Chapter 4

Genetic Control of Cadmium Concentration in Soybean Seeds

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Additional information is available at the end of the chapter

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Abstract

Cadmium (Cd) is a chemical element present in the soil. At high concentrations Cd can cause physiological and morphological damages to plants and it is highly toxic to human beings. Minimizing the intake of Cd and other heavy metals from food consumption is an important health issue. Efforts have been made to identify genetic elements that are involved in Cd detoxification in plants. Heavy metal transporter 3 (HMA3) plays a role in sequestration of Cd into vacuoles in soybean (*Glycine max*). Inheritance studies revealed that low Cd accumulation in soybean seed is controlled by a major gene (*Cda*1) with the allele for low accumulation being dominant. Major QTL for seed Cd accumulation, *Cda*1 and *cd*1, have been identified independently for low Cd accumulation and both mapped to the same location as on LG-K (Chromosome 9) with simple sequence repeat (SSR) markers. A single nucleotide substitution causing a loss of function of the ATPase was found. The SSR markers linked to the *Cda*1 and *Cd*1gene(s)/or QTLs and the SNP marker in the P1B-ATPase metal ion transporter gene in soybean can be utilized in marker assisted selection (MAS) for developing food grade soybean varieties.

Keywords: cadmium, soybean, SSR markers, QTLs, marker-assisted selection

1. Introduction

Cadmium (Cd) is a highly toxic element for human beings because of its extremely long biological half-life. Vast areas of agricultural soils are contaminated with Cd through the use of super phosphate fertilizers, sewage sludge, and inputs from the mining and smelting

industries [1]. Cd^{2+} is readily taken up by roots and can be translocated into aerial organs, where it affects photosynthesis and consequently root and shoot growth. At high concentrations, Cd can cause severe physiological and morphological damages to plants, such as stunted root and shoot growth [2–4], chlorosis, decreased reproducibility [5], and reduced water and nutrient uptake [6]. Cd stress can affect enzyme activities [3, 7], alter membrane permeability [6], and disrupt cell transport processes [8]. Cd stress can also disturb cellular redox control [9], damage the light-harvesting complex II [10] and photosystems I and II [11], and decrease carbon assimilation and chlorophyll content [12]. Soybean has long been a staple food for Asians, especially as soymilk, tofu, and oil [13]. Many soybean cultivars can accumulate high Cd concentration in seed when grown on Cd-polluted soil [2, 14].

Cd can accumulate in the human body over time from ingestion of food containing Cd, leading to a risk of chronic toxicity with excessive intake. In humans, it can damage kidneys, causing a loss of calcium and associated osteoporosis [15]. It is desirable to limit the concentration of Cd in crops used for human consumption to reduce potential health risks. Due to growing concern about safety of foods and human health, Codex Alimentarius Commission of Food and Agriculture Organization/World Health Organization (FAO/WHO) has proposed an upper limit of 0.2 mg kg^{-1} for Cd concentration in soybean grain [16]. The results of a large-scale survey of domestic agricultural products revealed that the Cd concentration of 16.7% of soybean seeds exceeded the international allowable limit of 0.2 mg kg^{-1} proposed by the Codex Committee until 2001, which is much higher than that of other upland crops [17].

Considering the health issues due to the intake of Cd and other heavy metals through food grains, cultivars with reduced uptake of these metals are needed for human health. Breeding cultivar with reduced Cd is an attractive method for changing the element profile of crops as the benefit will persist in the seed that can reduce the requirement for other management practices [18]. The amount of Cd that enters the human diet from a crop depends on the amount of Cd accumulated in the portion of the plant that is edible rather than solely on total plant uptake. Both accumulation and distribution of Cd in the plant differ depending on the species, the cultivar, and the growing conditions. In general, the distribution of Cd within the plant is influenced by transport from roots to the shoots via the xylem, transfer from the xylem to the phloem, and transport through the phloem from sources to sinks and other environmental factors [19].

2. Genetic variation for Cd uptake

Natural variation occurs in the uptake and distribution of essential and nonessential trace elements among crop species and among cultivars within species. Plant breeding can be an important tool to either increase the concentration of desirable trace elements or reduce that of potentially harmful trace elements such as Cd. Since the Cd trait is highly heritable, incorporation of the genes influencing low Cd accumulation can help to reduce the average grain Cd to levels below the recommended international limit. The allele for low Cd concentration can be incorporated into other cultivars through breeding program without affecting

other agronomic traits [20]. Cd uptake depends both on the Cd concentration in the soil and on the characteristics of the specific cultivars. Accumulation of large amounts of Cd in the root may limit the accumulation of Cd in edible aboveground portions of the plant. It was reported that Cd concentration in soybean seeds was reduced when high accumulating soybean lines (rootstock) were grafted with low accumulating lines. This indicated that the Cd accumulation in the seed was reduced by high accumulation in the root and was controlled by the rootstock cultivar [21]. Differences in seed Cd concentration among different varieties may be in part related to differences in the abilities of plants to control movement of Cd from the xylem into the phloem, and via the phloem to the soybean seeds [2, 22, 23]. There was also considerable genetic variation observed among soybean cultivars [2, 23–26], with low Cd cultivars appearing to retain more Cd in the root and translocate less to the seed than high Cd cultivars [22].

In field-grown soybean, a wide range of Cd concentrations varying from 0.08 to 1.1 mg kg^{-1} in seed have been reported depending on growing environment and soybean genotype [2, 27–29]. Low soil pH, vicinity to mining sites or sludge applications, has contributed to an increased Cd level in soybean seed [28–30]. In most studies, soybean Cd levels were considerably higher in roots, stems, leaves, or pods than in seeds. Moreover, a high soil Cd concentration is also toxic to soybean reducing plant growth and photosynthesis apart from other effects [31]. Due to genetic differences in soybean cultivar for seed Cd accumulation, a three- to sixfold Cd concentration increase was observed between lowest and highest accumulating genotypes. It was reported that the variation in the Cd accumulation level between genotypes was due to differences in both uptake and Cd retention of the roots [2]. Cadmium concentration in roots showed far higher than that in shoots of soybean genotypes. The root morphological traits such as the total root length (RL), root surface area (SA), and root volume (RV) were closely related to Cd tolerance at young seedlings under Cd treatments [26].

Genotypic differences in Cd uptake and distribution were observed in soybeans cultivated in pot and under low Cd concentrations in the field [2]. Cultivars with low Cd uptake accumulated much higher Cd in their roots than those of the cultivar with high Cd uptake [32]. Decreasing soil Cd concentration reduced Cd concentration in soybean seeds [33]. Interaction of Cd and nitrogen resulted in decreased Cd uptake by soybean seedling roots cultivated at a high nitrogen nutrition level [34]. Cd adversely affected soybean growth, nodulation, and N_2 fixation as a function of time and increase in Cd concentration [35]. The risk of toxicity from Cd in food is influenced not only by Cd concentration but also by concentrations of other trace elements such as Zn and iron (Fe) [36]. Breeding programs are underway to increase the concentration of essential trace elements to enhance the nutritional value of staple crops. Breeding programs to increase concentrations of essential trace elements would have the combined benefit of enhancing the nutritional value of staple crops while reducing the bioavailability of Cd, particularly if low Cd was included as a selection criterion [20].

Growth stage or the age of the plants and the time of exposure to the heavy metal also affect Cd absorption and distribution between different cultivars and between plant parts. The soybean cultivar "Doko" showed an increase in Cd concentration in the roots from the VC (cotyledon stage) to V2 (second node) stage while the cultivar "Bossier" showed the opposite trend in roots. The Cd content of both cultivars (cvs) in stems, however, did not change much

from VC to V2. The highest Cd concentration in roots, stems, and leaves was found approximately at the 8th, 10th, and 13th day after Cd addition, respectively. After these maxima, Cd concentration remained approximately constant in the stem and the leaves but decreased in the roots of both cvs [37]. Using tracer Cd, it was reported that Cd transported to seeds was absorbed before full seed stage and Cd absorbed at the beginning of growing stage was accumulated in leaves [38, 39]. The growing stage where Cd concentration in seeds becomes the highest was from full pod to full seed stage [40]. The relationship between Cd concentration in soil and soybean seeds was different among cultivars. There were significant differences of Cd uptake among soybean cultivars cultivated in the same upland fields. The order of Cd concentration in green beans and in matured soybean seeds was Enrei < Tsurunoko < Tsukui. The translocation of Cd to mature seeds increased rapidly after green seed formation [41].

3. Genetic control of Cd accumulation

Higher plants possess six possible ways to overcome heavy metal exposure at the cellular level: control metal influx, reduce metal bioavailability, chelate metals, promote metal efflux, compartmentalize and sequester metals, and detoxify metal-induced reactive oxygen species (ROS) [42–47]. Efforts have been made to identify gene(s) that are involved in Cd detoxification in plants. Cadmium accumulation in grain may be affected by the uptake by roots, xylem-loading-mediated translocation to shoots, and further transportation to seed via the phloem [48]. Cd translocation from roots to shoots is driven by transpiration in leaves [49]. Cd accumulation in the edible parts is thus likely to be controlled by the general translocation properties of leaves, stems, and roots via the xylem and phloem. Genetic variability for Cd uptake has been reported in soybean [2, 22, 23, 25, 50–52]. The seed Cd concentration of certain genotypes was consistently low under all field and soil conditions. Cd concentration in young tissue of the soybean correlated well to the final Cd concentration of the mature seed, which would facilitate breeding [23]. However, limited efforts have been made in the past to utilize the genetic variability for reduced Cd accumulation in crops. Now, because of market requirements and/or concerns for human health, researchers have placed greater emphasis on producing low Cd cultivars [20]. In soybean, inheritance studies using an $F_{2:3}$ population showed that low Cd accumulation in soybean seed is under the control of a major gene (*Cda*1) with the allele for low accumulation being dominant [53]. Genetic control of Cd accumulation in soybean cultivars was also reported from a field experiment, where 32 soybean cultivars were cultivated on three fields with high Cd content. Evaluation for the seed Cd accumulation revealed that 14 cultivars had an average Cd accumulation of 0.135 mg kg^{-1} or less (0.0936–0.1326) and 18 cultivars had an average Cd accumulation of 0.285 mg kg^{-1} or more (0.2852–0.4452), while none accumulated between 0.135 and 0.285 mg kg^{-1} Cd in the seed. This also suggested that a major gene played a role in controlling Cd accumulation in soybean seed [53].

Genetic control of Cd accumulation was also evaluated in a recombinant inbred line (RIL) population ($F_{6:8}$) derived from the cross between soybean genotype AC Hime (high Cd accumulator in seeds) and Westag-97 (low Cd accumulator). The amount of Cd accumulation in the seeds of the parents AC Hime (0.537 ± 0.046 mg kg^{-1}) and Westag-97 (0.170 ± 0.01 mg

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kg^{-1}) differed significantly ($P < 0.0004$, $F = 7.70$). Cd concentrations in the RILs ranged from 0.067 to 0.898 mg kg^{-1}, with a mean of 0.268 ± 0.013 mg kg^{-1}. Of the 166 RILs analyzed for seed Cd concentration, 87 had ≤0.2 mg kg^{-1} and 79 had ≥0.21 mg kg^{-1}. Treated in this manner, the Cd concentration in the soybean seed segregated in a 1:1 ratio, giving a χ^2-value of 0.386 ($P = 0.534$) (**Figure 1**). Transgressive segregation indicates that some minor genes or QTLs may be involved in influencing Cd accumulation in the AC Hime 9 × Westag-97 populations [53].

4. Breeding for low Cd accumulation

Genetic variation in Cd uptake and translocation had been found in crop plants. Plant-breeding approaches became feasible for the selection of genotypes with reduced Cd accumulation. Genetic variability for Cd accumulation within a species provides an opportunity to utilize plant breeding to select for genetically low Cd concentration. Cultivar selection is an important way to limit Cd uptake and accumulation in crops. Breeder should study the genetic variability for seed Cd concentration in germplasm. An understanding of the heritability of the genetic variability is essential in designing the breeding strategy. It would help in incorporation of the low Cd accumulation trait with suitable modern cultivars. However, identifying low Cd phenotypes by analysis of the grain is challenging due to the high cost of analysis [20]. Developing inexpensive methods would assist in transferring the low Cd accumulation traits with other desirable traits. In soybean grain, Cd concentration was found to be controlled by a single gene, with low Cd dominant in the crosses studied [53]. Lines with the low Cd trait had restricted root-to-shoot translocation, which limited the Cd accumulation in the grain. Genetic variability in soybean [2, 22, 23, 25, 51, 54] has been reported.

Based on the importance of soybean as a staple food crop, the development of low Cd soybean cultivars should be a priority [2, 22, 23, 52]. Inclusion of low Cd as a selection criterion adds an additional trait to an already lengthy list of characteristics that need to be incorporated into a potential new cultivar. The basic characteristics of yield, seed quality, biotic, and abiotic resistance should always be considered. Breeding for low Cd accumulation trait should be assessed based on time and resources available for other characters while determining the priorities. However, care should be taken when considering certain selection activities that may indirectly influence seed cadmium concentration. For breeding aluminum tolerance in crops growing on acid soils and selecting for improved bioavailability of zinc, it may be necessary to incorporate genes to limit the high Cd uptake that would occur at high pH soils (pH of <5.5) and uptake by plants due to similarity of these elements with Zn, respectively [20]. The Cd concentration in both low and high Cd cultivars can increase, if environmental factors, soil salinity, high Cl irrigation water, or management practices increase the phytoavailable Cd. Correction of Zn deficiencies, flooding of rice paddies combined with the application of organic matter and possibly limiting or addition of organic residues can reduce Cd uptake by crops [55]. Low Cd-accumulating cultivars combined with management practices would be more effective in decreasing Cd movement into the food chain than growing low Cd cultivars alone. Although appropriate cultivars and management practices can decrease Cd in crops, the risk of long-term accumulation of phytoavailable Cd in agricultural soils may exist, which could

increase the Cd concentration in both low and high Cd cultivars. Cultivar selection can be effective in reducing the potential Cd concentration in crops. However, the availability of an inexpensive methods to detect and select for genetic differences in Cd concentration at an early developmental stage will reduce the time and cost of a breeding program [23, 51, 56, 57].

5. Marker-assisted selection for low Cd accumulation in soybean

5.1. Developing markers for marker-assisted selection of low Cd accumulation

Marker-assisted selection (MAS), the use of molecular markers linked to or located at a desired gene locus, could be an alternative to phenotypic selection. In soybean, DNA markers linked to low Cd accumulation were identified using recombinant inbred line population ($F_{6:8}$) derived from the cross AC Hime (high Cd accumulation in seeds) and Westag-97 (low Cd accumulation in seeds). The distribution of Cd concentration of 166 RILs ranged from 0.067 to 0.898 mg kg^{-1}, with a mean of 0.268 ± 0.013 mg kg^{-1}. Of the166 RILs analyzed, 87 had ≤0.2 mg kg^{-1} and 79 had ≥0.21 mg kg^{-1} (**Figure 1**). Using the RIL population, seven simple sequence repeat (SSR) markers, SatK138, SatK139, SatK140 (0.5 cM), SatK147, SacK149, SaatK150, and SattK152 (0.3 cM), were reported to be linked to *Cda1* in soybean seed (**Table 1**). It was also reported that all the linked markers were mapped to the same linkage group (LG) K, indicating that a major gene affecting Cd accumulation could be located in the region (**Figure 2**).

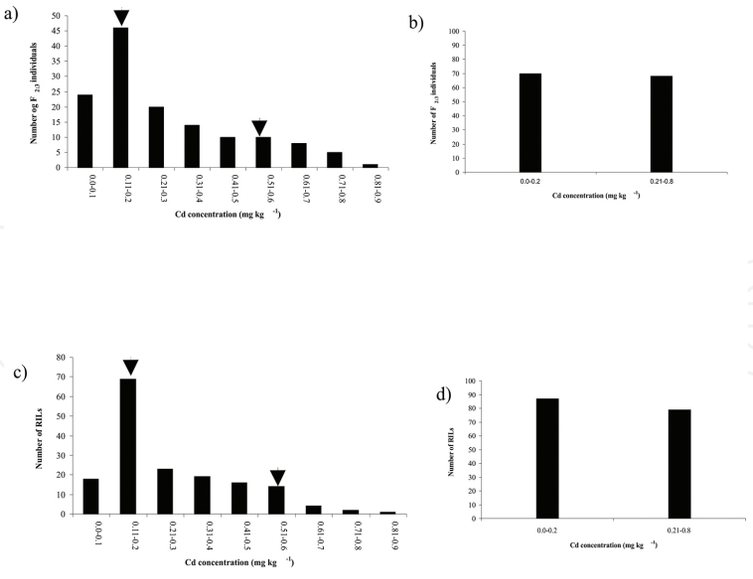

Figure 1. Frequency distribution of seed cadmium concentration in the AC Hime × Westag-97 population. (a) Frequency distribution in the $F_{2:3}$ population in 2005. (b) Lines with low Cd (≤0.2 mg kg^{-1}) and high Cd concentration (≥0.2 mg kg^{-1}) in the $F_{2:3}$ population. (c) Frequency distribution in $F_{6:8}$ RIL population in 2008. (d) Lines with low Cd (≤0.2 mg kg^{-1}) and high Cd concentration (≥0.2 mg kg^{-1}) in the $F_{6:8}$ RIL population. The arrow indicates the level of the parental lines. Solid and dashed arrows indicate the AC Hime and the Westag-97 parent, respectively.

Primer	Primer sequence (5′–3′)	Repeat DNA	Size (bp)	Reference
SatK 138F	AATGAATGTGATGTGATTTGTCA	$(AT)_{29}$	313	Jegadeesan et al. [53]
SatK 138R	TGAGTTAGGTAAGATGGTCATTAAAA			
SatK 139F	AACTAAACAATGTAATGTGATTTGTCA	$(AT)_{25}$	201	
SatK 139R	AAGTTAAACCTTAATTCAAGAAATGTG			
SatK 140F	AACTTTAATCGAAAAGTTATTGCTGA	$(AT)_{13}$	200	
SatK 140R	CAGCTAGAACCTAGAAGATTACGC			
SatK 147F	CCATGGATATCTCCTAATCTCCTG	$(AT)_{18}$	203	
SatK 147R	TCTGCAAATTAAAACTTAGAGGGTG			
SacK 149F	TGAACACATGCTCAACTTGTCA	$(AC)_{18}$	236	
SacK 149R	CGTGTGGTTGCTATTAACTAAATGA			
SaatK 150F	TGATGTCTCCGTACATAAAAGATCAC	$(AAT)_{8}$	286	
SaatK150R	CTTCAACCATACGCTTGTGAA			
SattK 152F	AAAATGTGACCAAACGGGAC	$(ATT)_{20}$	205	
SattK 152R	CACGCCAGTAAATCAAAACTCA			
Gm09: 4770663-F	AAAGCACGGCTGCTTATATAGTT			Benitez et al. [27]
Gm09: 4770663-F	CGTCGTGCATGTGTTATATATTATT			
Gm09:4790483-F	AAGCCCACGATTAGTACTTGGA			
Gm09:4790483-R	ACCAGGCATGTAGTTTCTGTAGC			
Gm-dCAPS-HMA1-F	TGACATCGGTATCTCACTGG		90	Benitez et al. [74]
Gm-dCAPS-HMA1-R	ATGACATTCTCAATTAGCTTTC			
GmHMA3w-F	GCTGACATCGGTATCTCA			Wang et al. [61] (**Figure 5**)
GmHMA3w-R	GCATTGCCTGTTTCATTTG			

Table 1. DNA markers linked to low cadmium accumulating locus Cda1 and Cd1 located on soybean linkage group K (Gm:09).

The closest flanking SSR markers linked to *Cda1* were validated using diverse soybean cultivars and a parallel population (RILs) involving Leo 9 × Westag-97. SSR markers SatK147, SacK149, and SattK152 clearly differentiated the high and low Cd-accumulating genotypes tested in soybean [53]. In order to identify QTL affecting Cd accumulation, a linkage map constructed with 161 markers identified a major QTL associated with low Cd concentration in the soybean seeds. The QTL for low Cd accumulation was also reported to be mapped on the same location as *Cda*1 on LG-K, and accounted for 57.3% of the phenotypic variation [53]. SSR markers closely linked to *Cda*1 in soybean seeds have the potential to be used for MAS to develop low Cd-accumulating cultivars in a breeding program. In a similar mapping approach, Benitez et al. [27] reported a major QTL cd1 affecting seed Cd content using RILs derived from

a cross between two cultivars: Harosoy (with high seed Cd content) and Fukuyutaka (with low Cd content). This major QTL, cd1, was identified on chromosome 9 (LG-K) across years and generations which accounted for 82, 57, and 75% of the genetic variation. Near isogenic lines (NILs) were used to confirm the effect of the QTL and the peak of the QTL that was located in the vicinity of two SSR markers, Gm09:4770663 and Gm09:4790483 (**Table 1**). The separate studies revealed a major QTL for seed *Cd* content, *Cda*1 at a similar genomic location, suggesting that *cd*1 and *Cda1* may be identical.

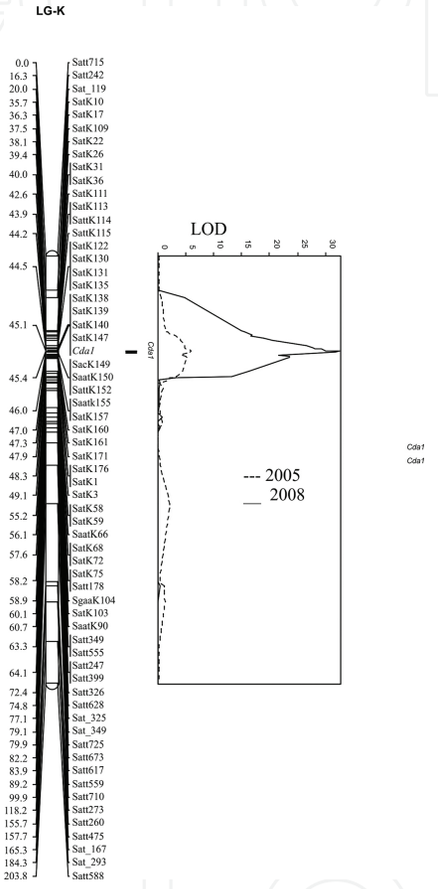

Figure 2. Linkage group-K which corresponds to chromosome 9 (Gm: 09) indicating the location of the newly developed SSR markers and the location of the major gene Cda1 or QTL controlling low Cd accumulation in soybean seed. Location of the major QTL associated with low Cd accumulation mapped on the LG-K with its LOD score values are shown for the AC Hime × Westag-97 $F_{2:3}$ (2005) and $F_{6:8}$ RIL (2008) populations.

5.2. Validation of markers linked to low Cd accumulation

SSR markers linked to low Cd accumulation were validated using diverse soybean genotypes differing in their seed Cd concentration. Of the 12 primers evaluated, three (SatK 147, SatK 149, and SattK 152) effectively differentiated all the high and low Cd genotypes and could be used effectively in MAS for identifying low Cd-accumulating genotype in soybean seed [53]. The reliability of these linked SSR markers was also tested using another RIL population (95 lines) involving Leo 9 × Westag-97. Leo 9 and Westag-97 had seed Cd concentrations of 0.435

± 0.046 and 0.170 ± 0.001 mg kg^{-1}, respectively. The concentration of Cd in the seeds of the Leo × Westag-97 population varied from 0.065 to 0.878 mg kg^{-1}, with a mean of 0.305 ± 0.019 mg kg^{-1}. Of the 95 lines analyzed, 42 were in the low (≤0.2 mg kg^{-1}) and 53 were in the high (≥0.21 mg kg^{-1}) category. Eight SSR primer pairs (SatK 122, SatK 131, SatK 140, SatK 147, SatK 149, SaatK 150, SattK 152, and SaatK 155) were found to be linked to the *Cda*1 gene [53]. It was found that the relative positions of the markers were found to be the same as was found in the AC Hime × Westag-97 population with minor variation in the distances, which often occurs with different mapping populations [58].

Furthermore, these SSR markers were validated for their suitability to discriminate the low and high Cd-accumulating soybean genotypes grown in Europe [59]. The reliability of the SSR markers for the *Cda1* gene revealed that more than half (12) of the examined soybean cultivars carried the allele for low Cd accumulation. The SSR analysis identified soybean cultivars with potential health risk when grown in metal-polluted areas, regardless of their natural tolerance [59]. Vollmann et al. [60] validated the low seed Cd accumulation trait based on the *Cda*1 locus and the associated Sack149 marker. Out of 48 genotypes evaluated, 19 exhibited the allele associated with low and 29 with high Cd accumulation in the seed. SSR marker Sack149 amplified a single polymerase chain reaction (PCR) product was visible in each of the accessions, and no other alleles than the two described for the SacK149 marker were found in any of the genotypes analyzed. Sack149 marker is clearly effective over a range of different genotypes, and thus soybean lines with reduced seed Cd concentration could be selected without the need for extensive and costly field testing in locations with Cd-contaminated soils.

6. Candidate gene(s) controlling Cd accumulation in soybean

Soybean genome sequence available from phytozome (http://www.phytozome.net/soybean.php) via SoyBase (http://soybase.org/gbrowse/cgi-bin/gbrowse/gmax1.01/) was analyzed to identify the candidate genes located between the tightly linked flanking markers (SatK 140 and SaatK 155). Three potential genes homologous to serine-threonine protein kinase, plant type (nt. 4909157–4913830) and two homologous to cation-transporting ATPase (nt. 4918664–4926453 and 5011045–5020110) were identified based on the predicted gene model for the DNA sequence from nt.4909157 to nt.5020110, flanked by SatK 140 and SaatK 155. "Moreover, 13 soybean ESTs, including TA47883_3847 [plasma membrane H^+-ATPase (*Sesbania rostrata*)], TA65152_3847 [Protein kinase, (*Medicago truncatula*)], and AW152957 [Adagio-like protein 1 (*Oryza sativa*)], were also aligned to this genomic region" [53]. There are four SSR markers (SatK 147, SacK 149, SaatK 150, and SattK 152) found in the vicinity of the genes. Of these SSR markers, SattK 152 is reported to be located in the candidate gene plasma membrane H^+-ATPase [53].

In another parallel study, the evaluation of the *Cda* 1 locus and the SSR marker genotype indicated that the candidate gene should be located in the 184.3-kb genomic region between the SatK130 and SacK 149 markers [61]. According to the gene annotation in the SoyBase (http://soybase.org/) [62], six annotated genes in this region were found (**Figure 3**).

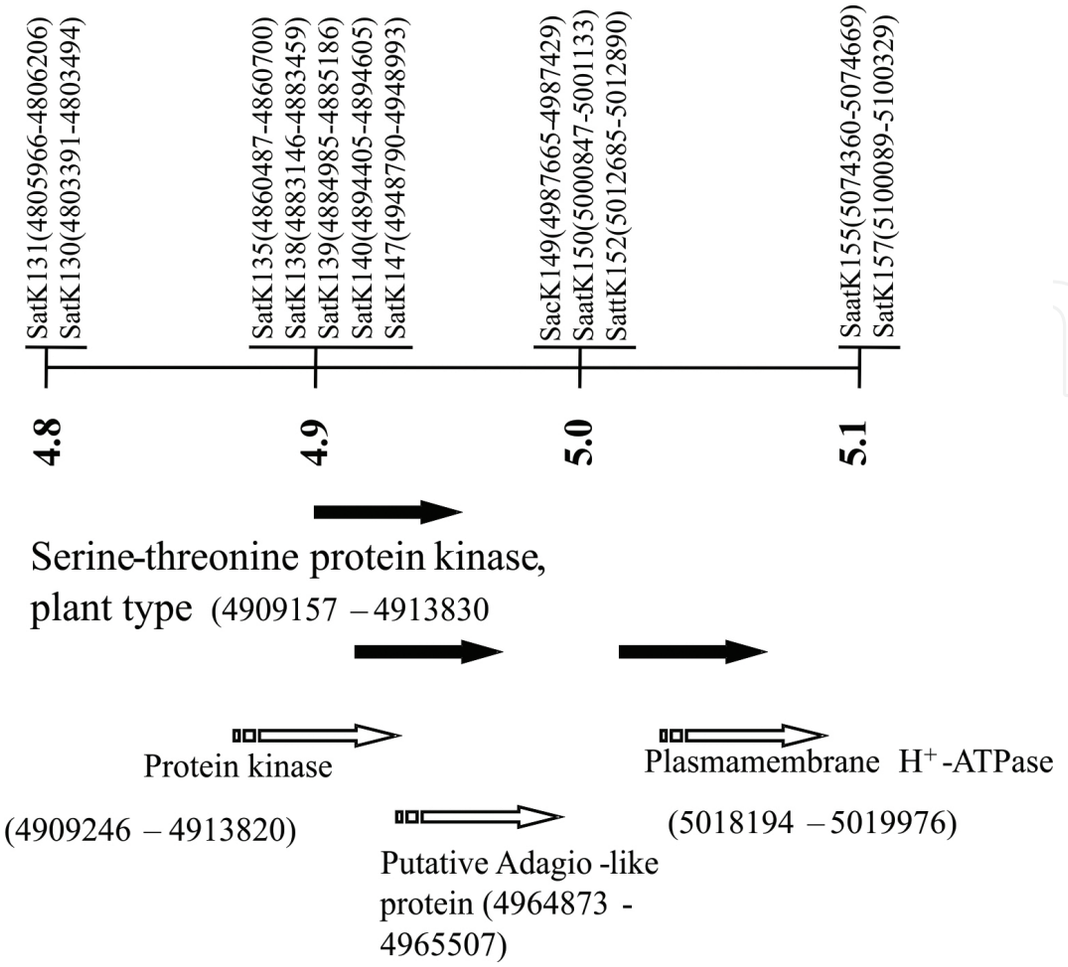

Figure 3. Physical location of the SSR markers in Gm:09 tightly linked to Cda1 controlling low Cd accumulation in soybean seeds. Putative genes located in the vicinity of the tightly linked markers (http://soybase.org/) based on the predicted and known gene function with EST support for soybean genomic sequences are shown.

Among them, Glyma09g06170 encodes a putative heavy-metal transporter (GmHMA3). Its homologs, AtHMA3 and OsHMA3, which belong to P1B-ATPases and localized on the vacuolar membrane in *Arabidopsis thaliana* and rice, were reported to have potential to sequester Cd into vacuoles to limit Cd transport to the xylem [63, 64]. On comparing the full-length cDNA sequence of GmHMA3a from AC Hime (a high Cd accumulator in seeds, GI# JN187676) and GmHMA3w from Westag 97 (a low Cd accumulator in seeds, GI# JN187675), it was found that the two gene sequences are identical and have nine exons and eight introns except for a single nucleotide polymorphism (SNP) at nucleotide position 1823 in GmHMA3a. This single nucleotide change from G to A resulted in the substitution of glutamic acid (E) with glycine (G) at position 608, which is highly conserved in AtHMA3 and OsHMA3, even in AtHMA2 and AtHMA4 [61]. HRM (high-resolution melt) analysis genotyped the SNP in AC Hime, Westag 97, and the 166 RILs; the results indicated GmHMA3w (0.3 cM away from *Cda*1) is significantly associated with low seed Cd concentration in the RILs. To validate the SNP, 20 diverse soybean cultivars were genotyped and confirmed by sequencing. It was found that the 13 high Cd accumulators had the GmHMA3a allele while the seven low Cd accumu-

lators had the GmHMA3w allele. GmHMA3w was found to be associated with low Cd level in soybean seeds and the SNP marker effectively differentiated high from low Cd phenotype [61]. Gene expression studies revealed that GmHMA3 expressed only in roots of AC Hime and Westag 97 (**Figure 4**), indicating that GmHMA3 plays an essential role in the transport of some divalent metals in roots [61].

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GmHMA3a     (1) MVENIKRSSFEVLGMCCATEEALVERILKPLRGVKDVSVIVPTRTVTVVHDVLLISESQIADALNAARLEASLRLQGETDNEKKWPDLTT
GmHMA3w     (1) MVENIKRSSFEVLGMCCATEEALVERILKPLRGVKDVSVIVPTRTVTVVHDVLLISESQIADALNAARLEASLRLQGETDNEKKWPDLTT

GmHMA3a    (91) MVCGLLLALSFLKYAYQPLGWLALGSVVIGFPKVLLRAIASIKALTLNINILVLLAVCGTAALQDFWEAGIIIFLFSIAQWLETRATHKA
GmHMA3w    (91) MVCGLLLALSFLKYAYQPLGWLALGSVVIGFPKVLLRAIASIKALTLNINILVLLAVCGTAALQDFWEAGIIIFLFSIAQWLETRATHKA

GmHMA3a   (181) MVAMSSLTSMAPQKAVIAETGELVDVNDVKINTILAVKAGDAIPLDGIVVEGKCEVDEKMLTGESLPVTKELDSVVWAGTINVNGYISVK
GmHMA3w   (181) MVAMSSLTSMAPQKAVIAETGELVDVNDVKINTILAVKAGDAIPLDGIVVEGKCEVDEKMLTGESLPVTKELDSVVWAGTINVNGYISVK

GmHMA3a   (271) TTVLAKDTVVARMSKLVEEASSRKSRTQRFIDHFAKYYIPAVVLISASIAVVPAALKVPNIKPWFHLAIVVLLSACPCALILSTPVAIFC
GmHMA3w   (271) TTVLAKDTVVARMSKLVEEASSRKSRTQRFIDHFAKYYIPAVVLISASIAVVPAALKVPNIKPWFHLAIVVLLSACPCALILSTPVAIFC

GmHMA3a   (361) ALTKAAISGLLLKGGDYIETLSGIKTVAFDKTGTITRGEFTVTDFSVSVDDISIETLLYWVSSVESKSSHPMAAALVEYGMLNSVKPIPE
GmHMA3w   (361) ALTKAAISGLLLKGGDYIETLSGIKTVAFDKTGTITRGEFTVTDFSVSVDDISIETLLYWVSSVESKSSHPMAAALVEYGMLNSVKPIPE

GmHMA3a   (451) NVENFQNFPGEGVYGIINGKDIYIGNRRIGARAGSERVDCRTQCQSPEISTPNQCCGPTLVGVFRLADTCRSGALEAIEELKLLGVRSVM
GmHMA3w   (451) NVENFQNFPGEGVYGIINGKDIYIGNRRIGARAGSERVDCRTQCQSPEISTPNQCCGPTLVGVFRLADTCRSGALEAIEELKLLGVRSVM

GmHMA3a   (541) LTGDSSQAAMYAQSQLNHALDIVHAELLPAEKAVIIENFKKDGLIAMIGDGMNDAPALATADIGISMEISGSALANETGNAILMSNDIRK
GmHMA3w   (541) LTGDSSQAAMYAQSQLNHALDIVHAELLPAEKAVIIENFKKDGLIAMIGDGMNDAPALATADIGISMGISGSALANETGNAILMSNDIRK

GmHMA3a   (631) IPEAIRLARKTTRKLIENVIISIGFKSVILALAIAGYPIVWLAVLTDVGTCLLVILNSMLILQEKTKYERKSTSSKYGTFSEDMTTALLD
GmHMA3w   (631) IPEAIRLARKTTRKLIENVIISIGFKSVILALAIAGYPIVWLAVLTDVGTCLLVILNSMLILQEKTKYERKSTSSKYGTFSEDMTTALLD

GmHMA3a   (721) KKSNSNENKAVLSAEKCGKDCCKNDTYREATTNKNESSGLSKLSSLKGNHNGNLVSIEVHIVKPCNGCGLGKVKMCEDFSCRTNNSSSDC
GmHMA3w   (721) KKSNSNENKAVLSAEKCGKDCCKNDTYREATTNKNESSGLSKLSSLKGNHNGNLVSIEVHIVKPCNGCGLGKVKMCEDFSCRTNNSSSDC

GmHMA3a   (811) CQEQSKTEKSDTGSIVTQEASIATLESDGYKGKSMDISKLSGTSVTPKCCKNLCCNDSVNNISNLSLSQPEIVIE
GmHMA3w   (811) CQEQSKTEKSDTGSIVTQEASIATLESDGYKGKSMDISKLSGTSVTPKCCKNLCCNDSVNNISNLSLSQPEIVIE
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Figure 4. Alignment of two allelic amino acid sequences (GmHMA3a and GmHMA3w) from AC Hime and Westag 97, respectively. Blue box indicated the single amino acid mutation. Red boxes showed all typical motifs of P_{1B}-HMA. Transmembrane domains were underlined with black lines.

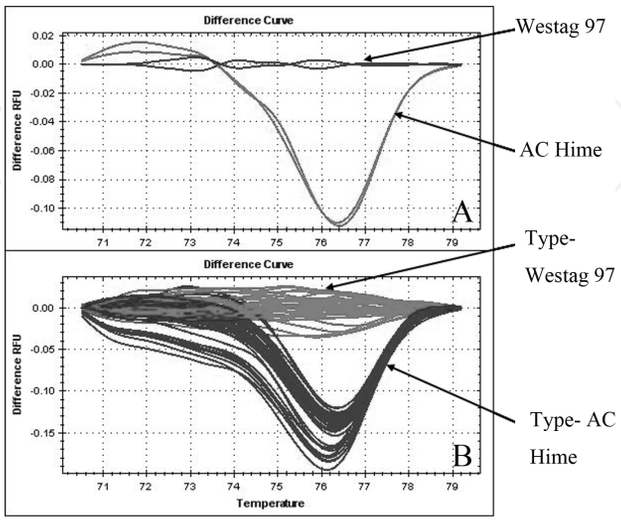

Figure 5. HRM analysis of the SNP for the parents (A) and the 166 RILs (B).

The regulation of metal homeostasis is complex and controlled by several metal-specific and metal nonspecific genes located in different membranes and long-distance transport systems to move throughout the plant. The presence of higher levels of heavy metal ions in the soil triggers a wide range of cellular responses including the synthesis of metal-detoxifying peptides and change in gene expression. Cd- and copper-responsive genes have been shown to code for signal transduction components, such as *Arabidopsis* mitogen-activated protein kinase kinase kinase (MAPKKK) MEKK1, stress-induced proteins, transcription factors, proteins participating in protein folding, and sulfur and glutathione metabolism [65–67]. MAPKs are pro-directed Ser/Thr kinases phosphorylating numerous substrates in different cellular compartments and thereby shown to involve in the signal transduction in the form of a phosphorylation cascade from upstream kinase to downstream targets. Cd ion-activated distinct mitogen-activated protein kinases were reported in alfalfa seedlings [68].

In soybean, candidate genes related to heavy metal transport or homeostasis were located in the vicinity of the identified QTL (Cda1). Protein kinase, putative adagio-like protein, and plasma membrane H^+-ATPase were found in the QTL vicinity. Genes uniquely induced by Cd ions in *Arabidopsis* showed a high percentage of genes with "kinase activity" (16.7%) [69]. In soybean, the influx of Cd across the plasma membrane of root cells has been shown to occur via a concentration-dependent process exhibiting saturable kinetics, indicative of metabolically mediated membrane transport process [70]. Cd seems to have differential-inhibiting effects on ATPase activity and proton transport activity in oat roots [71]. Evidence from previous studies suggests that protein kinases modulate the plant plasma membrane ATPase activity, and the ATPase probably contains multiple phosphorylation sites that may affect its activity in different ways [72]. The presence of protein kinase, and plasma membrane H^+-ATPase genes near the tightly linked SSR markers, suggests that the regulation of this enzyme may play a vital role in Cd stress [53]. This was later supported by a major QTL-controlling Cd concentration (*cd*1) identified in soybean [27]. Analysis of the genome sequence of Williams 82 from Sat_119 (Gm09:3585450) to Satt178 (Gm09:5438776) that flanks the *cd1* revealed the presence of P_{1B}-ATPase gene (Glyma09g06170.1, Gm09:4918664 to Gm09:4926453) in the vicinity, which had been implicated in the transport of a range of essential and also potentially toxic metals across cell membranes (e.g., Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}) [73].

The gene was designated as *GmHMA1*, and cDNA sequence analysis revealed the presence of two types of transcript candidates with different lengths (3719 and 3929 bp) that were identified for this gene and designated as *GmHMA1a* and *GmHMA1b* [74]. *GmHMA1a* sequence revealed the presence of nine exons and eight introns which are similar to the one identified in AC Hime [61]. In *GmHMA1b*, however, 210-bp nucleotides corresponding to the eighth intron were retained as part of an exon (i.e., were not spliced out), resulting in a structure with eight exons and seven introns. Hence, it was concluded for the existence of alternative splicing in the *GmHMA1*gene of soybean [74]. Evidence for such alternative splicing events of the intron-retention type in *GmHMA*1 has also been reported in soybean blue light photoreceptors (cryptochrome multigene family genes) GmCRY1b, GmCRY1c, GmCRY1d, and GmCRY2a [75]. The open-reading frame of *GmHMA*1a contained 2655 nucleotides with 1064 bp of the 3′-untranslated region. The putative polypeptide of *GmHMA1a* consisted of 885 amino acids with

molecular mass and isoelectric points of 95,135, and 5.86, respectively. In *GmHMA1*b, translation was prematurely terminated, resulting in a polypeptide consisting of 559 amino acids due to an alternative splicing that generated a stop codon around the middle of the region corresponding to the eighth intron. In *GmHMA1a* one base substitution from G to A at nucleotide position 2095 resulted in changed amino acid from glycine to glutamic acid at amino acid number 608, but it did not affect the putative amino acid translation of *GmHMA1b* because alternative splicing generated a stop codon upstream of the base substitution [74]. No catalytic domains have been ascribed to the region of amino acid substitution, but it was located immediately downstream of the ATP-binding domain. The glycine residue at the site of amino acid substitution was fully conserved in *AtHMA*3, *AtHMA*4, *AtHMA*6, and *AtHMA*7 [76, 77], suggesting that *GmHMA1a* of Fukuyutaka is the wild type. Similar to *AtHMA*3 and *AtHMA*4, the expression of *GmHMA1* was substantially lower than actin and was predominant in roots compared with leaves [76, 77]. Using the SNP location, dCAPS primers were designed to produce a 95-bp fragment in Harosoy, Fukuyutaka, and the NILs. After *BmrI* digestion, a shorter band of 70-bp fragments observed in Fukuyutaka and the NIL of Fukuyutaka type was designated as Gm-dCAPSHMA1 [74]. Linkage mapping revealed that the marker (Gm-dCAPSHMA1) was assigned to a position identical to Gm09:4790483 and located around the three markers, Gm09:4770663, Gm09:4790483, and Gm-dCAPS-HMA1, spanning 0.6 cM. The genotype of Gm-dCAPS-HMA1 was significantly associated with seed Cd concentration. The genotype and Cd concentration completely co-segregated in the RILs. The presence of P1B-ATPases near the marker location suggested that it may be present at the intracellular membranes and be responsible for compartmentation of metals, for example, sequestration in the vacuole, Golgi, or endoplasmic reticulum, or they may be present at the plasma membrane and function as efflux pumps removing potentially toxic metals from the cytoplasm [73, 76, 77].

Wang et al. [78] studied gene expression pattern of the low and high Cd-accumulating soybean genotypes Westag-97 and AC Hime and reported different expression levels of five metal nonspecific genes, a receptor-like serine/threonine-protein kinase (RSTK, glyma09g06160), a plasma membrane H^+-transporting ATPase (H^+-ATPase, glyma09g06250), an iron-sulfur cluster scaffold protein nfu-related (ISCP, glyma09g06300), and two uncharacterized conserved protein (UCP1, glyma09g06220, and UCP2, glyma09g06310), which were previously found at the *Cda1* locus [53]. The responses of the five genes at the *Cda1* locus to Cd treatment were studied using soybean genotypes differing in Cd sequestration and translocation. Westag 97, a low seed Cd accumulator that sequestrates Cd in roots and restricts it from loading into xylem and transporting to leaves and seeds, and AC Hime, a high seed Cd accumulator that has a smaller capacity of Cd accumulation in roots but translocates and stores more Cd in stems and leaves, were used for gene expression studies. The transcriptional levels of the five genes in both AC Hime and Westag 97 were altered in response to the external Cd treatment [78].The expression levels of RSTK were significantly increased by Cd in AC Hime but were decreased in Westag 97. These results indicated that the RSTK is probably involved in Cd transportation. RSTK can boost Cd transporting into stems and leaves in AC Hime through elevating its expression levels and limiting Cd transporting into leaves and stems in Westag 97 through reducing its expression level. The RSTK family is involved in signal transduction pathways in plants and interacts with membrane receptor proteins. Several studies have shown that the

expression levels of RSTKs are readily influenced by some biotic/abiotic stresses. H^+-ATPase, the only proton-pump operator in plasma membranes, not only regulates the ion homeostasis but also regulates the growth and development processes in plants. Although the Cd accumulation capacity differs in leaves and stems between AC Hime and Westag 97, the expression trends of H^+-ATPase in both leaves and stems of the two cultivars were similar. The expression levels were different in roots between AC Hime and Westag 97, which consisted of different Cd capacity. These results indicated that cultivars' effect on the expression of the soybean H^+-ATPase exposed to Cd and the soybean H^+-ATPase is probably involved in Cd transporting to root vacuoles in Westag 97 [78]. The gene expression levels of ISCP were also regulated by Cd. Cd significantly reduced the gene expression level in roots of both AC Hime and Westag 97. Similar to the RSTK, the expression patterns of ISCP in leaves and stems were opposite between AC Hime and Westag 97, which indicated that Cd caused some changes of fundamental life process. According to the different expression levels of RSTK, ISCP, and H^+-ATPase between Westag 97 and AC Hime, RSTK may be involved in transporting Cd into stems and leaves, H^+-ATPase may be correlated to the capacity of Cd accumulation in roots, and Cd caused some changes of fundamental life process which led to the different expression patterns of ISCP between Westag 97 and AC Hime [78]. In ATPase gene, a single nucleotide substitution causing a loss of function due to an amino acid substitution was reported; the functional isoform of the protein is present in the low Cd accumulating genotype that is considered as the wild-type allele [61, 74]. It was found that the expression of the ATPase gene is limited to the plant root only [61]. Wang et al. [79] evaluated the independent effect of the three Cd concentrations on the reference genes (RGs) using quantitative real time PCR (qRT-PCR). It was reported that the effect of increased Cd concentration on the expression levels of the four RGs (ACT3, PP2A, ELF1B, and F-box) is less than that on the other candidate RGs. The four genes may not be involved in any of the cellular processes associated with Cd uptake and translocation. Soybean has a complex network of homeostatic mechanisms that controls Cd uptake, accumulation, and trafficking, and some genes such as ACT3, PP2A, ELF1B, and F-box can self-regulate well when under metal stress and recommended them as most stable RGs in these gene expression studies.

7. Role of miRNAs in cadmium tolerance

Many abiotic conditions including heavy metal result in oxidative stress in plants [80]. Recently, increasing evidences have revealed that miRNAs played the crucial role on the regulation of plant genes at the posttranscriptional level in responding to metal stresses. Several miRNAs are involved in the regulation of genes responsible for antioxidation. MiR398 is the first miRNA identified to regulate plant responses to oxidative stress [81]. MiRNAs are small, non-protein coding single-stranded RNA, around 22–24 nucleotides in higher plants, which regulate gene expression at the posttranscriptional and translational levels [82–84]. Several studies have demonstrated that miRNAs involved in most of the essential physiological processes in plants, including signal transduction, development regulation, and stress

responses [85, 86]. MiRNAs are of importance for plant to respond to heavy metal stress [87–89]. Novel miRNAs responsive to Cd were reported in Brassica and rice [90–93].

Similarly, to study the regulatory mechanism of miRNAs in response to Cd treatment in soybean, a miRNAs microarray chip was used to detect the expression of miRNAs in HX3 and ZH24 roots with Cd stress or Cd-free. Under Cd stress, 26 Cd-responsive miRNAs were found [94]. Of these 26 miRNAs identified, gma-miR1535b, which was detected as being up-regulated in HX3 and down-regulated in ZH24 and all other miRNA, showed similar expression patterns in HX3 and ZH24. This suggested that miRNA regulation may represent the fundamental mechanism of adapting to Cd exposure [94]. Further, it was reported that miR397a, miR408, and miRNA398c showed almost the similar up-regulated alteration in response to Cd exposure, which might imply that SPL7 (SQUAMOSA promoter-binding protein-like 7) is involved in the regulation of Cu deficiency and Cd response in soybean [94]. To evaluate the target transcripts of the miRNAs, a high-throughput degradome sequencing was adopted using a small RNA library. Fifty-five targets cleaved by 14 Cd-responsive miRNAs were identified. In addition, a number of Cd-responsive miRNAs and target mRNAs in soybean have been validated by quantitative RT-PCR [94]. It is well established that lignin provides structural support and contributes to plant defense mechanism against both biotic and abiotic stresses [95]. Several studies reported on an increased lignin synthesis upon metal treatment [96, 97], and reported that lignification is one defense mechanism under Cd exposure in soybean root [98, 99]. One novel soybean Cd-responsive miRNA, miR1535b, was illustrated to cleave Glyma07g38620.1 and Glyma07g38620.1 encoding isopentyl transferase (IPT). It was shown that IPT catalyses the rate-limiting first step in de novo cytokinin (CK) biosynthesis and promotes the formation of isopentenyladenosine-59-monophosphate (iPa) [100, 101]. Overexpression of *ipt* in leaves and roots can promote stress tolerance in *Agrostis stolonifera* [102]. CK was reported to inhibit primary root elongation in *A. Thaliana* [103]. Under Cd exposure, Glyma07g38620.1 displayed an apparent up-regulation in HX3 and slight down-regulation in ZH24, so does the CK, which probably explain why HX3 show higher tolerance and distinctly primary root elongation inhibition than that of ZH24 [94].

8. Conclusion

Genetic variation for Cd accumulation in soybean genotypes provides an opportunity to develop varieties with low Cd content. Breeding programs are underway to produce low Cd cultivars of soybean. The low Cd accumulation in soybean seed was reported to be genetically controlled by a major gene *Cda1*. The SSR markers linked to the *Cda*1 gene in soybean would help in MAS to incorporate this trait with other agronomic traits. Candidate gene was found for seed Cd concentration in soybean using populations and NILs derived from a single cross and a dCAPS marker based on the base substitution was developed using *Cd*1 locus. A survey of various genetic resources with different seed Cd levels may be necessary to ascertain the prevalence of the base substitution, the existence of different genetic polymorphisms associated with seed Cd concentration, and the usefulness of the dCAPS marker. Marker based on the SNP in the P1B-ATPase metal ion transporter gene could be utilized as a precise gene-based

marker along with the linked Sack149 SSR marker, which will also reduce the cost involved in the Cd analysis. The cost involved in the MAS for one sample will be approximately $1–2, compared to $10–23 for Cd analysis in an established laboratory. In conclusion, the low seed Cd accumulation trait from the *Cda1* locus and its tightly linked SSR and SNP markers were clearly effective over a range of different genotypes, and thus soybean lines with reduced seed Cd concentration could be selected without the need for extensive and costly field testing in locations with Cd-contaminated soils. In addition, there is a possibility to study further mechanisms of controlling seed Cd concentration either on the root or on the shoot level, which is inferred from significant variation in seed Cd concentration within the two marker locus classes of the *Cda*1 gene and transgressive segregation.

Transgenic experiments may be necessary to determine the function of *GmHMA*1a and to verify whether the amino acid substitution affected transport and accumulation of Cd in seeds. Considering the human health issues due to Cd accumulation, the utilization of the soybean *Cda*1 locus for the selection of genotypes low in seed Cd is particularly of importance for food-grade soybean. Due to the current expansion of soybean production to new production regions with partly unknown heavy metal concentration of soils, the cultivation of low Cd-accumulating varieties would contribute to better food safety for soy food products.

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