biochar decrease Cd availability in soil through increasing soil pH, thereby decreasing Cd uptake and accumulation in rice grain (Fig. 1A; Cui *et al*., 2011; Suksabye *et al*., 2016; Chen *et al*., 2018; Zhang *et al*., 2019). Flooding during grain filling stage effectively decreases Cd accumulation in rice grain (Fig. 1B; Arao *et al*., 2009). This effect is also attributed to decreased Cd availability in soil due to formation of CdS as described above. On the other hand, using rice cultivars with high Cd accumulation has been reported to be effective to clean up soil contaminated with Cd at low to moderate levels (Murakami *et al*., 2009). Cultivation with a high Cd-accumulating indica variety, Choukokoku for two years, resulted in 38% reduction of total soil Cd content (Murakami *et al*., 2009). However, these approaches are not cost-effective and time-consuming. As a sustainable approach for remediation of Cd-contaminated soil, transgenic attempts have been made to breed crop cultivars with low Cd accumulation, especially in rice.

Since OsNramp5 is a major transporter for Cd uptake (Sasaki *et al*., 2012), knockout of this gene has found to be very effective in reducing Cd accumulation in rice grain in several studies (Sasaki *et al*., 2012; Ishikawa *et al*., 2012; Tang *et al*., 2017; Liu *et al*., 2019; Yang *et al*., 2019). For example, knockout of *OsNram5* in rice resulted in a remarkable decrease of Cd accumulation in the brown rice (Fig. 1C; Sasaki *et al*., 2012). A rice mutant with loss of function of *OsNramp5* generated by ion-beam irradiation showed nearly undetectable Cd in the grains when grown in Cd-contaminated paddy field (Ishikawa *et al*., 2012). Knockout of *OsNramp5* by CRISPR/Cas9 also resulted in 90% reduction in grain Cd in hybrid rice (Tang *et al*., 2017). However, contradictory results on the rice yield have been reported. In several studies, knockout of *OsNramp5* resulted in significant reduction in the growth and yield (Sasaki *et al*., 2012; Liu *et al*., 2019; Yang *et al*., 2019). This is because OsNramp5 is also a transporter for Mn uptake (Sasaki *et al*., 2012). Knockout of *OsNramp5* also resulted in decreased Mn uptake, causing growth inhibition and grain yield loss due to Mn-deficiency. By contrast, two studies reported that knockout of *OsNramp5* did not show yield penalty (Ishikawa *et al*., 2012; Tang *et al*., 2017). The exact reason for this inconsistency is unknown, but one possibility could be different mutation point in the OsNramp5 protein and different growth conditions. Similarly, knockdown of Hv*Nramp5* also significantly reduced Cd accumulation in barley (Wu *et al*., 2016). However, in wheat and maize, the effect of *Nramp5* knockout on Cd accumulation has not been examined. Besides, knockout of *OsCd1* also decreased Cd uptake and accumulation in rice (Yan *et al*., 2019). However, the rice yield was also negatively influenced because OsCd1 is also involved in Mn uptake (Yan *et al*., 2019).

By contrast, it was consistently reported that over-expression of *OsHMA3* in rice resulted in significant reduction of Cd accumulation in the grains without yield penalty. The expression of native *OsHMA3* is quite low in rice roots (Ueno *et al*., 2010), therefore over-expression of functional *OsHMA3* increases vacuolar sequestration of Cd in the root cells, resulting in decreased root-to-shoot translocation of Cd (Ueno *et al*., 2010; Sasaki *et al*. 2014). The over-expression lines of *OsHMA3* only contained 0.3 mg kg^{-1} Cd in the brown rice in contrast to 6 mg kg^{-1} Cd in the wild-type rice when grown in a Cd-contaminated soil (Fig. 1D; Ueno *et al*., 2010). Furthermore, the concentration of Zn and Fe was not affected in the over-expression lines. Overexpression of *OsHMA3* in an *Indica* rice cultivar decreased 94 to 98% Cd in brown rice compared to the wild type without loss of grain yield (Lu *et al*., 2019). A recent study showed that OsHMA3 is also responsible for vacuolar sequestration of Zn in root cells (Sasaki *et al*., 2014; Cai *et al*., 2019). However, it seems that Zn homeostasis in the shoots is maintained by up-regulating five genes related with Zn transport (Sasaki *et al*., 2014). In contrast to rice, little is known about the effect of *HMA3* over-expression on Cd accumulation in other cereal crops although *HvHMA3* has been implicated in Cd accumulation in barley grains by genome-wide association mapping (Wu *et al*., 2015).

In terms of root-to-shoot step, knockout of *OsHMA2* also resulted in significant decrease in Cd accumulation in rice grain (Yamaji *et al*., 2013). However, the knockout lines showed decreased rice yield because OsHMA2 is also required for Zn transport (Yamaji *et al*., 2013). Since *OsHMA2* is also expressed in the node in addition to the roots and showed high expression, expression of *OsHMA3* under the control of *OsHMA2* resulted in effective and selective reduction of Cd accumulation in the rice grain through increased vacuolar sequestration of Cd not only in the roots, but also in the node before loading to the grain (Shao *et al*., 2018). Knockout of *OsCAL1* decreased root-to-shoot translocation, but did not affect Cd accumulation in the grain (Luo *et al*., 2018).

Manipulation of transporters involved in Cd distribution in the nodes has also succeeded to reduce Cd in rice grain. For example, knockout of *OsCCX2* decreased Cd concentration in rice grain by about half (Hao *et al*., 2018). Knockdown of *OsLCT1* also resulted in decreased Cd accumulation in the rice grain (Uraguchi *et al*., 2011).

FUTURE PROSPECTIVE

During last decade, great progresses have been made in understanding of molecular mechanism of Cd accumulation in rice. Several transporters involved in the uptake, vacuolar sequestration, translocation and distribution of Cd have been identified in rice as described above. However, little is known on the molecular mechanisms of Cd accumulation in barley, maize, and wheat. Furthermore, even for rice, we are far from fully understanding of the molecular mechanism underlying the Cd accumulation. Many transporters are still missing and remain to be identified in future by using different approaches. For example, Cd efflux transporters in the root exodermis and endodermis cells are not functionally characterized, and transporters involved in xylem unloading and phloem loading of Cd are also unknown.

Since Cd is a non-essential element for plant growth, it seems that there is no transporter specific for Cd. Transport of Cd in plants is mediated by transporters for essential metals as described above. For example, Cd uptake is mediated by OsNramp5, a Mn transporter (Sasaki *et al*., 2012), while OsHMA2 and OsHMA3 are transporters for Zn (Ueno *et al*., 2010; Yamaji *et al*., 2013; Cai *et al*., 2019). Therefore, knockout of these genes except for *OsHMA3* resulted in growth inhibition due to deficiency of these essential metals. This means that these genes cannot be used for breeding rice cultivars with low Cd accumulation. Although over-expression of *OsHMA3* effectively and selectively reduces Cd accumulation in the rice grain (Ueno *et al*., 2010; Shao *et al*., 2018), there is still public acceptance problem on transgenic plants. Therefore, screening of the new alleles with different selectivity for Cd and essential metals may provide useful materials for breeding low-Cd cultivars in future.

There are great genotypic variations in Cd accumulation in rice, barley, wheat, and maize (Ueno *et al*., 2009; Wu *et al*., 2015; Zaid *et al*., 2018; Zhao *et al*., 2018). Several QTL for Cd accumulation have been detected (Ueno *et al*., 2009; Zaid *et al*. 2018; Zhao *et al*., 2018; Wang *et al*., 2019b). Identification and pyramiding of these QTL genes into elite cultivars will contribute to breed safe cereal crops with low Cd accumulation through marker assisted selection in future.

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