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ORIGINAL PAPER

Mapping and validation of simple sequence repeat markers linked to a major gene controlling seed cadmium accumulation in soybean [*Glycine max* (L.) Merr]

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Abstract Daily consumption of cadmium (Cd) contaminated foods poses a risk to human health. Cultivar selection is an important method to limit Cd uptake and accumulation, however, analyzing grain Cd concentration is costly and time-consuming. Developing markers for low Cd accumulation will facilitate marker assisted selection (MAS). Inheritance studies using a threshold value of 0.2 mg kg^{-1} for low and high and an $F_{2:3}$ population showed that low Cd accumulation in soybean seed is under the control of a major gene (*Cda1*, proposed name) with the allele for low accumulation being dominant. A recombinant inbred line (RIL) population ($F_{6:8}$) derived from the cross AC Hime (high Cd accumulation) and Westag-97 (low Cd accumulation) was used to identify the DNA markers linked to *Cda* gene(s) or quantitative trait loci (QTLs) controlling low Cd accumulation. We screened 171 simple sequence repeat (SSR) primers that showed polymorphism between parents on the 166 RILs. Of these, 40 primers were newly developed from the soybean genomic DNA sequence. Seven SSR markers, SatK138, SatK139, SatK140 (0.5 cM), SatK147, SacK149, SaatK150 and SattK152 (0.3 cM), were linked to *Cda1* in soybean seed. All the linked markers were mapped to the same linkage group (LG) K. The closest flanking SSR markers linked to *Cda1* were validated using a parallel population (RILs) involving Leo × Westag-97. Linked markers were also validated with diverse soybean genotypes differing in their seed Cd concentration and showed that SSR markers SatK147, SacK149, and SattK152 clearly differentiated the high and low Cd accumulating genotypes tested. To treat Cd uptake as a quantitative trait, QTL analysis using a linkage map constructed with 161 markers identified a major QTL associated with low Cd concentration in the seeds. The QTL was also mapped to the same location as *Cda1* on LG-K. This QTL accounted for 57.3% of the phenotypic variation. Potential candidate genes (genes with known or predicted function that could influence the seed Cd concentration) like protein kinase, putative Adagio-like protein, and plasma membrane H^+-ATPase were found to be located in the locus of interest. Of the four SSR markers located in the region, SattK152 was localized in the plasma membrane H^+-ATPase gene. SSR markers closely linked to *Cda1* in seeds of soybean were identified and have potential to be used for MAS to develop low Cd accumulating cultivars in a breeding program.

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Introduction

Cadmium (Cd) is a major pollutant metal that is highly toxic to living organisms. Vast areas of agricultural soils are contaminated with Cd through the use of super phosphate fertilizers and sewage sludge, and inputs from mining and smelting industries (McGrath et al. 2001). 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. Cadmium can accumulate in the human body leading to a risk of chronic toxicity with excessive intake (http://www.accessscience.com/studycenter.aspx?main=9&questionID=4978). In humans it can damage kidneys, causing a loss of calcium and associated osteoporosis (Kazantzis 2004). The international standard for cadmium concentration in foods has been discussed by the Codex Alimentarius Commission of FAO/WHO due to the growing concern about the safety of foods and human health. The Codex committee on food additives and contaminants has proposed an upper limit of 0.2 mg kg^{-1} for cadmium concentration in soybean grain (Codex Alimentarius Commission 2001); thus, minimizing the intake of cadmium and other heavy metals from food grains is an important health issue. Cultivar selection is an attractive method for changing the trace element profile of crops, as the benefit will persist in the seed and can reduce the requirement for other management techniques such as fertilization management or crop rotations. Furthermore, supplies of high-quality, low Cd phosphate rock is limited and its use has economic trade-offs (Cupit et al. 2002).

The level of Cd in grain may be affected by the three transport processes most likely to mediate Cd accumulation in the shoots and, subsequently, in the seeds: (i) uptake by roots, (ii) xylem-loading-mediated translocation to shoots, and (iii) further translocation to seeds via the phloem (Clemens et al. 2002). Cd translocation from roots to shoots is driven by transpiration in leaves (Salt et al. 1995). In durum wheat, it is suggested that Cd accumulation in grains may occur *via* phloem (Hart et al. 1998). High Cd accumulation levels in durum wheat grain may be partially due to the elevated translocation of Cd from leaves and stems to maturing grain (Harris and Taylor 2001; Chan and Hale 2004) or directly from root to shoots (Greger and Löfstedt 2004). Cadmium concentration in rice grain is positively correlated with Cd accumulation in the plant and the Cd distribution ratio from aerial parts to the grain (Liu et al. 2007; Kashiwagi et al. 2009). Florijn and Van Beusichem (1993) distinguished two groups of maize inbred lines; a group with low shoot but high root Cd concentrations ('shoot Cd excluder') and a group with similar shoot and root cadmium concentrations ('non-shoot Cd excluder'). 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.

The fact that genetic variability exists within a species with the tendency to accumulate Cd provides an opportunity to utilize plant breeding to select for genetically low Cd concentration. Although genotypic differences in Cd concentrations have been studied in a range of crops, only limited efforts have been made in the past to utilize plant selection or breeding to reduce Cd in crops. Now, because of market forces and concerns for human health, researchers have placed greater emphasis on producing low Cd cultivars of several grain crops (Grant et al. 2008). Genetic variability for cadmium uptake has been reported in soybean (Reddy and Dunn 1986; Bell et al. 1997; Arao and Ae 2001; Arao et al. 2003; Ishikawa et al. 2005a; Morrison 2005; Arao and Ishikawa 2006). The seed cadmium concentration of certain genotypes was found consistently low under all field and soil conditions. Cadmium concentration in young tissue of the soybean correlated well to the final Cd concentration of the mature seed, which would facilitate breeding (Arao and Ishikawa 2006). Based on the importance of soybean as a staple food crop, development of low Cd soybean cultivars should be a priority (Arao et al. 2003; Ishikawa et al. 2005a; Morrison 2005; Arao and Ishikawa 2006).

While cultivar selection can be effective in reducing the potential Cd concentration in crops, there are still constraints in utilizing this approach to produce low Cd cultivars. Development and testing of a new cultivar is time-consuming because the low Cd characteristic must be incorporated into a cultivar that has acceptable characteristics for yield, agronomic suitability, quality and disease resistance. Moreover, the chemical analysis required to select for low Cd accumulation is expensive. An ability to detect and select for genetic differences in Cd concentration at an early growth stage will reduce the time and cost of a breeding program (Arao and Ae 2001; Archambault et al. 2001; Arao and Ishikawa 2006, Stolt et al. 2006). Marker assisted selection (MAS), the use of molecular markers linked to a desired gene, could be an alternative to phenotyping.

Simple sequence repeats (SSRs), also known as microsatellites, have become important tools for a broad range of application such as genome mapping and genetic diversity studies. More than 1,810 genetically mapped SSR markers are available to soybean breeders and geneticists (Cregan et al. 1999; Song et al. 2004, Hwang et al. 2009). Molecular markers can be used for linkage mapping with bulked-segregant analysis (BSA; Michelmore et al. 1991). BSA was successfully employed in identifying two RAPD (random amplified polymorphic DNA) markers linked to a single dominant gene controlling grain Cd concentration in durum wheat (Penner et al. 1995) and the major gene (*Cdu*1) was mapped to the long arm of wheat chromosome

5B (Knox et al. 2009). Markers have also been found associated with a major gene affecting Cd accumulation in oat (Tanhuanpää et al. 2007). Quantitative trait loci (QTLs) controlling Cd concentration were reported in brown rice (Ishikawa et al. 2005b; Kashiwagi et al. 2009). QTLs affecting Cd concentration in roots and shoots were mapped in *Thlaspi caerulescens,* a natural Zn, Cd, and Ni hyperaccumulating species belonging to the Brassicaceae family (Deniau et al. 2006).

The objectives of the present study were to: (1) identify closely linked SSR markers in association with gene(s) or QTL controlling low Cd accumulation in soybean seeds from a RIL population by both simple marker analysis and interval QTL mapping; (2) validate the markers in another population and several soybean genotypes differing in seed Cd concentration; and (3) identify the candidate genes underlying the major Cd QTL by reference to the Williams 82 soybean genome.

Materials and methods

Mapping populations

Three soybean cultivars, AC Hime (Poysa and Buzzell 2001), Leo (Poysa et al. 2005) and Westag-97 (Ablett et al. 1999), were used as parents in this study for their contrasting seed cadmium concentrations, 0.537 ± 0.046, 0.435 ± 0.03 and 0.170 ± 0.01 mg kg^{-1} respectively. A population of 166 $F_{6:8}$ RILs was derived from a cross between AC Hime × Westag-97. A parallel population of 95 $F_{6:8}$ RILs was developed simultaneously from Leo × Westag-97. Fourteen diverse soybean genotypes differing in seed Cd concentration were used for marker validation (Table 1). Plant populations were grown in Woodslee, Ontario on a Brookston clay loam soil with a high Cd concentration (250 ppm) and a pH of 6.2. The $F_{2:3}$ AC Hime × Westag-97 and Leo × Westag-97 populations were grown in 2005 as unreplicated hill plots with 5 seed per hill and 0.6 m intra-row spacing and 1.0 m inter-row spacing. Ten hills of each parent were included as checks. The $F_{2:3}$ populations were advanced by single seed descent to develop the $F_{6:8}$ RILs. The $F_{6:8}$ AC Hime × Westag-97 and the $F_{6:8}$ Leo × Westag-97 RILs were grown in 2008 in separate two replicate completely randomized block design trials with single row plots 1.5 m long with 60 cm inter-row spacing. Seeds from each replicate were bulked for Cd analysis. Normal agronomic practices were followed.

Cadmium analysis

Thirty grams of soybean seed (15 g from each replicate) was ground for 15 s in a high speed (20,000 rpm) mill (Knifetec Sample Mill; FOSS, Eden Prairie, MN, USA) equipped with a water-cooled grinding chamber to reduce clumping and ensure a uniform particle size (<0.5 mm). The ground samples were transferred to plastic bags for storage and shipping. Closed vessel microwave acid digestion was used for sample preparation (Gawalko et al. 1997). The individual samples were weighed-out in 1 g sample size into fluoropolymer PFA decomposition vessels and 10 ml of ultrapure grade nitric acid was added to each sample. The decomposition vessels were placed inside ceramic outer vessels, sealed with a vented screw cap, and placed in a 16-sample rotor in a programmable microwave digester (Multiwave 3000, PerkenElmer, Shelton, CT, USA). The digester was programmed to ramp the digestion solution temperature (T) to 90°C across a 10 min period, hold that T for 2 min prior to a 5 min ramp to 110°C which was held for 3 min prior to a final 5 min ramp to 140°C which was held for 2.5 min. Temperature and pressure in the decompositions vessels were monitored by a contactless probe attached to the microwave control unit. After digestion, the vessels were allowed to cool prior to the sample being decanted into 50 ml polypropylene tubes (Digitubes, SCP Sciences, Montreal, Canada) and brought to a volume of 50 ml with double deionized water. Duplicates were also prepared through-out the batch of samples as part of our quality control (qc) protocol. In-house qc samples and certified reference materials (NIST, Gaithersburg, MD, USA) were included as part of this protocol. The precision or repeatability of the complete

Table 1 Validation of the SSR markers associated with low-Cd accumulation in diverse commercial Ontario soybean cultivars

Cultivar	Cd (mg kg^{-1})	SatK 147	SacK 149	Markers SaatK 150	SattK 152
Cultivar 1	0.537 ± 0.046	−	−	−	−
Cultivar 2	0.170 ± 0.010	+	+	+	+
Cultivar 3	0.093 ± 0.017	+	+	+	+
Cultivar 4	0.094 ± 0.015	+	+	−	+
Cultivar 5	0.114 ± 0.024	+	+	+	+
Cultivar 6	0.125 ± 0.037	+	+	+	+
Cultivar 7	0.127 ± 0.022	+	+	−	+
Cultivar 8	0.096 ± 0.017	+	+	−	+
Cultivar 9	0.397 ± 0.073	−	−	−	−
Cultivar 10	0.330 ± 0.050	−	−	−	−
Cultivar 11	0.395 ± 0.058	−	−	−	−
Cultivar 12	0.397 ± 0.067	−	−	−	−
Cultivar 13	0.424 ± 0.073	−	−	−	−
Cultivar 14	0.431 ± 0.066	−	−	−	-
Cultivar 15	0.442 ± 0.063	−	−	−	−
Cultivar 16	0.444 ± 0.066	−	−	−	−

+, SSR allele specific to low-Cd concentration; −, SSR allele specific to high Cd concentration in the soybean seeds

process is within a relative standard deviation (RSD) of 10%. Cd concentration in each sample was analyzed by graphite furnace atomic absorbance (SIMMA, PerkinElmer) following the recommended procedure of the instrument (Gawalko et al. 2009). Cd concentration for each line was the mean of the two replicate with an RSD of less than 10%.

DNA isolation and marker analysis

Young leaf samples (100 mg) at third leaf stage were frozen in liquid nitrogen and ground using an AutoGrinder 48 (AutoGen Inc., Holliston, MA, USA). After incubation with plant lysis buffer (AutoGen AG00121) at 65°C for 30 min, DNA was automatically extracted using an AutoGen 850 alpha DNA automatic system following the manufacturer's manual (AutoGen Inc.). The extracted DNA from each line was quantified using Nanodrop Spectrophotometer ND1000, and then diluted to 25 ng/µl.

SSR markers from all 20 linkage groups (LG) were selected from the soybean genetic map (Song et al. 2004).

Primer design and examination

To develop microsatellite markers targeted to areas lacking SSR markers, as well as to saturate the genomic region of interest, soybean DNA sequence information (http://www.phytozome.net/soybean.php) accessed via SoyBase (http://soybase.org/gbrowse/cgi-bin/gbrowse/gmax1.01/) was used for developing new SSR markers. The DNA sequence retrieved from SoyBase was used to search for repeat DNA using Tandem Repeats Finder program (Benson 1999). PCR primers, between 19 and 30 bp in length and flanking each of the repeated sequences, were designed using the NCBI (National Center for Biotechnology Information) primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) which uses primer3 (Rozen and Skaletsky 2000) to design PCR primers and then submits them to a BLAST search against a user-selected database. Primers were selected to have a $T_m \geq 55$°C at a salt concentration of 50 mM with an expected fragment size of 200 and 300 bp. Primers were synthesized by SIGMA GENOSYS (Oakville, ON, Canada). Each primer pair was tested for polymorphism using AC Hime and Westag-97. Primer sequence information is given in Electronic Supplemental Table 1.

SSR analysis

SSR analysis was performed in 10 µl containing 2 µl genomic DNA (25 ng/µl), 0.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTP mixtures (5 mM), 1 µl 10× PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl) 0.3 µl SSR primer (1.5 µM), 0.2 µl *Taq* polymerase (5 units/µl), 5.5 µl double-distilled water. The amplification conditions were 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 47°C, 30 s at 72°C, then 5 min at 72°C. After amplification, the PCR products were mixed with loading buffer (95% formamide, 0.01% bromophenol blue, 0.01% cyanol blue). The PCR products were denatured for 5 min at 94°C in a Gene Amp PCR 9600 system and snap cooled in ice for 5 min. The denatured PCR products were separated on a 6% (w/v) denaturing polyacrylamide gel with a DNA Analyzer 4200 (LICOR Co.). The primer pairs producing the polymorphic bands between the parents were used for the entire RIL population from the cross AC Hime × Westag-97.

Linkage and QTL analysis

To detect the genomic region linked with low cadmium concentration in soybean seeds, the recombinant inbred lines were classified as high and low types based on the parental value ± standard error. However, the mean value of two replications with an RSD of less than 10% for the RIL was used for QTL analysis because the cost of analysis prohibited us from doing more than 2 replications. Lines with Cd concentration $\leq$0.2 mg kg^{-1} were classified as low type and above this limit lines were classified as high type. Bulked segregant analysis was performed by pooling 10 of each high and low Cd lines from the $F_{2:3}$ population. The $F_{2:3}$ population was harvested individually and portion of the F_3 seeds were used for Cd analysis. The segregation pattern of the Cd concentration in $F_{2:3}$ and RIL populations was tested using χ^2 test. Genetic maps were constructed with Mapmaker 3.0 (Lander et al. 1987) using LOD = 3.0 and Kosambi's mapping function (Kosambi 1944) to convert recombination frequencies into map distances (cM). QTL analyses were conducted using WinQTLCart employing composite interval mapping (Zeng 1994; Wang et al. 2004). The genome wide LOD score threshold for QTL detection was determined using the permutation test (1,000 replications) at a P value of 0.05. According to the permutation results, the LOD score threshold was set at 3.0 for the trait to declare the presence of a QTL. The estimated percentage of variance explained by the QTL affecting the trait was obtained by the software in the CIM model. Genetic maps were generated with MapChart version 2.2 (Voorrips 2002). The microsatellite markers used in this study have been mapped (Cregan et al. 1999; Song et al. 2004) in other soybean populations, therefore, markers were anchored on the linkage group on the basis of their expected locations. Newly developed SSRs from the DNA sequence of LG-K (Gm:09) were assigned with the name S + repeat constituents + K, where S is for soybean and K is for the location, LG-K.

Candidate gene search

The soybean database SoyBase (http://soybase.org) provides access to genetic and physical maps and molecular markers, genome browser and annotation. For the genes governing low Cd concentration, the region lying between the tightly linked markers was considered for the identification of putative genes based on the predicted and known functions.

Results

A preliminary study of 32 soybean cultivars grown on three fields conducive to expressing high Cd accumulation in seed showed that 14 cultivars had an average Cd accumulation in the seed 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 suggested a major gene played a major role in controlling Cd accumulation in soybean seed and that on high Cd soils a dividing level of around 0.2 mg kg^{-1} could be used to group genotypes.

Cd concentration in parents and RILs

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 kg^{-1}) differed significantly ($P < 0.0004$, $F = 7.70$). The distribution of Cd concentration of the $F_{2:3}$ population and the $F_{6:8}$ RILs were the same with a P value < 0.0001 for both populations (Fig. 1). In the $F_{2:3}$ population, of the 138 individuals analyzed, 70 were in the low (≤0.2 mg kg^{-1}) and 68 were in the high category (≥0.21 mg kg^{-1}). The concentration of Cd in the seeds of the AC Hime × Westag-97 $F_{2:3}$ progeny varied from 0.010 mg kg^{-1} to 0.878 mg kg^{-1}, with a mean of 0.235 ± 0.022 mg kg^{-1}. The distribution suggested that Cd accumulation is controlled by a major gene with the allele for low accumulation being dominant. A similar trend was also observed for the RILs. Of the 166 RILs tested for seed cadmium concentration 87 had ≤0.2 mg kg^{-1} and 79 had ≥0.21 mg kg^{-1}. Cadmium 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}. 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 ($P = 0.534$). Transgressive segregation suggests that some

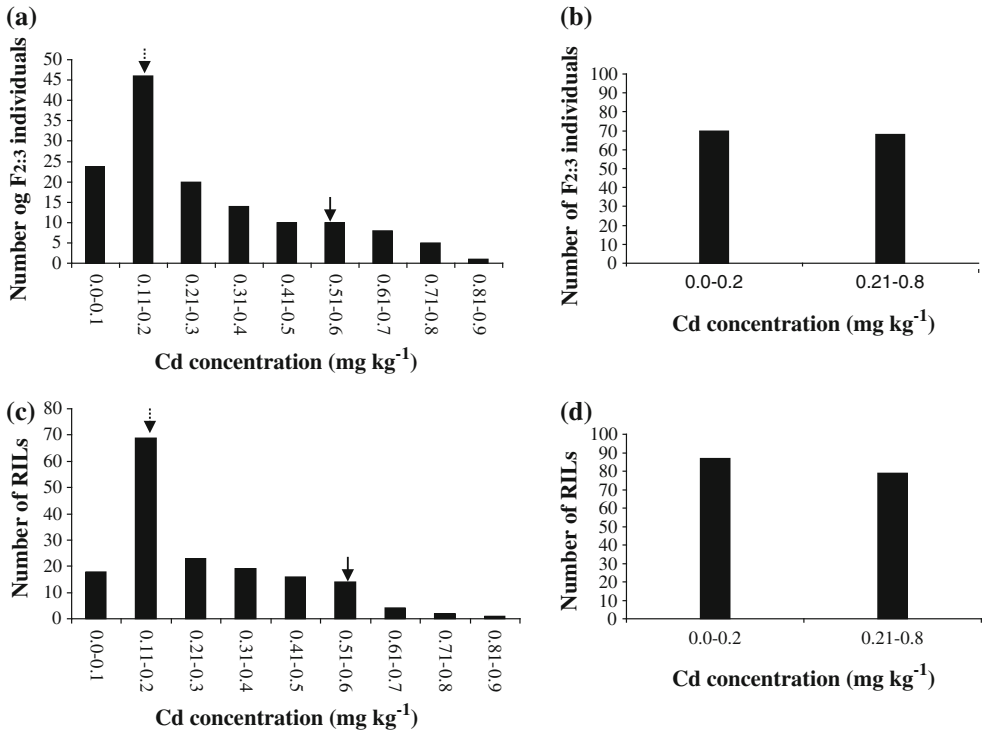

Fig. 1 Frequency distribution of seed cadmium concentration in the AC Hime × Westag-97 population. **a** Frequency distribution in the $F_{2:3}$ population in 2005. **b** *Lines* with low Cd (≤0.2 mg kg^{-1}) and high Cd concentration (≥0.2 mg kg^{-1}) in the $F_{2:3}$ population. **c** Frequency distribution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

minor genes or QTLs may be involved in influencing Cd accumulation in the AC Hime × Westag-97 population.

Mapping of locus controlling low Cd accumulation in seed

A total of 497 SSR markers covering all 20 linkage groups of soybean were tested for polymorphism between AC Hime and Westag-97. Of the 497 primers tested, 179 (36%) were found to be polymorphic between the two parents. BSA was performed in the $F_{2:3}$ individuals and it identified three primers (Satt 260, Satt 725 and Satt 559) polymorphic between the bulks. The primers which showed polymorphism between parents and bulks were screened with 138 $F_{2:3}$ individuals of AC Hime × Westag-97. In two point linkage analysis between each SSR marker and seed Cd concentration phenotype ($F_{2:3}$), three SSR markers (Satt 260, Satt 725 and Satt 559) were found to be linked. The closest marker linked to Cd accumulation was Satt 559 with a distance of 37.9 cM (LOD = 3.0).

To identify the tightly linked markers and the QTL associated with low Cd accumulation, further analyses were done using the RIL population. A total of 131 SSR primers were screened on 166 RILs. Based on the two point linkage analysis, 9 SSR markers (Satt 617, Satt 326, Satt 247 Satt 628, Satt 349, Sat_325, Satt 260, Satt 725 and Satt 559) were found to be linked with low Cd concentration (LOD = 3.0). Of these 9 markers, three (Satt 260, Satt 725 and Satt 559) were previously identified in the $F_{2:3}$ population. The closest marker to the major Cd accumulation gene (*Cda1*, proposed name) was Satt 349 with a distance of 18.1 cM. Since all the linked markers were from the same LG-K, all the available SSR markers from that LG were tested. The closest flanking markers to the *Cda1* gene were identified as Satt 178 (8.8 cM) and Satt 242 (26.5 cM). The low Cd accumulating gene *Cda1* was found to be located in the gene rich region of the LG-K where few SSR markers are mapped. Furthermore, there were no polymorphic SSR primers found between Satt 178 and Satt 242.

Targeted SSR marker development

To saturate the gap between Satt 178 and Satt 242, soybean genomic DNA sequences were searched for new SSRs (http://soybase.org/gbrowse/cgi-bin/gbrowse/gmax1.01/). Dinucleotide and trinucleotide SSRs were identified in the soybean genomic sequence from Gm09:nt. 3.3 to 6.9 Mbp flanked by the markers Satt 242 and Satt 178. A total of 48 SSR loci distributed in this region were targeted for primer design. Primer pairs were designed for sequences with AT/TA, GT/TG, and various other repeat motifs. Of the 48 primer pairs tested, only 20 (41.6%) showed polymorphism between parents AC Hime and Westag-97 and were subsequently mapped in the RILs. Two markers, SatK 1 and SatK 36, were found to be closely linked with the *Cda1* gene at distances of 3.5 and 8.0 cM, respectively. To further narrow the gap between the marker and the *Cda1* gene, the soybean genomic sequence from nt 3858897 to nt 5369175 in Gm09 (LG-K) was targeted. Fifty-seven primer pairs were designed, of which 46 primer pairs were designed for sequence with an AT/TA, and 11 primer pairs for various other repeats. Of the 57 primer pairs, 20 showed polymorphism between the parents and were mapped in the RIL population. Linkage analysis identified SatK 138, SatK 139, SatK 140, SatK 147 (0.5 cM) and SacK 149, SaatK 150, SattK 152 (0.3 cM) as tightly linked markers flanking *Cda1* in soybean.

QTL mapping of the low Cd accumulation in seed

Of the 171 markers scored in the RIL population, 161 markers formed 20 linkage groups representing the 20 known LG. On average, 8 markers were placed on each linkage group. The number of markers ranged from 2 for LG D1b, F and M to 62 on LG-K. The total map spanned 1040.6 cM with an average of 52.0 cM per linkage group excluding the 10 unlinked markers.

To identify other possible genes with major and minor effects on low cadmium accumulation, RIL data on seed Cd concentration was analyzed by WinQTL. The locations of the significant QTL with their LOD support interval are presented in Fig. 2. A major QTL located on LG-K was found to be associated with low Cd accumulation in soybean seeds. The regions on LG-K which showed significant association were flanked by SatK 113 and SatK 58 explaining 57.3% of the phenotypic variance.

Validation of linked markers in different genetic backgrounds

To test the reliability of the linked SSR markers identified using the AC Hime × Westag-97 population, a parallel RIL population (95 lines) involving Leo × Westag-97 was used. Leo and Westag-97 had seed Cd concentrations of 0.435 ± 0.046 and 0.170 ± 0.001 mg kg^{-1} respectively. Frequency distribution of seed Cd concentration in the Leo × Westag-97 RIL ($F_{6:8}$) population was similar to that of the AC Hime × Westag-97 population (Fig. 3). Of the 95 lines analyzed, 42 were in the low ($\leq$0.2 mg kg^{-1}) and 53 were in the high ($\geq$0.21 mg kg^{-1}) category. 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}. Eight SSR primer pairs (SatK 122, SatK 131, SatK 140, SatK 147, SatK 149, SaatK 150, SattK 152 and SaatK 155) were screened on the 95 RILs. Two point linkage analyses showed that all the

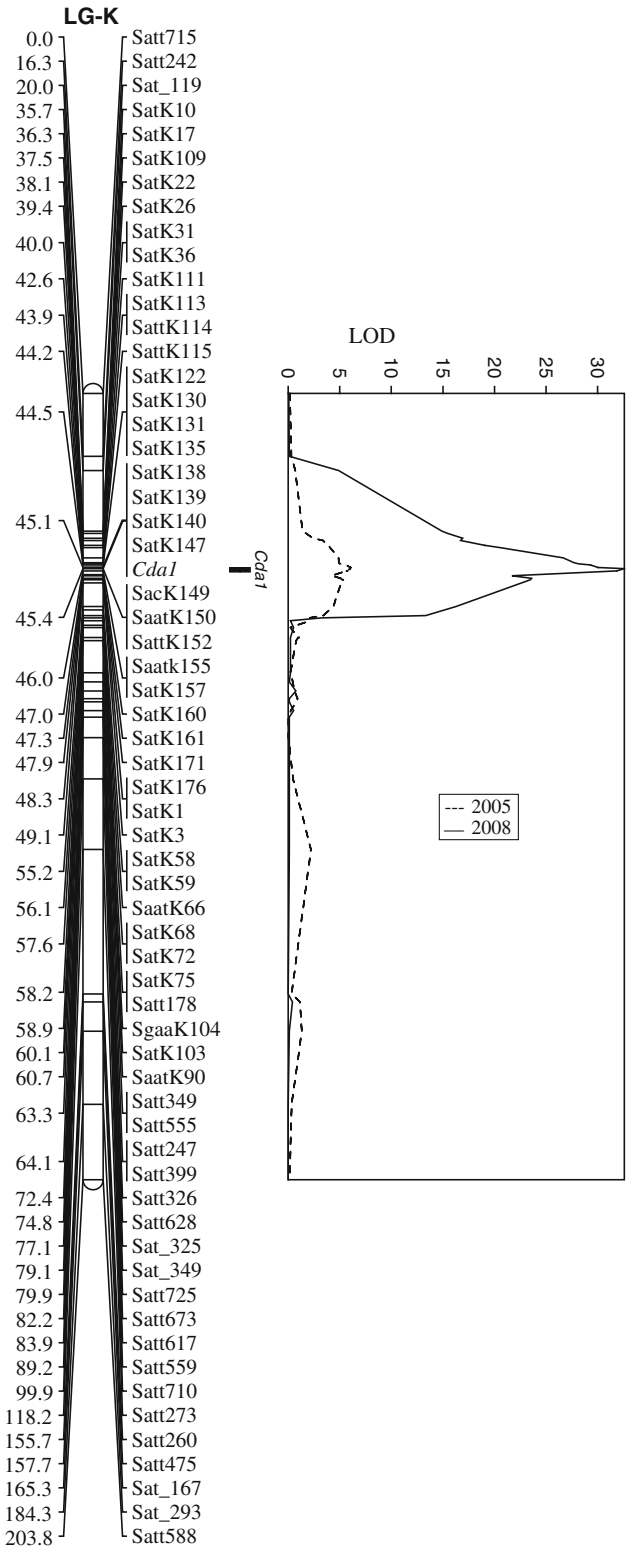

Fig. 2 Linkage group-K which corresponds to the chromosome 9 (Gm: 09) indicating the location of the newly developed SSR markers and the location of the major gene *Cda1* or QTL controlling low Cd accumulation in soybean seed. Location of the major QTL associated with low Cd accumulation mapped on the 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

markers were found to be linked to the *Cda1* gene controlling low Cd accumulation in seed (Fig. 4a). The relative position of the markers was found to be the same as was found in the AC Hime × Westag-97 population with small variation in the distances, which often occurs with different mapping populations (Cregan et al. 1999).

To validate the usefulness of the linked SSR markers in MAS, 14 diverse soybean genotypes differing in their seed Cd concentration were screened (Fig. 4b). Of the 12 primers tested, three (SatK 147, SatK 149, and SattK 152) effectively differentiated all the high and low Cd genotypes (Table 1) and could be used effectively in MAS for low Cd concentration in soybean seed.

Identification of putative genes at the *Cda1* locus

Based on the soybean genome sequence available from phytozome (http://www.phytozome.net/soybean.php) via SoyBase (http://soybase.org/gbrowse/cgi-bin/gbrowse/gmax1.01/), candidate genes located between the tightly linked flanking markers (SatK 140 and SaatK 155) were identified (Fig. 5). Based on the predicted gene model for the DNA sequence from nt.4909157 to nt.5020110, flanked by SatK 140 and SaatK 155, there were 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). Moreover, 13 soybean ESTs, including TA65152_3847 [Protein kinase, *Medicago truncatula*], AW152957 [Adagio-like protein 1 (*Oryza sativa*)] and TA47883_3847 [plasma membrane H^+-ATPase (*Sesbania rostrata*)], were also aligned to this genomic segment. Of the four SSR markers (SatK 147, SacK 149, SaatK 150 and SattK 152) found in the vicinity of the genes, SattK 152 is located in the candidate gene plasma membrane H^+-ATPase.

Discussion

In the present study, segregation of seed Cd concentration in the $F_{2:3}$ and $F_{6:8}$ RIL populations indicates that low Cd uptake is controlled by a major gene, *Cda1*. There were significant differences in the Cd concentration among the $F_{2:3}$ and the RIL populations derived from AC Hime × Westag-97. Segregation of lines in $F_{2:3}$ and RILs showed a similar trend towards the low accumulating parent indicating that *Cda1* was dominant. Transgressive segregation, however, suggests that some minor genes may also influence Cd accumulation. Similarly a dominant major gene controlling low cadmium uptake was also observed in wheat (Clarke et al. 1997) and oat (Tanhuanpää et al. 2007). There is very limited information on genetic analysis of Cd accumulation in soybean seed; results from this

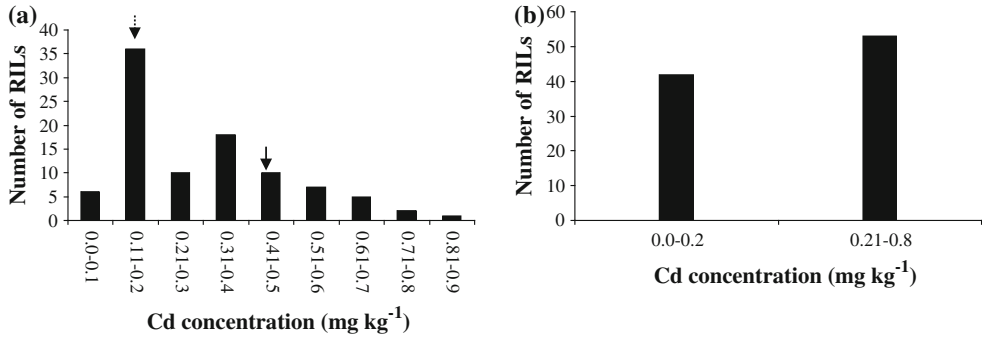

Fig. 3 **a** Frequency distribution of seed cadmium concentration in the Leo × Westag-97 $F_{6:8}$ RIL population in 2008. **b** *Lines* with low Cd (≤0.2 mg kg^{-1}) and high Cd concentration (≥0.2 mg kg^{-1}). *Arrow* indicates the level of the *parental lines*. *Solid* and *dashed* arrows indicate the Leo and the Westag-97 parent respectively

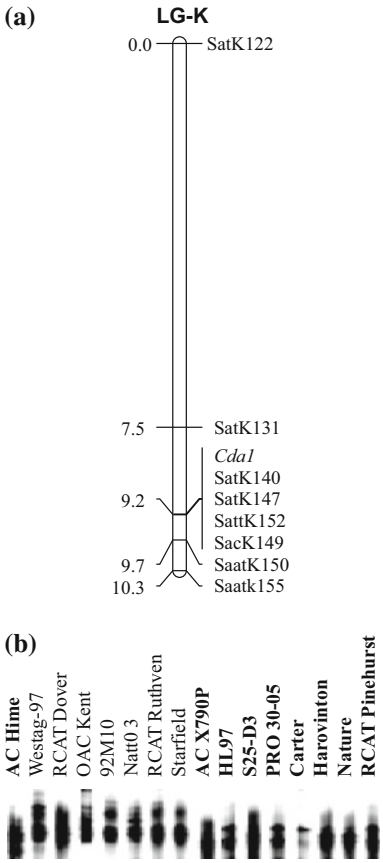

Fig. 4 Marker validation using SSR markers linked to *Cda1* controlling low Cd accumulation in soybean seeds. **a** Location of the tightly linked SSR markers in Leo × Westag-97 population. **b** Validation of tightly linked marker SattK 149 in diverse soybean genotypes differing in their seed Cd concentration. Genotypes in *bold* and *normal* indicate genotypes with high and low Cd concentration respectively in seeds

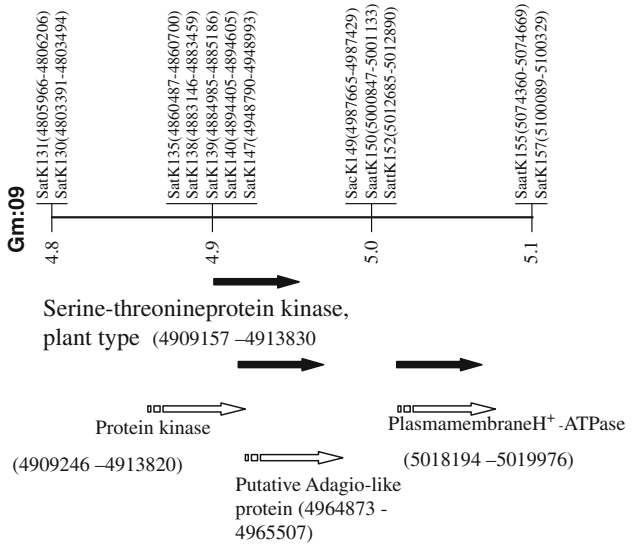

Fig. 5 Physical location of the SSR markers in Gm:09 tightly linked to *Cda1* controlling low Cd accumulation in soybean seeds. Putative genes located in the vicinity of the tightly linked markers (http://soybase.org/) based on the predicted and known gene function with EST support for soybean genomic sequences are shown

study provide a fundamental basis for future research on genetic improvement of seed Cd concentration in soybean.

One hundred and twelve new SSR primer pairs derived from the published soybean genomic sequences were designed for the sequence with microsatellite repeats, but only 40 (35.7%) of the primer pairs produced useful polymorphic markers for this population. The percentage of polymorphic SSR markers from this genomic region is comparable to previously reported SSR markers in soybean (Song et al. 2004). The soybean genome sequence information, which was made available to the public in 2008, has led to the development of targeted SSR markers to saturate a gap of more than 10 cM left from the earlier published soybean map (Cregan et al. 1999; Song et al. 2004). This has helped to identify the markers tightly linked to *Cda1*. A high proportion of SSRs appeared to be in very close proximity (0.3–0.5 cM) to the gene and indicated a moderately strong relationship between SSR density and gene density in soybean (Choi et al. 2007).

The *Cda1* gene was found to be located in the LG-K. Seven SSR markers (SatK 138, SatK 139, SatK 140, SatK 147 and SacK 149, SaatK 150, SattK 152 (0.3 cM)) were linked to *Cda1*. SSR markers associated with other elements like calcium concentration (Zhang et al. 2009), iron deficiency chlorosis (Wang et al. 2008), and manganese toxicity (Kassem et al. 2003) have also been reported in soybean. Genetic analysis of Cd accumulation has been reported in cereals. PCR based markers linked to a gene responsible for cadmium accumulation has been reported in durum wheat (Penner et al. 1995), and in oat (Tanhuanpää et al. 2007). SSR markers linked to low Cd accumulation in soybean seeds will assist in MAS to introgress the gene into different genetic backgrounds, which will reduce the cost compared to seed Cd analysis.

The transport and accumulation of minerals in seeds is a complex process involving several genes. QTL mapping has enabled the identification of genomic regions involved in seed mineral (Ca, Cu, Fe, K, Mg, Mn, P, and Zn) concentration in the model legume *Medicago truncatula* (Renuka et al. 2009). QTL mapping for Zn and Cd hyperaccumulation in the hyperaccumulator species *Thlaspi caerulescens* revealed two QTLs were responsible for Cd accumulation in roots, while one QTL was involved in Cd accumulation in shoots (Deniau et al. 2006). In *Arabidopsis halleri* sp. *halleri* three QTLs were identified for Cd tolerance and the gene for heavy metal transporting ATPase4 (HMA4) was found to be co-localized with the a major QTL whose transcript levels were shown to be higher in the roots and shoots of *A. halleri* (Courbot et al. 2007). Cd tolerance and Cd accumulation, however, were shown to be independent characters, as was the case for Zn (Macnair et al. 1999). Genotypes with the most and the least Cd tolerance as well as Cd accumulating plants were selected in the backcross population involving *A. halleri* (Bert et al. 2003). QTLs controlling the translocation of Cd from source to sink regions have been reported in rice (Ishikawa et al. 2005b; Kashiwagi et al. 2009). In the present study, treating the Cd concentration in the seed as a quantitative character has led to the identification of a major QTL located in the LG-K, the region where the *Cda1* gene was identified using linkage analysis. The height of the peak observed for the 2008 data was high with very high LOD scores (32.5). The height of peak was low for the 2005 data which could be due to: a) the difference in the population type ($F_{2:3}$ in 2005 vs. $F_{6:8}$ RIL in 2008); (b) the difference in the population size (136 in 2005 vs. 166 in 2008); (c) differences in weather and environmental factors in 2005 vs. 2008; and (d) experimental errors. QTLs with minor effects were not detected in this study, possibly due to lower number of markers present in other linkage groups. A number of factors such as genetic variation in the individuals, environmental variation, population size, number of markers and experimental error can influence the detection of QTL in a segregating population (Collard et al. 2005). In both populations, the trait enhancing allele was found to originate from the low Cd accumulating genotype, Westag-97.

For a number of metals, including zinc, copper and arsenic, genetic analysis has shown that tolerance is controlled by a small number (one or two) of major genes, with additional modifiers determining the level of tolerance displayed (Schat et al. 1993; Smith and Macnair 1998; van Hoof et al. 2001). It will be interesting to further investigate if any such mechanism operates in soybean for the low Cd accumulation, which was shown to be under the control of a major gene in this study. At the same time two or three major genes with additive effect could not be rejected as observed for the Cd tolerance in *A. halleri* (Bert et al. 2003).

The regulation of metal homeostasis is complex, requiring transport proteins located in different membranes as well as long distance transport systems to move the nutrients throughout the plant. These short and long distance systems are tightly controlled by several metal-specific and metal non-specific genes. Candidate genes related to heavy metal transport or homeostasis were located in the vicinity of the identified QTL. Protein kinase, putative Adagio-like protein and plasma membrane H^+-ATPase were found in the QTL vicinity. The environmental presence of elevated levels of heavy metal ions triggers a wide range of cellular responses including change in gene expression and synthesis of metal-detoxifying peptides. Cadmium- and copper-responsive genes have been shown to code for signal transduction components, such as Arabidopsis mitogen-activated protein kinase kinase kinase (MAPKKK) MEKK1, transcription factors, stress induced proteins, proteins participating in protein folding, and sulfur and glutathione metabolism (Xiang and Oliver 1998; Suzuki et al. 2001; Louie et al. 2003). MAPKKKs are Ser/Thr protein kinases that phosphorylate and thereby activate MAPKKS. MAPKKs in turn are dual-specific kinases that phosphorylate MAPKs on Thr and Tyr residues. The dual phosphorylation of MAPKs renders the enzyme active. MAPKs are pro-directed Ser/Thr kinases phosphorylating numerous substrates in different cellular compartments. In this way, information is transduced in the form of a phosphorylation cascade from upstream kinase to downstream targets. Cadmium ion activated distinct mitogen-activated protein kinases were observed in alfalfa seedlings (Claudia et al. 2004). Genes uniquely induced by Cd ions in Arabidopsis showed a high percent of genes with "Kinase activity" (16.7%) (Cheng-Ri et al. 2009).

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 (Cataldo et al. 1983). Astolfi et al. (2003) showed that Cd seems to have differential inhibiting effects on proton transport activity and ATPase activity in oat roots. Several lines of evidence suggest that plant plasma membrane ATPase activity is probably modulated by protein Kinases, and the ATPase probably contains multiple phosphorylation sites which may affect its activity in different ways (Serrano 1989). Presence of protein kinase, and plasma membrane H^+-ATPase genes near the tightly linked markers, suggest that regulation of this enzyme may play an important role in Cd stress. It was also suggested that Cd accumulation in grain might relate to phloem (Hart et al. 1998; Cieslieski et al. 1996; Tanaka et al. 2007) and xylem (Uraguchi et al. 2009) mediated Cd translocation. The Cd translocation pathways in soybean need to be studied in order to understand which of the pathways identified in other crops such as wheat (Hart et al. 1998; Cieslieski et al. 1996; Harris and Taylor 2001) and rice (Kashiwagi et al. 2009; Uraguchi et al. 2009) operates in the low Cd accumulating soybean cultivars. The putative heavy metal transport related genes near the tightly linked markers are good candidate genes for further investigation. They may explain the transport and accumulation of differential Cd levels in the soybean seeds.

The present study shows that low Cd accumulation in soybean seed is genetically controlled by a major gene *Cda1*, which has the potential to produce low Cd cultivars. The SSR markers linked to the *Cda1* gene in soybean will help in MAS to combine this trait with other agronomic traits. It 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 lab. Moreover, it will also reduce the time required for the identification of low Cd lines in breeding programs. The SSR markers identified in the present study have been validated using a different population and other soybean genotypes differing in their Cd accumulation. The location of these markers in the soybean genome map can be used to identify the gene(s) responsible for low accumulation, which will be useful both for developing functional markers and for characterizing the gene's role in the low Cd accumulation.

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References

Ablett GR, Stirling BT, Fischer JD (1999) Westag 97 soybean. Can J Plant Sci 79:371–372

Arao T, Ae N (2001) Screening of genotypes with low cadmium content in soybean seed and rice grains, Plant nutrition: food security and sustainability of agro-ecosystems through basic and applied research. Fourteenth International Plant Nutrition *Colloquium.* Hannover, Germany, pp 292–293

Arao T, Ishikawa S (2006) Genotypic differences in cadmium concentration and distribution of soybeans and rice. JARQ Jpn Agric Res Q 40:21–30

Arao T, Ae N, Sugiyama M, Takahashi M (2003) Genotypic differences in cadmium uptake and distribution in soybeans. Plant Soil 251:247–253

Archambault DJ, Marentes E, Buckley W, Clarke J, Taylor GJ (2001) A rapid seedling-based bioassay for identifying low cadmium-accumulating individuals of durum wheat (*Triticum turgidum* L.). Euphytica 117:175–182

Astolfi S, Zuchi S, Chiani A, Passera C (2003) In vivo and in vitro effects of cadmium on H^+ ATPase activity of plasma membrane vesicle from oat (*Avena sativa* L.) roots. J Plant Physiol 160:387–393

Bell MJ, McLaughlin MJ, Wright GC, Cruickshank A (1997) Inter- and intra-specific variation in accumulation of cadmium by peanut, soybean and navy bean. Aust J Agric Resour Econ 48:1151–1160

Benson G (1999) Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res 27:573–580

Bert V, Meerts P, Saumitou-Laprade P, Salis P, Gruber W, Verbruggen N (2003) Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. Plant Soil 249:9–18

Cataldo DA, Garland TR, Wildung RE (1983) Cadmium uptake kinetics in intact soybean plants. Plant Physiol 73:844–848

Chan DY, Hale BA (2004) Differential accumulation of Cd in durum wheat cultivars: uptake and translocation as sources of variation. J Exp Bot 55:2571–2579

Cheng-Ri Z, Takashi I, Yoshiharu S, Yuriko K, Yuji S, Takashi H, Shigeru S, Nozomu S, Daisuke S, Hiroyuki K (2009) Comparative transcriptomic characterization of aluminium, sodium chloride, cadmium and copper rhizotoxicities in *Arabidopsis thaliana*. BMC Plant Biol 9:32. doi:10.1186/1471-2229-9-32

Choi IY, Hyten DL, Matukumalli LK, Song Q, Chaky JM, Quigley CV, Chase K, Lark KG, Reiter RS, Yoon MS, Hwang EY, Yi SI, Young ND, Shoemaker RC, van Tassell CP, Spech JE, Cregan PB (2007) A soybean transcript map: gene distribution, haplotype and single-nucleotide polymorphism analysis. Genetics 176:685–696

Cieslieski G, Van Rees KCJ, Huang PM, Kozak LM, Rostad HPW, Knot DR (1996) Cadmium uptake and bioaccumulation in selected cultivars of durum wheat and flax as affected by soil type. Plant Soil 182:115–124

Clarke JM, Leisle D, Kopytko GL (1997) Inheritance of cadmium concentration in five durum wheat crosses. Crop Sci 37:1722–1726

Claudia J, Hirofumi N, Heribert H (2004) Heavy metal stress. Activation of distinct mitogen-activated protein kinase pathways by copper and cadmium. Plant Physiol 136:3276–3283

Clemens S, Palmgren MG, Kramer U (2002) A long way ahead: understanding and engineering plant metal accumulation. Trends in Plant Sci 7:309–315

Codex Alimentarius Commission. (2001) Report of the 33rd Session of the codex committee on food additives and contaminants, Hague, The Netherlands 12–16 March 2001, p 285

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica 142:169–196

Courbot M, Willems G, Motte P, Arvidsson S, Roosens N, Saumitou-Laprade P, Verbruggen N (2007) A major quantitative trait locus

for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase[1[OA]]. Plant Phys 144:1052:1065

Cregan PB, Jarvick T, Bush AL, Shoemaker RC, Lark KG, Kahler AL, Kaya N, Van Toai TT, Lohnes DG, Chung J, Specht JE (1999) An integrated genetic map of the soybean genome. Crop Sci 39:1464–1490

Cupit M, Larsson O, de Meeus C, Eduljee GH, Hutton M (2002) Assessment and management of risks arising from exposure to cadmium in fertilisers: II. Sci Total Environ 291:189–206

Deniau AX, Pieper B, Ten Bookum WM, Lindhout P, Aarts MGM, Schat H (2006) QTL analysis of cadmium and zinc accumulation in the heavy metal hyperaccumulator *Thlaspi caerulescens*. Theor Appl Genet 113:907–920

Florijn PJ, Van Beusichem ML (1993) Uptake and distribution of cadmium in maize inbred lines. Plant Soil 150:25–32

Gawalko EJ, Nowicki TW, Babb J, Tkachuk R (1997) Comparison of closed vessel and focused open-vessel microwave dissolution of cadmium, copper, lead and selenium in wheat, wheat products, corn bran and rice flour by transverse-heated graphite furnace atomic absorption spectrometry. AOAC Int 80:379–387

Gawalko EJ, Garrett RG, Warkentin T, Wang N, Richter A (2009) Trace elements in Canadian field peas: a grain safety assurance perspective. Food Add Contam 26:1002–1012

Grant CA, Clarke JM, Duguid S, Chaney RL (2008) Selection and breeding of plant cultivars to minimize cadmium accumulation. Sci Total Environ 390:301–310

Greger M, Löfstedt M (2004) Comparison of uptake and distribution of cadmium in different cultivars of bread and durum wheat. Crop Sci 44:501–507

Harris NS, Taylor GJ (2001) Remobilization of cadmium in maturing shoots of near isogenic lines of durum wheat that differ in grain cadmium accumulation. J Exp Bot 52:1473–1481

Hart JJ, Welch RM, Norvell WA, Sullivan LA, Kochian LV (1998) Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. Plant Physiol 116:1413–1420

Hwang T-Y, Sayama T, Takahashi M, Takada Y, Nakamoto Y, Funatsuki H, Hisano H, Sasamoto S, Sato S, Tabata S, Kono I, Hoshi M, Hanawa M, Yano C, Xia Z, Harada K, Kitamura K, Ishimoto M (2009) High-density integrated linkage map based on SSR markers in soybean. DNA Res 16:213–225

Ishikawa S, Ae N, Sugiyama M, Murakami M, Arao T (2005a) Genotypic variation in shoot cadmium concentration in rice and soybean in soils with different levels of cadmium contamination. Soil Sci Plant Nutr 51:101–108

Ishikawa S, Ae N, Yano M (2005b) Chromosomal regions with quantitative trait loci controlling cadmium concentration in brown rice (*Oryza sativa*). New Phytol 168:345–350

Kashiwagi T, Shindoh K, Hirotsu N, Ishimaru K (2009) Evidence for separate pathways in determining cadmium accumulation in grain and aerial plant parts in rice. BMC Plant Biol 9:8. doi: 10.1186/147-2229-9-8

Kassem MA, Meksem K, Kang CH, Njiti VN, Kilo V, Wood AJ, Lightfoot DA (2003) Loci underlying resistance to manganese toxicity mapped in a soybean recombinant inbred line population of essex × forrest. Plant Soil 0:1–8

Kazantzis G (2004) Cadmium, osteoporosis and calcium metabolism. Biometals 17:493–498

Knox RE, Pozniak CJ, Clarke FR, Clarke JM, Houshmand S, Singh AK (2009) Chromosomal location of the cadmium uptake gene (*Cdu*1) in durum wheat. Genome 52:741–747

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER, an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Liu JG, Qian M, Cai GL, Yang JC, Zhu QS (2007) Uptake and translocation of Cd in different rice cultivars and the relation with Cd accumulation in rice grain. J Hazard Mater 143:443–447

Louie M, Kondor N, Dewitt JG (2003) Gene expression in cadmium tolerant *Datura inoxia*: detection and characterization of cDNAs induced in response to Cd^{2+}. Plant Mol Biol 52:81–89

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D (1999) Zinc tolerance and hyperaccumulation are genetically independent characters. Proc R Soc Lond B 266:2175–2179

McGrath SP, Zhao FJ, Lombi E (2001) Plant and rhizosphere processes involved in phytoremidiation of metal-contaminated soils. Plant Soil 232:207–214

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Morrison MJ (2005) Cadmium concentration in Canadian soybean, The Pulse Beat. Manitoba Pulse Growers Assoc 47:33–34

Penner GA, Clarke J, Bezte LJ, Leisle D (1995) Identification of RAPD markers linked to a gene governing cadmium uptake in durum wheat. Genome 38:543–547

Poysa V, Buzzell RI (2001) AC Hime soybean. Can J Plant Sci 81:443–444

Poysa V, Woodrow L, Yu K (2005) Leo soybean. Can J Plant Sci 85:389–391

Reddy MR, Dunn SJ (1986) Heavy metal absorption by soybean on sewage sludge treated soil. J Agric Food Chem 34:750–753

Renuka PS, Thierry H, Michael AG (2009) Identification of QTL affecting seed mineral concentrations and content in the model legume *Medicago truncatula*. Theor Appl Genet 119:241–253

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, NJ, pp 365–386

Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanism of cadmium mobility and accumulation in Indian mustard. Plant Physiol 109:1427–1433

Schat H, Kuiper E, Ten Bookum WM, Vooijs R (1993) A general model for the genetic control of copper tolerance in Silenne vulgaris: evidence from cross between plants from different tolerant populations. Heredity 70:142–147

Serrano R (1989) Structure and function of plasma membrane ATPase. Plant Mol Biol 40:61–94

Smith SE, Macnair MR (1998) Hypostatic modifiers cause variation in degree of copper tolerance in *Mimulus guttatus*. Heredity 80:760–768

Song QJ, Marek LF, Shoemaker RC, Lark KG, Concibido VC, Delannay X, Specht JE, Cregan PB (2004) A new integrated genetic linkage map of the soybean. Theor Appl Genet 109:122–128

Stolt P, Asp H, Hultin S (2006) Genetic variation in wheat cadmium accumulation on soils with different cadmium concentrations. J Agron Crop Sci 192:201–208

Suzuki N, Koizumi N, Sano H (2001) Screening of cadmium-responsive genes in *Arabidopsis thaliana* reveals protein denaturation and oxidative stresses to be critical components of cadmium toxicity. Plant Cell Environ 24:1177–1188

Tanaka K, Fujimaki S, Fujiwara T, Yoneyama T, Hayashi H (2007) Quantitative estimation of the contribution of the phloem in cadmium transport to grains in rice plants (*Oyza sativa* L.). Soil Sci Plant Nutr 53:72–77

Tanhuanpää P, Kalendar R, Schulman AH, Kiviharju E (2007) a major gene for grain cadmium accumulation in oat (*Avena sativa* L.). Genome 50:588–594

Uraguchi S, Mori S, Kuramata M, Kawasaki A, Aroa T, Ishikawa S (2009) Root-to-shoot translocation via the xylem is the major process determining shoot and grain cadmium accumulation in rice. J Exp Bot 60:2677–2688

Van Hoof NALM, Hassinen VH, Hakvoort HW, Ballintijn KF, Schat H, Verkleji JAC, Ernst WHO, Karenlampi SO, Terverhanta AI (2001) Enhanced copper tolerance in Silene vulgaris (Moench) Garcke populations from copper mine is associated with increased transcript levels of 2b-type metallothionein gene. Plant Physiol 126:1519–1526

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTL. J Hered 93:77–78

Wang S, Basten CJ, Zeng Z-B (2004) *Windows QTL Cartographer 2.0*. Department of Statistics, North Carolina State University, Raleigh, NC. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

Wang J, McClean PE, Lee R, Goos RJ, Helms T (2008) Association mapping of iron chlorosis loci in soybean (*Glycine max* L. Merr.) advanced breeding lines. Theor Appl Genet 116:777–787

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis. Plant Cell 10:1539–1550

Zeng Z-B (1994) Precision mapping of quantitative trait loci. Genetics 136:1457–1468

Zhang B, Chen P, Shi A, Hou A, Ishibashi T, Wang D (2009) Putative quantitative trait loci associated with calcium content in soybean seed. J Hered 100:263–269