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SCREENING SOYBEAN GERMPLASM FOR PRESENCE OF *Cda1* ALLELE INVOLVED IN LOW CADMIUM ACCUMULATION USING MOLECULAR MARKERS

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ABSTRACT

Soybean is an important source of plant protein used in human diets. Cadmium (Cd) from agricultural soil is a toxic metal for plants, including soybean. Furthermore, the cadmium is taken up to seeds of soybean and becomes a risk for animals and human by feed and food chain. A method to avoid this risk is represented by the selection and breeding soybean lines/cultivars with genetic potential to minimized cadmium accumulation. This work reports an approach assisted by DNA-markers for selection of soybean cultivars with low Cd-accumulation. In this study, we screened the alleles of *Cda1* gene, in 22 soybean cultivars, using three DNA markers (SSR-Sack149, Gm-dCAPS-HMA1 and Cda1-KASP). The results showed that 11 genotypes carried the allele for low cadmium accumulation, 8 genotypes had the allele for high cadmium accumulation and three genotypes were heterozygous/heterogenous. All three markers classified the soybean genotypes in the same manner but KASP marker is more efficient in marker-assisted selection/breeding for *Cda1*. This study offers valuable information to breeders and other researches regarding the selection of soybean germplasm with low cadmium accumulation in the pursuit of reducing the Cd-accumulation and assuring the food safety worldwide.

Keywords: soybean, Cd accumulation, *Cda1*, P_{1B}-ATPase, KASP.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is a source of plant protein and an important crop worldwide, originating in the northeast of China. The area cultivated with soybeans in the European Union decreased by 0.8% in 2021, compared to the previous year. Also, in Romania the area cultivated with soybean decreased in 2021, reaching at 139,6 thousand hectares, with 29,3 thousand hectares less than 2020 but the average yield was 2489 kg/ha with 582 kg/ha more than average yield in 2020. Romania ranked third in the area cultivated with soybeans, after Italy and France (https://insse.ro/..... productia_vegetala_la_ principalele_culturi_in_anul_2021_0.pdf). Worldwide, this crop is cultivated on 644,323 thousand hectares dedicated to organic product (Dorđević et al., 2019).

Soil pollution with heavy metals due to anthropogenic activities (mining, refining, fertilizers, etc.), the use of industrial wastewater for irrigation, or natural sources such as volcanic activity and weathering of rocks represents a serious concern of environmental protection and for food security (Bardáčová et al., 2017;).

Among the heavy metals that pollute the soil, cadmium (Cd) has become of the most toxic metal for plants and animals. Cadmium binds particularly to the liver and kidney, causing organ failure and cancer, and its toxicity represents one of the most discussed safety issues in many European countries (Huff et al., 2007; Djordjevic et al., 2019).

In 2022, Abdelwaheb et al., assessed the adsorption of copper and cadmium in two agricultural soils from Tunisia and Romania to evaluate the risk of water pollution and in their study the elemental analysis performed with SEM (scanning electron microscope) showed a very low amount of cadmium and copper in both soils (0.01%) before adsorption, but after that, the Romanian soil retained both pollutants more than the

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Tunisian soil. Also, the study showed that the retention mechanism of copper and cadmium was mainly related to the surface charge (electrostatic interaction) and the presence of phosphorus, sulfur, chloride and carbon in the soil (precipitation and complexation reaction). Cd is taken up in plants by roots and can be translocated into aerial organs, where it affects photosystems I and II and can cause severe physiological and morphological damages to plants, reduced water and nutrient uptake, affect enzyme activities (Smeets et al., 2008), alter membrane permeability, disrupt cell transport processes (Arao et al., 2003; Jegadeesan et al., 2016), and in special for soybean, it affects nodulation and N2 fixation (Sheirdil et al., 2012).

Cd concentration in soybean seeds was found to be higher in the experiment of comparing the Cd enrichment factor among wheat, rice, soybean, and corn when Cd concentration with 0.5 mg kg^{-1} or 1.5 mg kg^{-1} was added to soil (Wang and Hu, 1998). In other study, the Cd concentration in seeds of 20 soybean cultivars varied between 0.23 mg kg^{-1} to 2.33 mg kg^{-1} when Cd concentration in soil was 1.98 mg kg^{-1} (Siqi et al., 2017). Therefore, the uptake of cadmium by plants varies not only among plant species but also among cultivars.

Low Cd accumulation in soybean seed is under the control of a major gene (*Cda1*) with the allele for low accumulation being dominant, that was mapped on soybean molecular linkage group K using simple sequence repeat (SSR) markers (Jegadeesan et al., 2010). Vollmann et al. (2015), validated the low seed Cd accumulation trait based on the *Cda1* locus and the associated Sack149 marker. Benitez et al. (2010) analyzed the recombinant inbred line population identified a major QTL controlling Cd concentration (cd1) at the same genomic region as mentioned above. So, these results suggests that the two loci reported may be the same. Later, Benitez et al. (2012), described a candidate gene for the cd1 locus, a P_{1B}-ATP-ase, involve in metal ion transport across cellular membranes and a single-base substitution (SNP - Single Nucleotide Polymorphisms) led to an amino acid substitution from E to G at amino acid position 608 that make the difference between low and high Cd concentration. This researchers team developed a derived cleaved amplified polymorphic sequence (dCAPS) marker to detect the base substitution, and this dCAPS marker was successfully associated with seed Cd concentration. Recently, Nissan et al. (2022), developed a KASP (Kompetitive Allele Specific PCR) marker, based on the SNP sequence, for efficient marker-assisted breeding for *Cda1*. Also, in the same study, a novel minor QTL was identified on chromosome 13 in the X4050 population between SSR markers Satt522 and Satt218.

The soybean genome has 20 HMA (metal ATPase) family members, presented as 10 paralogous pairs, which is significantly more than in Arabidopsis and rice (Fang et al., 2016).

Based on COMMISSION REGULATION (EU) 2021/1323 of 10 August 2021 amending Regulation (EC) No 1881/2006 as regards to the maximum levels of cadmium in certain foodstuffs, for soybeans the maximum level accepted is 0,20 mg/kg wet weight.

The goal of the present study was the marker-assisted selection of soybean genotypes for low cadmium accumulation that could contribute in the pursuit of reducing the cadmium accumulation in soybean and assure the food safety.

MATERIAL AND METHODS

The plant material consisted of 22 soybean cultivars, from different group of maturity, grown at NARDI Fundulea, Romania (Table 1).

Genomic DNA was isolated from two-three seeds (according to the dimensions of seeds), using SDS1 method by Cristina et al. (2017) with slight changes (SDS -2,5% instead of 1,5%).

DNA amplification

Amplification with SSR marker Sack149 was carried out with DreamTaq Green DNA Polymerase (Thermo Scientific) in a 15µl final volume reaction, containing 1x buffer mix, 0,3 µM each primer and about 90-100 ng

DNA template. Reactions were performed in an ABI ProFlex™ 3 × 32-well PCR System with the following amplification programme: initial denaturation at 95°C for 3 min, 35 cycles (95°C for 30 s, 57°C annealing temperature for 30 s, 72°C for 30 s) and 10 min final extension.

Gel electrophoresis for the separation of the amplicons was carried out with 3% "high resolution" agarose (CleverGEL-Clever Scientific), stained with ethidium bromide and visualized on UV light with Uvidoc HD6 system (Uvitec).

In case of the *GmHMA1/ P_{1B}-ATPase* gene, the amplification with the dCAPS marker was performed similar to the Sack149 reaction, except the annealing temperature - 53°C and the number of cycles (38). After the amplification, the PCR product (10 µl) was cleaved with *Bmr*I (NEB) restriction enzyme (3U), at 37°C for 2,5 h. Gel electrophoresis for the separation of digested PCR products was carried out in a 2,5% "routine use" agarose gel (CleverGEL-Clever Scientific).

KASP assay was performed in 10.2 µl mixtures containing 2 µL DNA (30 ng/µl), 5 µl of 1x KASP-TF V4.0 2X Master Mix (KBS-1050-012; LGC-Biosearch Technologies), 0.2 µL of primer mixture, and 3 µl ddH_2O.

KASP assay was carried out in an Eppendorf Mastercycler ep Gradient S with the following amplification programme: denaturation at 95°C for 15 min, followed by 10 touchdown cycles (95°C for 20 s; 61-55°C for 60 s, decreasing by 0.6°C per cycle) and 28+10 additional cycles (95°C for 20 s; 55°C for 60 s). The plate fluorescent readings were performed in a FLUOstar Omega Microplate Reader (BMG LABTECH). The genotyping data analysis and reporting was processed with KlusterCaller software (LGC, Biosearch Technologies).

The KASP primers (development by Nissan et al., 2022) carry a standard FAM tail (5′-GAAGGTGACCAAGTTCATGCT-3′) and HEX tail (5′-GAAGGTCGGAGTCAACG GATT-3′) with different fluorescence signals.

RESULTS AND DISCUSSION

Markers used in this study were all polymorphic and clearly differentiated the genetic variants present in the analyzed soybean germplasm either low or high Cd accumulation, as showed in Table 1. The classification of the genotypes was identical for each marker analyzed.

The SSR marker (SacK149) had previously been identified through a QTL analysis as described by Jegadeesan et al. (2010) and used in other studies (Vollmann and Losak, 2016; Bardáčová et al., 2017). Similar to these researchers, using SSR-Sack149 we observed two alleles, one for high Cd uptake ~225bp, and one for low Cd uptake ~237bp (Figure 1).

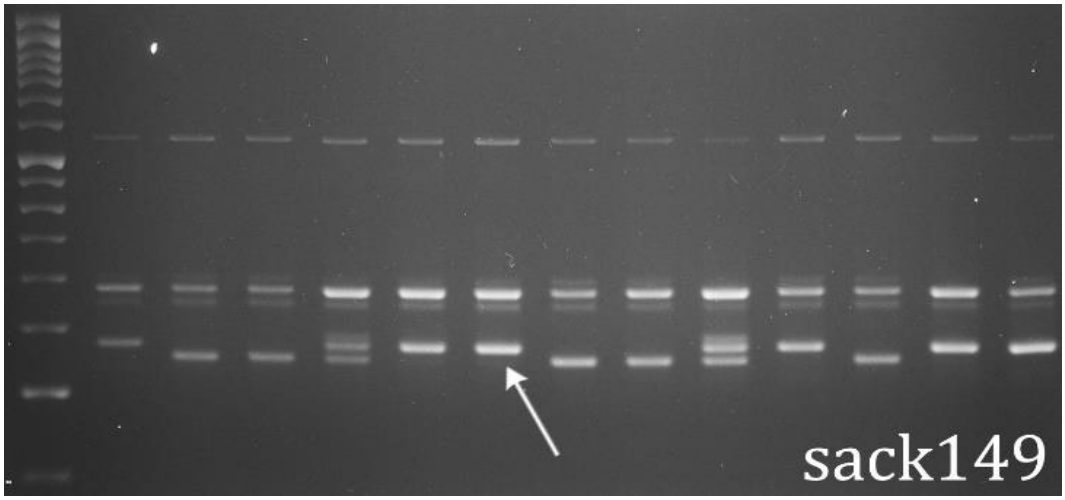

Figure 1. Electrophoretic profile obtained with SSR-Sack149. The arrow denotes PCR product for the "low Cd accumulation" allele. M-50 bp DNA Ladder. 1. Ovidiu F; 2. Camelia F; 3. Miruna TD; 4. Daciana; 5. Columna; 6. S7-F14-997; 7. Crina F; 8. Oana F; 9. Fabiana F; 10. Steara; 11. Eider; 12. S9-F08-1674; 13. Larisa

The second marker used in this study, Gm-dCAPS-HMA1, produced a ~145bp PCR product and after *Bmr*I digestion, two fragments of 25bp + 120bp resulted in case of the genotypes that belong to low Cd-accumulation type (Benitez et al., 2012).

In our screening, results obtained with this marker, were identical to the ones obtained with the SSR marker Sack149, respectively, all the genotypes with the digested PCR product being classified into the low Cd-accumulation type group.

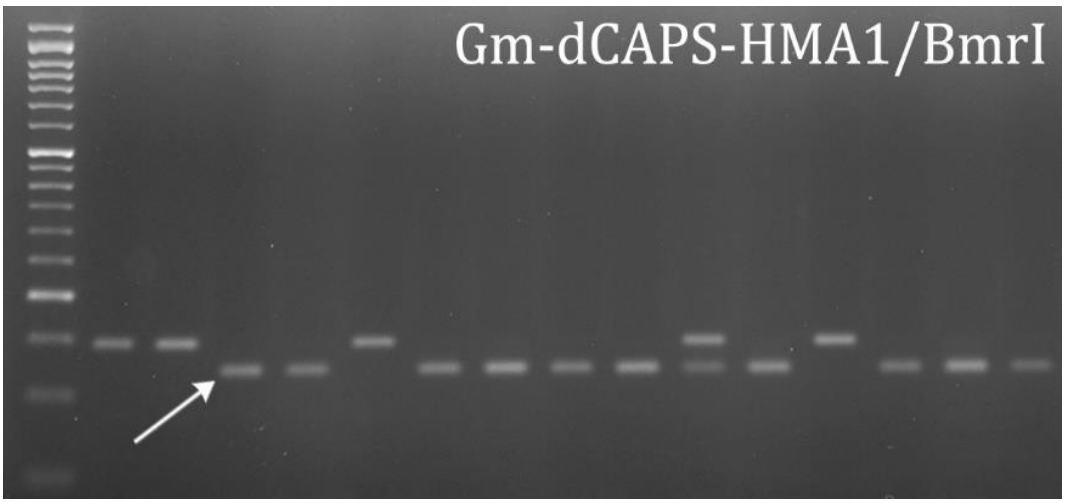

Figure 2. Electrophoretic profile obtained with Gm-CAPS-HMA1 (2.5% agarose gel).
The arrow denotes PCR product for allele "low Cd accumulation". M-50 bp DNA Ladder. 1. Advisor; 2. Lenka; 3. Columna; 4. S7-F14-997; 5. Crina F; 6. Christine; 7. Larisa; 8. Teo TD; 9. Darina TD; 10. Neve; 11. Maximus; 12. GL Melanie; 13. Steara; 14. Ovidiu F; 15. S9-F08-1674

Another approach for the screening of soybean germplasm, regarding the classification into low or high Cd-accumulation, was the KASP assay with a marker developed by Nissan et al. (2022).

The results obtained with this technique are presented in Figure 3 and Table 1. The genotypes were clearly separated and classified into low Cd (G allele) and high Cd (A allele). Results were identical with the other markers analyzed in this study in terms of the classification of the genotypes into low or high Cd accumulation. Nevertheless, KASP marker is a faster and more robust alternative to classical methods that involve more steps (digestion with restriction enzymes and/or gels for the separation of PCR fragments).

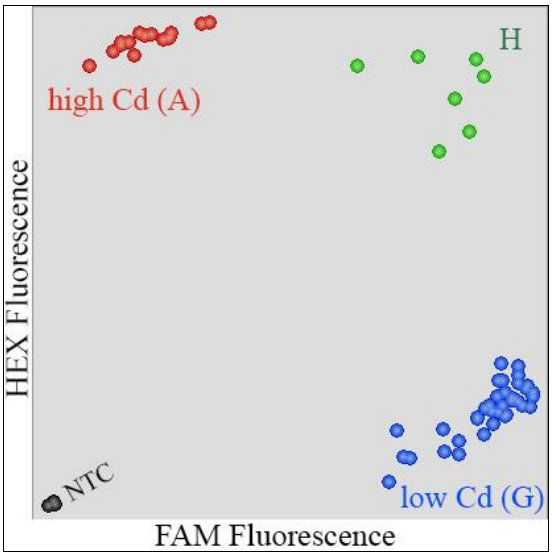

Figure 3. KASP assay for *Cda1* alleles differentiation

The results obtained in this study showed that there are 11 genotypes classified by molecular markers assays with low cadmium accumulation, 8 genotypes with high cadmium uptake and three genotypes heterozygous/heterogenous. All three markers can be utilized in marker assisted selection (MAS) for developing food grade soybean cultivars which is particularly important for environments with unknown cadmium status of soil. Therefore, DNA marker-assisted selection may be an effective method to incorporate this trait into breeding lines/cultivars of soybean with excellent agronomic traits.

Table 1. Molecular markers result for Cda1 gene screening

No.	Genotypes	Cadmium tolerance			
		SacK149	GmHMA1	KASP (G/A)	
1	Ovidiu F	low	low	G	low
2	Camelia F	high	high	A	high
3	Miruna TD	high	high	A	high
4	Daciana	H	H	Het	H
5	Columna	low	low	G	low
6	S7-F14-997	low	low	G	low
7	Crina F	high	high	A	high
8	Oana F	high	high	A	high
9	Fabiana F	H	H	Het	H
10	Steara	low	low	G	low
11	Eider	high	high	A	high
12	S9-F08-1674	low	low	G	low
13	Larisa	low	low	G	low
14	Teo TD	low	low	G	low
15	Darina TD	low	low	G	low
16	Neve	H	H	Het	H
17	Maximus	low	low	G	low
18	Advisor	high	high	A	high
19	GL Melanie	high	high	A	high
20	Lenka	high	high	A	high
21	Christine	low	low	G	low
22	Mercury	low	low	G	low

Characterization of soybean germplasm in terms of genetic variability at *Cda1* locus, involved in cadmium accumulation, gives breeders the opportunity to better choose the cultivars that guarantee food safety. Our screening based on molecular markers lay the groundwork for future research on seed Cd accumulation in soybean, but, for a better selection and validation of these results, it is necessary to study the cadmium uptake in these cultivars. On the other hand, the genotypes with high Cd accumulation could be used in studies for soil remediation.

CONCLUSIONS

Molecular markers assay showed that there are 11 soybean cultivars with low cadmium accumulation allelic variant in the studied soybean germplasm.

This study highlights the value of KASP assay for a faster selection of soybean cultivars with low cadmium accumulation, selection that aims to minimize the transfer of cadmium from soil *via* soybean seeds into the human food chain, and thus fulfilling international trade requirements.

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