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Note

Isolation and characterization of induced mutants in the gene associated with seed cadmium accumulation in soybean

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Food contamination by cadmium (Cd) is a serious threat to human health. Thus, it is imperative to prevent Cd accumulation in staple crops like soybean. The development of low Cd accumulating cultivars is an effective solution. To this end, it is essential to identify the gene(s) controlling seed Cd accumulation. Although *Glyma.09G055600* (*GmHMA3*) seems to be associated with Cd accumulation in soybean, it has not been established if it is responsible for seed Cd accumulation. In the present study, the effect of *GmHMA3* on seed Cd accumulation in soybean was validated using three independent *GmHMA3* mutants isolated from an ethyl methanesulfonate-induced soybean mutant library. Each of mutant had an amino acid substitution in GmHMA3 and segregating progenies were developed by crossing the original cultivar with each of the three mutants. The relationship between these three mutations and seed Cd accumulation was investigated. While two of them significantly increased seed Cd accumulation corresponding to previous reports of a natural missense mutation in *GmHMA3*, the other slightly decreased seed Cd accumulation. Overall, these results indicate that *GmHMA3* is responsible for seed Cd accumulation in soybean.

Key Words: soybean, cadmium (Cd), *GmHMA3*, mutant, amino acid substitution.

Introduction

Cadmium (Cd) is a heavy metal highly toxic to many organisms including humans. Excess Cd intake can damage kidneys, lungs, and bone (Godt *et al.* 2006). In Japan, the itai-itai disease is recognized as a chronic toxicity caused by excess Cd intake from polluted water and food crops (Kobayashi *et al.* 2009). Soybean [*Glycine max* (L.) Merr.] is a major crop used in oil production and as livestock feed worldwide. In Japan and other East Asian countries, it is processed into traditional foods like tofu, miso, and natto. These foods are growing in popularity in other countries. The Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), which are responsible for food safety and human health, proposed an upper limit of 0.2 mg kg^{-1} Cd in soybean seeds in 2001. Therefore, it is imperative to develop and apply techniques preventing the concentration of Cd in soybean. One effective approach is to produce low Cd accumulating cultivars.

In two independent studies, major quantitative trait loci (QTLs) associated with seed Cd accumulation were identified on chromosome 9 using recombinant inbred lines (RILs) derived from a cross between soybean cultivars with contrasting seed Cd accumulating phenotypes (Benitez *et al.* 2010, Jegadeesan *et al.* 2010). Jegadeesan *et al.* (2010) identified a QTL, *Cda1*, using RILs derived from a cross between 'AC Hime' and 'Westag-97'; in a subsequent study, Wang *et al.* (2012) suggested that *Glyma.09G055600* was associated with *Cda1*. The other QTL, *cd1*, which was identified using a different RIL population (Benitez *et al.* 2010), was also associated with *Glyma.09G055600* (Benitez *et al.* 2012). Both Benitez *et al.* (2012) and Wang *et al.* (2012) suggested that a single-base substitution resulting in a missense mutation in the ninth exon of *Glyma.09G055600* distinguished high Cd accumulating cultivars from low Cd accumulating cultivars. The cultivar 'Harosoy' (high Cd

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accumulating) showed approximately four times more seed Cd concentration than the cultivar 'Fukuyutaka' (low Cd accumulating) based on a four-experiment average (Benitez *et al.* 2010). A single-base substitution between 'Fukuyutaka' and 'Harosoy' resulted in an amino acid substitution from glycine (Gly) to glutamic acid (Glu) immediately downstream of the ATP-binding domain (GDGxNDx motif) of Glyma.09G055600 (Benitez *et al.* 2012). *Glyma.09G055600* was later designated as *GmHMA3* because of the homology to *AtHMA3* (Heavy Metal Associated) of *Arabidopsis thaliana* and *OsHMA3* of rice (*Oryza sativa* L.) (Morel *et al.* 2009, Ueno *et al.* 2010, Wang *et al.* 2012). Hence, the latter name is used in the present study. Using transgenic soybean plants overexpressing *GmHMA3*, it was found that this gene prevented Cd translocation from roots to stems (Wang *et al.* 2018). However, the authors assessed Cd concentration in young seedlings only, and they indicated that overexpression of wild type *GmHMA3* increased root Cd concentration and decreased stem Cd concentration but did not affect leaf Cd concentration (Wang *et al.* 2018). Although the studies mentioned above suggest that *GmHMA3* is associated with the control of Cd accumulation in soybean, it has not yet been determined whether *GmHMA3* is in fact the causal gene for seed Cd accumulation. Because the seeds are the edible portions of soybean plants, determining which gene is responsible for Cd accumulation in seeds is indispensable for breeding low seed Cd accumulating soybean cultivars.

Induced mutant libraries are powerful tools for the development of novel alleles, and they have been used in genetic and functional analyses of soybean (Anai 2012, Cooper *et al.* 2008, Tsuda *et al.* 2015). Thus, we used an induced mutant library to screen *GmHMA3* mutants. The purpose of screening *GmHMA3* mutants was to evaluate the effects of this gene on seed Cd accumulation.

Materials and Methods

Plant materials

An ethyl methanesulfonate (EMS)-induced soybean mutant library of 'Fukuyutaka' was used in screening. It was constructed at Saga University (Saga, Japan) and consisted of seeds and DNA preparations from approximately 3,900 lines (Anai 2012). This mutant library was used because 'Fukuyutaka' was reported as a low seed Cd accumulating cultivar harboring wild type *GmHMA3* (Benitez *et al.* 2012).

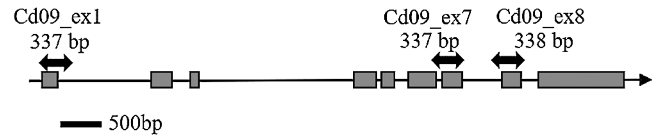

Fig. 1. Schematic structure of the *GmHMA3* regions amplified for mutant screening. Boxes, lines, and bold bidirectional arrows denote exons, introns, and amplified regions for mutant screening, respectively.

Screening GmHMA3 mutants from the mutant library

High resolution melting (HRM) analysis was performed to screen mutants according to a previously described method with a slight modification (Tsuda *et al.* 2015). The initial screening was performed on DNA pools from approximately 3,900 mutant lines in two 384-well plates using multiplex HRM analysis. Three *GmHMA3* target regions (**Fig. 1**) were screened simultaneously. This technique was applied in high-throughput target mutant screening. The primer sequences used in the multiplex HRM analysis are listed in **Table 1**. After a mutation was detected in a DNA pool containing five or six mixed DNA samples of mutant lines, each sample was screened separately by a simplex HRM analysis with three replications. The mutation-bearing DNA amplicons detected by the simplex HRM analysis and those of the original cultivar 'Fukuyutaka' were sequenced at a DNA sequencing service (Eurofins Genomics, Tokyo, Japan) to determine the mutated sequences. All nine exons of *GmHMA3* in 'Fukuyutaka' and *GmHMA3* mutants were also sequenced to confirm there was no mutation in *GmHMA3* other than that detected by HRM analysis (**Supplemental Table 1**). The DNA sequences of 'Fukuyutaka' and *GmHMA3* mutants were compared using the sequence alignment editor, Bio-Edit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Mutants selected in the preceding screening method were analyzed using eight simple sequence repeat (SSR) markers, namely Sat_087, Sat_301,

Table 1. Primers used for screening mutants and genotyping segregating progenies

	Primer name	Direction	Sequence	Restriction enzyme	Targeted mutant line
Screening	Cd09_ex1	Forward	TTCCTTTTGAAACGCATAGTG		
		Reverse	GAGTAACGCATCCGATAAATCC		
	Cd09_ex7	Forward	TGTGACAGATTTTTCTGTTTCTG		
		Reverse	AGAAAGTCTACGAAGGATGCAG		
	Cd09_ex8	Forward	AAGTACATTGTCCTCTTTGGGTAAC		
		Reverse	ATCATTTTGTCATAAGATTGTTGG		
Genotyping	Cd09_ex1	Forward	TTCCTTTTGAAACGCATAGTG	*Mbo* II	M1
	(CAPS marker)	Reverse	GAGTAACGCATCCGATAAATCC		
	Cd_dCAPS_ex1	Forward	TGAAGCCTCTTCGTGGAGTCGA	*Mbo* II	M3
	(dCAPS marker)	Reverse	GAGTAACGCATCCGATAAATCC		
	Cd09_ex1	Forward	TTCCTTTTGAAACGCATAGTG	*AlwN* I	M5
	(CAPS marker)	Reverse	GAGTAACGCATCCGATAAATCC		

Satt540, Sat_337, Sat_153, Satt631, BARCSOYSSR_11_0207, and BARCSOYSSR_20_1017 (Soybase; https://www.soybase.org/), and compared with 'Fukuyutaka' to eliminate outcrossing lines.

Development of materials to assess GmHMA3 mutation

A high mutation density was induced in the library used in the present study by twice exposure to the chemical mutagen EMS (Anai 2012). Therefore, seed Cd accumulation levels could not be compared between the original cultivar 'Fukuyutaka' and the *GmHMA3* mutants to assess mutant effects. Instead, the three selected *GmHMA3* mutants were backcrossed to 'Fukuyutaka' to reduce the effects of unrelated mutations. Segregating F_2 and F_3 progenies were used to determine the effects of *GmHMA3* mutations on seed Cd concentrations.

Field and greenhouse trial designs

Segregating F_2 progenies of the three selected *GmHMA3* mutants were cultivated in the field at Tsukuba [Institute of Crop Science, NARO (NICS), 36°00'N, 140°02'E] in 2014. For the field experiment, approximately 40 F_2 seeds were randomly selected from each of the three segregating progenies and sown on July 16, 2014. Seeds were sown in 70 cm wide rows at 13 cm intervals. All F_2 plants were harvested individually. N, P_2O_5, and K_2O basal fertilizers were applied at 30, 100, and 100 kg ha^{-1}, respectively. At the same condition, three plants of the original cultivar 'Fukuyutaka' were cultivated with two replications and harvested in bulk. To assess mutant effects on plant growth, the main stem lengths of F_2 progeny plants were measured at maturity.

Segregating F_2 and F_3 progenies of the three selected *GmHMA3* mutants were also cultivated in the greenhouse at Daisen [Tohoku Agricultural Research Center, NARO (TARC), 39°32'N, 140°22'E] in 2014 and 2015, respectively. For the greenhouse experiments, F_2 and F_3 seeds were sown in pots (1/2000a) on August 1, 2014 and on July 30, 2015, respectively. Fertilizers N, P_2O_5, and K_2O were applied at 0.6, 2, and 2 g pot^{-1}, respectively. One mutant type plant, one wild type plant, and one 'Fukuyutaka' plant were grown in one pot. Five and six replications were assessed in 2014 and 2015, respectively. Each plant was harvested individually. The pot soil Cd concentrations in the greenhouse experiment were approximately 1.15 and 1.05 mg kg^{-1} in 2014 and 2015, respectively.

Genotyping of GmHMA3

Cleaved amplified polymorphic sequence (CAPS) and derived cleaved amplified polymorphic sequence (dCAPS) markers were developed to detect single-base substitutions distinguishing 'Fukuyutaka' and the selected *GmHMA3* mutants. Primers and restriction enzymes used for each mutant line are listed in **Table 1** (the names of mutant lines were explained in Results). These markers were used to genotype seeds obtained from each F_2 plant grown in the NICS field experiment. Polymerase chain reaction (PCR) products were amplified using each primer pair and Go Taq Master Mix (Promega, Madison, WI, USA). An initial 2 min denaturation at 92°C was followed by 34 cycles of 1 min denaturation at 92°C, 30 s annealing at 58°C, and 1 min extension at 68°C, and a final 5 min extension at 72°C. The amplicons were digested with the appropriate restriction enzymes (**Table 1**) and the restriction fragments were separated on a 7.5% polyacrylamide gel.

Before sowing seeds for the TARC greenhouse experiment, their genotypes at the *GmHMA3* locus were determined by DNA sequencing. The PCR products were amplified by Cd09_ex1 under the conditions described above for the CAPS and dCAPS analyses. Amplicons were purified with DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA, USA) and sequenced by a DNA sequencing service (Eurofins Genomics, Tokyo, Japan).

Measurement of seed and soil Cd concentrations

Seed Cd concentrations (mg kg^{-1} dry weight) were determined by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7700x, Agilent Technologies, Tokyo, Japan) according to a previously described method with a slight modification (Takagi *et al.* 2015). A sample of 10 seeds from each plant was dried at 105°C for 20 to 24 h. The dried seeds were then ground into fine powder with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), and 20 mg ground samples were transferred into 15 mL metal-free centrifuge tubes (INA·OPTIKA, Osaka, Japan) and digested in 0.3 mL concentrated nitric acid at 95°C for 2 h. The volumes of the digests were then brought up to 10 mL with ultrapure water. A *t*-test was performed for each experiment to compare Cd concentrations between seeds of mutant *GmHMA3* individuals and seeds of wild type individuals.

The soils used in the greenhouse experiments at TARC in 2014 and 2015 were sampled before sowing. Their Cd concentrations were determined by an analytical service (Akita Bunseki Kagaku Center Ltd., Akita, Japan). Ten grams of air-dried soil samples were digested in 50 mL 0.1 M hydrochloric acid under continuous mixing at 30°C for 1 h. The digests were paper-filtered and Cd concentrations were determined by atomic absorption spectrophotometer.

Results

Efficient soybean mutant screening by multiplex HRM analysis

The high-throughput target mutant screening performed by multiplex HRM analysis used three sets of primers designed to amplify the first, seventh, and eighth exons of *GmHMA3* (total amplifying length: 1,012 bp) using a reference genome database, Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) (**Fig. 1**). These primer pairs were named Cd09_ex1, Cd09_ex7, and Cd09_ex8, respectively (**Table 1**). The first and seventh exons were selected because the amino acid sequence translated from them contained two P_{1B}-ATPase specific conserved regions, a putative

metal-binding domain in the *N*-terminal region, and a histidine-proline amino acid sequence (HP motif) (**Fig. 2**). Although the eighth exon did not contain P_{1B}-ATPase specific conserved regions, the unique melting point of Cd09_ex8 showed well combination with those of Cd09_ex1 and Cd09_ex7 for multiplex HRM analysis. Thus, Cd09_ex8 was used to screen a longer region of *GmHMA3*. All the PCR products were dissociated according to their unique melting points. Multiplex HRM analysis selected 11 candidate DNA pools from the two 384-well plates, and simplex HRM analysis revealed that 10 of them contained *GmHMA3* mutations. Therefore, multiplex HRM analysis can screen target mutants with low error rate and high degree of efficiency.

Isolation of the GmHMA3 mutants

Sequence analysis confirmed the presence of single-base substitutions in the 10 *GmHMA3* mutants selected by HRM analysis. These mutants were named M1 to M10 according to the position of the mutation in the coding sequence (CDS). Mutant data are summarized in **Table 2**. Nearly all

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First exon                  Metal binding domain
Fukuyutaka  1  MVENIKRSSFEVLGMCCATEEALVERILKPLRGVKDVSVIVPTRTVTVVHDVLLISESQI  60
M1          1  MVENIKRSSFEVLGMCCATKEALVERILKPLRGVKDVSVIVPTRTVTVVHDVLLISESQI  60
M3          1  MVENIKRSSFEVLGMCCATEEALVERILKPLRGVNDVSVIVPTRTVTVVHDVLLISESQI  60
M5          1  MVENIKRSSFEVLGMCCATEEALVERILKPLRGVKDVSVIVPTRTITVVHDVLLISESQI  60
Harosoy     1  MVENIKRSSFEVLGMCCATEEALVERILKPLRGVKDVSVIVPTRTVTVVHDVLLISESQI  60

Seventh exon          HP motif
Fukuyutaka  421  VSSVESKSSHPMAAALVEYGMLNSVKPIPENVENFQNFPGEGVYGIINGKDIYIGNRRIGARAGSER  487
M1          421  VSSVESKSSHPMAAALVEYGMLNSVKPIPENVENFQNFPGEGVYGIINGKDIYIGNRRIGARAGSER  487
M3          421  VSSVESKSSHPMAAALVEYGMLNSVKPIPENVENFQNFPGEGVYGIINGKDIYIGNRRIGARAGSER  487
M5          421  VSSVESKSSHPMAAALVEYGMLNSVKPIPENVENFQNFPGEGVYGIINGKDIYIGNRRIGARAGSER  487
Harosoy     421  VSSVESKSSHPMAAALVEYGMLNSVKPIPENVENFQNFPGEGVYGIINGKDIYIGNRRIGARAGSER  487

Eighth exon
Fukuyutaka  488  VDCRTQCQSPEISTPNQCCGPTLVGVFRLADTCRSGALEAIEELKLLGVRSVMLTGDSSQAAMYAQSQ  555
M1          488  VDCRTQCQSPEISTPNQCCGPTLVGVFRLADTCRSGALEAIEELKLLGVRSVMLTGDSSQAAMYAQSQ  555
M3          488  VDCRTQCQSPEISTPNQCCGPTLVGVFRLADTCRSGALEAIEELKLLGVRSVMLTGDSSQAAMYAQSQ  555
M5          488  VDCRTQCQSPEISTPNQCCGPTLVGVFRLADTCRSGALEAIEELKLLGVRSVMLTGDSSQAAMYAQSQ  555
Harosoy     488  VDCRTQCQSPEISTPNQCCGPTLVGVFRLADTCRSGALEAIEELKLLGVRSVMLTGDSSQAAMYAQSQ  555

Ninth exon                                    GDGxNDx motif
Fukuyutaka  556  LNHALDIVHAELLPAEKAVIIENFKKDGLIAMIGDGMNDAPALATADIGISMGISGSALANETGNAIL~ SLSQPEIVIE  885
M1          556  LNHALDIVHAELLPAEKAVIIENFKKDGLIAMIGDGMNDAPALATADIGISMGISGSALANETGNAIL~ SLSQPEIVIE  885
M3          556  LNHALDIVHAELLPAEKAVIIENFKKDGLIAMIGDGMNDAPALATADIGISMGISGSALANETGNAIL~ SLSQPEIVIE  885
M5          556  LNHALDIVHAELLPAEKAVIIENFKKDGLIAMIGDGMNDAPALATADIGISMGISGSALANETGNAIL~ SLSQPEIVIE  885
Harosoy     556  LNHALDIVHAELLPAEKAVIIENFKKDGLIAMIGDGMNDAPALATADIGISMEISGSALANETGNAIL~ SLSQPEIVIE  885
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Fig. 2. Amino acid alignments in the first, seventh, eighth, and ninth exons of GmHMA3 in 'Fukuyutaka', M1, M3, M5, and 'Harosoy'. Metal-binding domain, HP motif, and GDGxNDx motif are indicated in gray. Mutation points of M1, M3, M5, and 'Harosoy' in relation to 'Fukuyutaka' are indicated in boxes. Amino acid alignment of 'Harosoy' was obtained from Benitez *et al.* (2012).

Table 2. *GmHMA3* mutants selected from the EMS-induced mutant library of 'Fukuyutaka'

Screening primers	Mutant line	mode of mutation	base change	Position in CDS (bp)[b]	Amino acid substitution[c]	Seed availability for assesment of mutant effects
Cd09_ex1 (337 bp)[a]	M1	hetero	G>A	58	Glu 20 Lys	Yes
	M2	hetero	G>A	64	Ala 22 Thr	No
	M3	homo	A>C A>T	105 114	Lys 35 Asn Syn	Yes
	M4	homo	A>C A>T	105 114	Lys 35 Asn Syn	No
	M5	homo	G>A	136	Val 46 Ile	Yes
Cd09_ex7 (337 bp)[a]	M6	homo	G>A	1297	Ala 433 Thr	No
	M7	hetero	G>A	1433	Arg 478 Lys	No
Cd09_ex8 (338 bp)[a]	M8	hetero	G>A	1570	Ala 524 Thr	No
	M9	homo	G>A	1615	Val 539 Ile	No
	M10	homo	G>A	1615	Val 539 Ile	No
	Harosoy[d]		G>A	1823	Gly608Glu	

[a] Amplicon size excluding primer sequences.
[b] Positions in the coding sequence (CDS) were counted from *GmHMA3* start codon (ATG) cited in Phytozome.
[c] Syn: synonymous site at which a base substitution does not cause an amino acid substitution.
[d] Information of 'Harosoy' was obtained from Benitez *et al.* (2012).

single-base substitutions in the mutants were G to A; the exceptions were M3 and M4 (A to C and A to T) (**Table 2**). The base-change trends observed here corroborated those of previous studies concerning mutant libraries developed by EMS (Anai 2012, Tsuda *et al.* 2015). The single-base changes and mutant positions screened in the present study were the same between M3 and M4 and between M9 and M10 (**Table 2**). Because the mutant library used here was developed by repeated EMS treatments (Anai 2012), redundant mutants derived from the same events would be present in the library. Lines M3 and M4 had two single-base substitutions (A to C and A to T) in *GmHMA3*. The A to C mutation resulted in an amino acid substitution (Lys35Asn) but the A to T mutation was synonymous (**Table 2**). Overall, the eight independent *GmHMA3* mutants in the genetic background of 'Fukuyutaka' were detected, and all of them had missense mutation, and there was no nonsense mutation. Four mutant lines, M2, M7, M8, and M9, were not available for further analysis because of low seed quantities. Lines M1, M3, M5, and M6 were analyzed using the eight SSR markers to exclude outcrossing lines. This analysis eliminated M6, because of its possibility of outcrossing (data not shown). Therefore, the remaining three mutant lines, M1, M3, and M5, were used in subsequent experiments. No additional mutations were detected in the exons of the three *GmHMA3* mutants (data not shown).

Characterization of the GmHMA3 mutants

Lines M1, M3, and M5 were backcrossed to the original cultivar 'Fukuyutaka' to reduce the effects of unrelated mutations and to develop segregating progenies at the *GmHMA3* locus. The segregation ratios at the *GmHMA3* locus of the F_2 plants derived from the three cross combinations fitted well to the ratio mutant:heterozygous:wild type = 1:2:1 ($P > 0.05$) (**Table 3**).

Table 3. The segregation of *GmHMA3* mutant, heterozygous, and wild genotypes in F_2 plants cultivated at NICS derived from crosses between 'Fukuyutaka' and one of the three mutant lines, M1, M3, and M5

Cross combination	Number of plants			Expected ratios for 1:2:1			*P* value[a]
	mutant	hetero	wild	mutant	hetero	wild	
Fukuyutaka/M1	8	16	17	10.3	20.5	10.3	0.052
Fukuyutaka/M3	10	21	8	9.8	19.5	9.8	0.804
Fukuyutaka/M5	7	25	11	10.8	21.5	10.8	0.390

[a] χ^2 goodness-of-fit test was conducted to validate the segregating ratios.

The growth at maturity of F_2 plants derived from the three cross combinations between 'Fukuyutaka' and M1, M3, and M5 did not obviously differ from that of 'Fukuyutaka' at the NICS field experiment in 2014 (data not shown). Furthermore, the main stem lengths of mutant *GmHMA3* individuals did not significantly differ from those of wild type individuals derived from the three cross combinations (**Table 4**).

In the progeny test for seed Cd concentration, seeds of mutant *GmHMA3* individuals had 1.6- to 2.7-fold higher Cd concentrations than those of wild type individuals ($P < 0.001$ or $P < 0.01$ for each experiment) derived from the cross between 'Fukuyutaka' and M1 (**Table 4**). Seeds of mutant *GmHMA3* individuals also showed 17 to 22% higher Cd levels than those of wild type individuals ($P < 0.05$ or $P < 0.1$ for each experiment) derived from the cross between 'Fukuyutaka' and M5 (**Table 4**). In contrast, seeds of mutant *GmHMA3* individuals showed 12 to 19% lower Cd

Table 4. Seed Cd concentrations in *GmHMA3* segregating progenies derived from crosses between 'Fukuyutaka' and one of the three mutant lines, M1, M3, and M5

Cross combination	Genotype	NICS in 2014: Number of individuals assessed	NICS in 2014: Seed Cd concentration (mg/kg)[a]	NICS in 2014: Main stem length (cm)[b]	TARC in 2014: Number of individuals assessed	TARC in 2014: Seed Cd concentration (mg/kg)	TARC in 2015: Number of individuals assessed	TARC in 2015: Seed Cd concentration (mg/kg)
Fukuyutaka/M1	Mutant type	8	0.24 ± 0.03	69.1 ± 7.0	5	0.92 ± 0.03	6	1.57 ± 0.29
	Wild type	17	0.09 ± 0.01	70.6 ± 5.5	5	0.49 ± 0.06	6	0.99 ± 0.15
	Ratio (mutant type/wild type)		2.71	0.98		1.87		1.58
	Difference[c]		***	ns		***		**
Fukuyutaka/M3	Mutant type	10	0.10 ± 0.02	69.0 ± 8.7	5	0.59 ± 0.19	6	0.75 ± 0.08
	Wild type	8	0.11 ± 0.02	71.0 ± 6.3	5	0.73 ± 0.18	6	0.88 ± 0.17
	Ratio (mutant type/wild type)		0.88	0.97		0.81		0.85
	Difference		ns	ns		*		ns
Fukuyutaka/M5	Mutant type	7	0.12 ± 0.02	67.7 ± 6.3	5	0.89 ± 0.30	6	0.91 ± 0.07
	Wild type	11	0.10 ± 0.01	63.7 ± 9.6	5	0.73 ± 0.19	6	0.77 ± 0.13
	Ratio (mutant type/wild type)		1.20	1.06		1.22		1.17
	Difference		*	ns		+		*
	Fukuyutaka	2	0.08 ± 0.01	67.0 ± 1.4	15	0.70 ± 0.21	18	0.78 ± 0.09
Soil Cd concentration			No data			1.15 mg/kg		1.05 mg/kg

[a] Seed Cd concentrations were showed as average ± standard deviation in each plot.
[b] Main stem lengths were showed as average ± standard deviation in each plot.
[c] The *t*-test was conducted to compare seed Cd concentrations in mutant and wild type seeds. +, *, **, and *** indicate significant differences were detected by *t*-test at the 10, 5, 1, and 0.1% levels, respectively, and ns indicates significant differences was not detected.

levels than those of wild type individuals derived from the cross between ‘Fukuyutaka’ and M3 (**Table 4**). Nevertheless, significant difference was detected for one experiment at TARC in 2014 ($P < 0.05$). These results indicated that mutations at the *GmHMA3* locus significantly affect soybean seed Cd concentration.

Discussion

Plant mutant libraries play important roles in reverse genetic approaches towards target gene analysis. Therefore, various mutant libraries have been constructed and applied to soybean (Anai 2012, Dierking and Bilyeu 2009, Liu *et al.* 2012, Tsuda *et al.* 2015, Watanabe *et al.* 2011). In the present study, the ‘Fukuyutaka’ mutant library developed by Anai (2012) enabled us to determine if *GmHMA3* is responsible for seed Cd accumulation in soybean. The *GmHMA3* mutant screening by multiplex HRM analysis identified eight independent mutants, and the effects of mutations in M1, M3, and M5 on soybean seed Cd accumulation were assessed.

Compared to wild type siblings, mutations in M1 and M5 significantly increased seed Cd concentration. Based on its homology to AtHMA3 and OsHMA3, GmHMA3 is thought to have a P_{1B}-ATPase function (Benitez *et al.* 2012, Morel *et al.* 2009, Ueno *et al.* 2010, Wang *et al.* 2012). In *Arabidopsis thaliana*, AtHMA3 was found to play an important role in the detoxification of heavy metals such as Cd, zinc, cobalt, and lead by sequestering these metal ions in root cell vacuoles (Morel *et al.* 2009). In rice, functional OsHMA3 limited Cd translocation from roots to shoots by selectively sequestrating Cd into root vacuoles (Ueno *et al.* 2010). In soybean, it was indicated that wild type GmHMA3 transported Cd into the root endoplasmic reticulum, thereby limiting Cd translocation from roots to stems (Wang *et al.* 2018). Based on these previous results, it was hypothesized that functional HMA3 limits Cd translocation from roots by sequestering it into root tissues, and that the *GmHMA3* mutations in M1 and M5 might have resulted in the deterioration of function of Cd transport into root endoplasmic reticulum, promoting Cd translocation from roots to stems, and increasing Cd accumulation in seeds. However, additional studies are necessary for understanding the details of the mechanism of Cd translocation and accumulation in seeds. Interestingly, M1 in which glutamic acid (Glu) mutated to lysine (Lys) at the putative metal-binding domain in the *N*-terminal region of P_{1B}-ATPase (**Fig. 2**) (Williams and Mills 2005), showed a higher relative increase in seed Cd concentration than M5. Seeds of mutant individuals showed approximately 1.6- to 2.7-fold higher Cd concentrations than those of wild type individuals derived from M1. Benitez *et al.* (2012) reported that RILs with high seed Cd accumulating genotypes at the *GmHMA3* locus had approximately two times higher seed Cd concentrations than RILs with low seed Cd accumulating genotypes. They also discussed that the differences in seed Cd concentration between low and high seed Cd accumulating RILs were due to a single-base substitution in the ninth exon of *GmHMA3*, which resulted in an amino acid substitution from glycine (Gly) to glutamic acid (Glu) immediately downstream of the ATP-binding domain (GDGxNDx motif) (**Fig. 2**) (Benitez *et al.* 2012). Therefore, the putative metal-binding domain of GmHMA3 might be necessary for the prevention of Cd accumulation in soybean seeds, as the ATP-binding domain.

The single-base substitution observed in M1 changed glutamic acid (Glu) to lysine (Lys) at amino acid number 20 of GmHMA3, and these amino acids present different characteristics on electrically-charged side chains. The single-base substitution between the low Cd cultivar ‘Fukuyutaka’ and the high Cd cultivar ‘Harosoy’ changed glycine (Gly) to glutamic acid (Glu) at amino acid number 608 of GmHMA3 (Benitez *et al.* 2012), and the characteristics of these amino acids are also different. On the other hand, the single-base substitution observed in M5 changed valine (Val) to isoleucine (Ile) at amino acid number 46 of GmHMA3, and the characteristics of these amino acids are similar. Therefore, the characteristics of the amino acid mutated in M1 might also be important for preventing Cd accumulation in soybean seeds, as the mutation region.

The mutation of M3 was observed near those of M1 and M5 in the first exon of *GmHMA3* and consisted of changing lysine (Lys) to asparagine (Asn) at amino acid number 35 of GmHMA3. Although these amino acids present different characteristics, the effect of M3 on seed Cd accumulation was the smallest among the three mutants. And then, the mutation in M3 slightly decreased seed Cd accumulation. This mutant effect was opposite to those of M1 and M5. Different mutant effects were reported in *OsNRAMP5* associated with Cd accumulation in rice. The RNAi-induced silencing of *OsNRAMP5* in rice promoted Cd translocation to shoots (Ishimaru *et al.* 2012). However, three *OsNRAMP5* mutants found by Ishikawa *et al.* (2012) accumulated little Cd in their shoots and grains. These contradictory results indicated that bidirectional functional changes can occur in heavy metal transporters. Both OsNRAMP5 and OsHMA3 transport heavy metals; *OsNRAMP5* participates in Cd uptake by roots whereas *OsHMA3* is involved in Cd translocation from roots to shoots (Ishikawa *et al.* 2012, Ueno *et al.* 2010). Therefore, certain *GmHMA3* mutations might decrease seed Cd accumulation in soybean. To confirm whether the mutation in M3 really decreases seed Cd accumulation, additional studies are necessary because the mutant effect was not statistically significant in the present study.

In conclusion, we demonstrated that *GmHMA3* is responsible for seed Cd accumulation in soybean. Information on seed Cd accumulation is essential as the seed is the edible portion of soybean plants thereby affecting human health. Our results also showed that *GmHMA3* mutants detected in the present study didn’t affect plant growth. This conclusion enables soybean breeders to accurately develop low seed Cd accumulating cultivars using the allelic information of the *GmHMA3* locus.

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