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Original Paper

Variabily of *Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L. var. Plainsman After Cd and Si Treatment Detected by CDDP and PBA Marker Techniques

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During evolution, plants have developed complex mechanisms to cope with biotic and abiotic stresses. The study of heavy metal-plant interactions, particularly at the molecular genetic level, helps to understand heavy metal accumulation in plants and their resistance to heavy metal-induced stress. *Amaranthus* spp. have a potential in phytoremediation techniques. In this study was analysed the effects of cadmium (Cd) treatment and Cd treatment with addition of silicon (Si) on *Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L. var. plainsman, in leaves, in the context of the molecular genomic response using two different DNA marker techniques such as cytochrome P450 mono-oxygenase analogues (PBA) and conserved DNA-derived polymorphism (CDDP) for WRKY genes. The PBA marker technique consists of several primers pairs that have been designed for cytochrome P450 genes, and CDDP primers pairs have been designed on conserved regions of DNA. Both marker techniques have been shown to be able to detect the response to biotic and abiotic stresses. We detected higher variability in genomic profile with the CDDP marker systems compared to PBA. However, the highest number of loci (169) we detected using the BF (forward primer) + BR (reverse primer) of PBA.

Keywords: CDDP, PBA, heavy metals, phytoremediation, abiotic stress

1 Introduction

The concentration of Cd in soil varies greatly depending on the sources of contamination, which include natural processes and anthropogenic activities such as industrial emissions, urbanisation and the use of fertilisers in agriculture (Khan et al., 2017). Cd toxicity in plants occurs at several levels from morphological, physiological to molecular level (Baruah et al., 2023). Increase reactive oxygen species (ROS) production (Huang et al., 2017), also prevent the uptake of another important substance and has an impact on the growth (Srivastava and Srivastava, 2024). There are a several remediation methods that reduce Cd toxicity in soil (Zulfiqar et al., 2021). Phytoremediation, a plant-based technology, relies on the ability of plants to accumulate metals in different parts of the plant. *Amaranthus* spp., a well-studied plant, shows potential for metal accumulation. In the study Tőzsér et al. (2023), a meta-analysis was performed to investigate the accumulation capacity of heavy metals of different *Amaranthus* plant parts (root, stem, and leaf). The response of *Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L. var. plainsman to stress caused by different Cd concentrations has already been studied and changes in the expression of some genes which may be related to the ability to respond to this stress, have been detected (Lancíková et al., 2020). In order for phytoremedition to be most effective, there are ways to helps become more efficient and protect them from Cd toxicity. Si treatment improved *Oryza sativa* L., growth and stress tolerance by reducing Cd toxicity, improving root function, reducing metal uptake, modulating stress-related phytohormones, and regulating heavy metal and Si transporter gene expression (Kim et al., 2014).By identifying and studying the genetic diversity of plants,

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we are able to understand the molecular basis of various biological phenomena.

DNA markers are increasingly being used in basic genomic studies and applied plant breeding. The rationale for choosing a particular technique depends on the type of plant being studied, the objective of the research, and the availability of the necessary resources (Amiteye, 2021). Yamanaka et al. (2003) present a new PBA marker technique consisting of multiple primer sets designed for cytochrome P450 genes (CYPs) in the plant kingdom for the assessment of genetic diversity.

Variability is given by the random distribution of cytochrome P450 regions amplified by universal primers that targeting CYP or heme binding sites in plants (Amiteye, 2021).

Next-generation sequencing has revealed insights that genes for CYPs are affected by abiotic stress (Pandian et al., 2020). The next DNA marker technique introduced by Collard and Mackill in 2009, consist of a set of multiple primers designed for well-studied genes that are also known to affected by abiotic and biotic stress. CDDP primers have been designed on conserved regions of DNA and among the genes they have been designed on are genes for WRKY (Xie et al., 2005), a proto-oncogenic protein (MYB) (Jiang et al., 2004), auxin-binding protein (ABP1), MADS (Lim et al., 2000), homeobox genes that function as transcription factors with a unique homeodomain (KNOX) (Nagasaki et al., 2001), and ERFs (Gutterson & Reuber, 2004).

2 Material and Methods

2.1 Plant Material

Amaranthus hypochondriacus L. × *Amaranthus hybridus* L. var. plainsman was used in our study. Experiments on plant growth in both soil (AGRO gardening substrate with active humus) and hydroponic systems were carried out in a controlled environment inside a plant growth chamber, set to 23 °C with a 16-hour light and 8-hour dark cycle, and 50% humidity. In the hydroponic experiments, amaranth plants were initially grown in soil until they reached the 4–5 leaf stage. At this point, they were transferred to Hoagland hydroponic nutrient solution (Arnon & Hoagland, 1950) for 7 days acclimatisation period. After acclimatisation, the plants were exposed to Cd ($CdCl_2$) and Si (Na_2SiO_3) treatment for 14 days, giving in a total of 21 days in the hydroponic system. Only leaves were used for analysis. The following treatments were used for the analysis with the following concetration: 5 mg.L^{-1} Si, 10 mg.L^{-1} Si, 15 mg.L^{-1} Cd, 15 mg.L^{-1} Cd + 5 mg.L^{-1} Si and 15 mg.L^{-1} Cd + 10 mg.L^{-1} Si. Three biological replicates of each treatment were used in the analysis. The concentration 15 mg.L^{-1} is the highest concentration which did not cause damage in the plants. In the study Lancíková et al. (2020) tested also another concentrations. The highest concentrations (30 mg.L^{-1} and more) cause lethal effect. The plants treated with 20 mg.L^{-1} showed sign of damage.

2.2 DNA Preparation

EliGene® Plant DNA Isolation Kit (Elisabeth Pharmacon™) was used for isolation of DNA. After DNA extraction, the concentration and purity of the DNA was measured by NanoPhotometerTM spectrophotometer (Implen 360). The concentration of DNA in each sample was normalised to the same value 20 ng of total DNA in each sample prior to analysis. To verify the functionality of the isolated DNA, we focused on the internal transcribe sequence (ITS) by using following ITS primers: ITS 1 with the nucleotide sequence 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS 4 5'-TCC TCC GCT TAT TGA TAT CC-3' (White et al., 1990). For a total reaction volume of 10 µl, we used 5 µl of 2× Elizyme HS Robust Mix, 400 nM of both forward and reverse primer, 3.2 µl of nuclease free water and 20 ng of DNA.

2.3 PCR Conditions

For PBA analysis, 2× Elizyme HS Robust Mix was used. We used for one reaction of 5 µl 2× Elizyme HS Robust Mix, 1,000 nM of each forward and reverse primer and 3 µl of DNA (20 ng.µl^{-1}) was added to the reaction in total volume 10 µl. The thermal cycle consisted of the following steps: initial denaturation at 95 °C for 5 minutes, than 45 cycles of denaturation at 95 °C for 1 minute, hybridization of primers was optimal at 50 °C for 1 minute, elongation at 72 °C for 2 minutes,and final extension at 72 °C for 10 minutes.

For CDDP analysis, a 5 µl mastermix of 2× Elizyme HS Robust Mix was also used for a single reaction in a final volume of 10 µl, 1,000 nM of each forward and reverse primer, 2 µl of nuclease-free water, and 1 µl of DNA (20 ng. µl^{-1}). The thermal cycle consisted of the following steps: initial denaturation at 95 °C for 5 minutes, than 35 cycles of denaturation at 95 °C for 45 seconds, hybridization of primers at 54 °C for 45 seconds, extension at 72 °C for 1 minute and 30 seconds, and final extension at 72 °C for 10 minutes.

PCR reactions were performed using TProfessional Basic gradient XL (Biometra). After PCR amplification, samples were loaded on an agarose gel (2%) and after 3 hours the gel was visualised using a camera (OLYMPUS 7070). Binary matrices were generated from the electrophoreogram using the GelAnalyzer tool (GelAnalyzer 23.1.1).

Table 1 Nucleotide sequences of primers which were used in DNA marker techniques

Name of primer	Nucleotic sequence of primers	References
PBA		
CYP1A1F (AF)	5´-GCC AAG CTT TCT AAC AAT GC-3´	Amiteye, 2021
CYP1A1R (AR)	5´-AAG GAC ATG CTC TGA CCA TT-3´	
CYP2B6F (BF)	5´-GAC TCT TGC TAC TCC TGG TT-3´	
CYP2B6R (BR)	5´-CGA ATA CAG AGC TGA TGA GT-3´	
CYP2C19F (CF)	5´-TCC TTG TGC TCT GTC TCT CA-3´	
CYP2C19R (CR)	5´-CCA TCG ATT CTT GGT GTT CT-3´	
CDDP		
FORWARD1	5´-TGG CGS AAG TAG GGC CAG-3´	Xie et al., 2005
REVERSE 1	5´-GTG GTT GTG CTT GCC-3´	
REVERSE 2	5´-GCC CTC GTA SGT SGT-3´	
REVERSE 2B	5´-TGS TGS ATG CTC CCG-3´	
REVERSE 3	5´-GCA SGT GTG CTC GCC-3´	
R REVERSE B	5´-CCG CTC GTG TGS ACG-3´	

AF, BF, CF – forward primers, AR, BR

2.4 Bioinformatic analysis

For bioinformatic analysis, an online program Marker Efficiency Calculator (iMEC) was used (Amiryousefi et al., 2018) to calculate several indexes of variability from the binary matrix, for polymorphism assessment which include polymorphism information (PIC) and discriminatory power (D). These indices are based on codominant DNA markers (Amiryousefi et al., 2018). A Jaccard distance matrix was then constructed from the binary matrix in the R studio environment (R studio team, 2020) using package "vegan" version 2.6-4 (Oksanen et al., 2022) and using the "vegdist" function (Oksanen et al., 2022). Based on this distance matrices, a heat maps were created using the "pheatmap" function in the "pheatmap" package (Kolde, 2019) to display the distance, that represented disimilarities between the given samples. The clustering method "average" = UPGMA was used as the clustering method.

3 Results and Discussion

Two marker techniques, PBA and CDDP, were used for the detection of variability among the *Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L. var. plainsman plants treated with Cd and also with Si addition. In the following table (Table 2), the total number of fragments, D and PIC can be seen. The highest number of fragments was achieved using the BR + BF

Table 2 Primers, which was used in the study, number of fragments, which was amplified using two different markers techniques, and two indexes: PIC and D. AF, BF, CF means forward primer, AR, BR, CR means reverse primers, R1, R2, R2B, R3, R3B means reverse primers and F1 is a forward primer for CDDP

Primer	Number of fragments	D	PIC
PBA			
AF + AR	150	0.519	0.334
BF + BR	169	0.657	0.367
CF + CR	99	0.384	0.280
CDDP			
R1 + F1	103	0.489	0.324
R2 + F1	105	0.868	0.356
R2B + F1	167	0.703	0.373
R3 + F1	122	0.681	0.371
R3B + F1	97	0.711	0.373

D – dicsrimination power; PIC – polymorphic information power

primer combination. Up to 169 fragments were amplified. The lowest number was observed using the CDDP marker technique using the R3B primer. The value of PIC ranges from 0 to 1 and the closer the number is to 1, the higher the variability (Botstein et al., 1980). D tells us the probability that two randomly selected individuals show different numbers of fragments and are thus distinguishable from each other (Tessier et al., 1999). The values range from 0.280 to 0.373, indicating that there is not high variability among samples.

The threshold of Cd toxicity varies among plants and depends on several factors such as species, ecotype and cultivar (Ismael et al., 2018). The results show that there is only small variability between the samples using different primer combinations. From the study Lancíkova et al. (2020), we already known that *Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L. var. plainsman accumulate Cd with concetration 15 mg.L^{-1} mostly in the roots not in the leafs and translocation factor (TF) decreased with the increased amount of Cd. Treatments with 15 mg.L^{-1} Cd in combination with 5 mg.L^{-1} and 10 mg.L^{-1} Si showed distinct effects on plant profiles by using two different DNA markers techniques. Differences in response to these Si concentrations suggest that Si may modulate Cd toxicity in a concentration-dependent manner by influencing genomic variability. However, ultimately no very significant variability was found among the variants, which may be due to the fact that research on other plants has shown that the addition of Si causes Cd to accumulate mainly in the roots, preventing its transport to the aerial parts of the plant (Rizwan et al., 2012).

Different combinations of primer pairs were used to amplify different numbers of fragments. The heatmaps (Figure 1 and Figure 2) show the variability among the plants and the values inside describe how different the resulting profiles are.These distances calculated across variants through the distance matrix showed considerable variation depending on the primer pair used. By using primer combinations F1 + R2, R2B, R3 we obtained more variable profiles compared to the other combinations. We can also see differences among biological repeats. The profiles of the control plants differed to the profiles of the affected plants treated by Cd with the combination of Si. This highlights that different concentrations of Cd and Si can induce different genomic responses in variety Plainsman, and some treatments may have a more pronounced effect on the plant genome than others. It has been found that Cd toxicity can be mitigated by Si and in multiple ways (Hou et al., 2023). The ability of plants to retain Cd in the root increased after Si application and its concentration in the cell wall increased significantly. On the other hand,

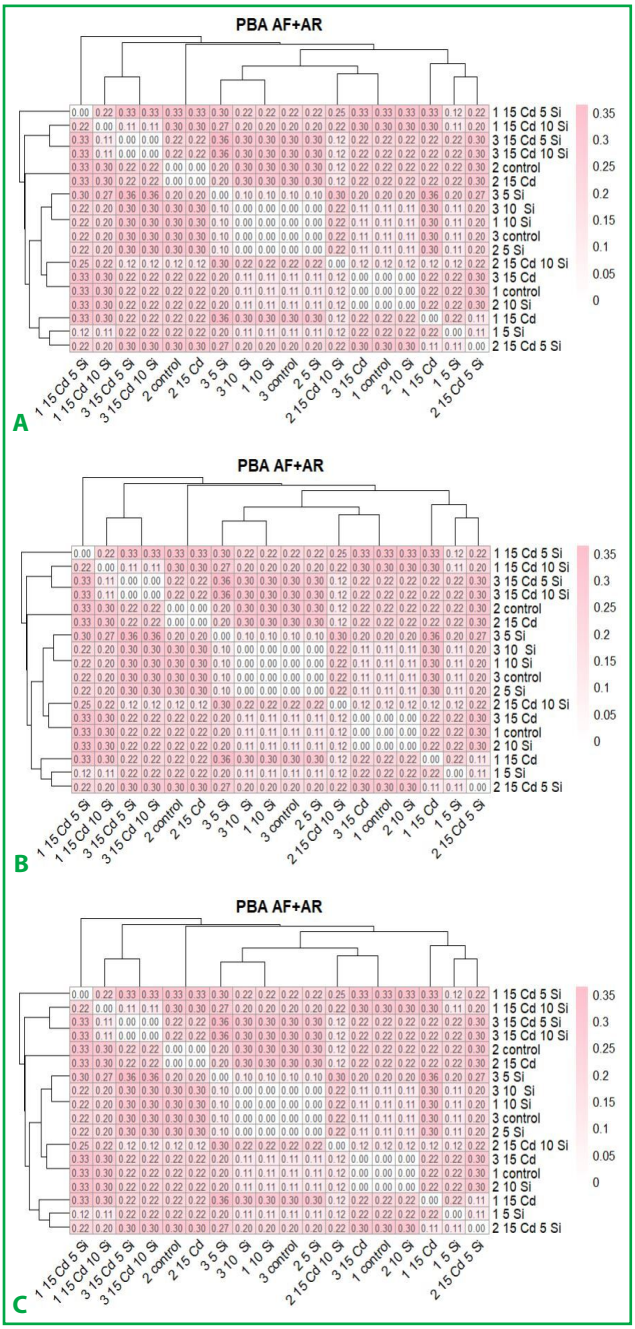

Figure 1 The heat maps shows the distances between treatments and gives an overview of the effect of Si in combination with Cd on plants using PBA markers technique

Number 1, 2, 3 at the beginning means the biological replication of the variant, the numbers 5,10,15 means the concentration of Cd (mg.L^{-1}) and Si (mg.L^{-1}), control means control samples, planted without treatment. On the top of heat maps are the names of primers which was used in analysis. Legen on the right side represents dissimilarity. The range is based on the dissimilarity matric. The distances range from 0.00 (identical profiles, highlighted in white) to 0.35 (the most divergent profiles, highlighted in pink)

the amount of Cd and apoplast and symplast decreased significantly (Cai et al., 2022).

By detecting the polymorphism using the PBA marker technique, with three combinations of primers AF + AR, BF + BR and CF + CR, were found, that there was low variability among the treatments in the *Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L. var. *plainsman* that was treatet by Cd with and without addition of Si. As we can see from the heatmaps (Figure 1), many times the value of 0.000 is repeated, which means that the given samples are identical in terms of the amount and size of the fragments that we were able to capture in the plants using the primer combinations. In the analysis, thee biological repeats for a given influence were used.

The analysis revealed variability in genomic profiles influenced by Cd and Si concentration. Using AF + AR (Figure 1A) primer combination, variability was minimal, with the largest observed distance (0.36) occuring between plants exposed to 15 mg.L^{-1} Cd with 5 mg.L^{-1} Si (3 15 Cd 5 Si) and 10 mg.L^{-1} Si (3 10 Si). Identical profiles (distance = 0.00) were observed in several comparisons, such as between biological variant 2 under control conditions (2 control) and plant exposed to 15 mg.L^{-1} Cd (2 15 Cd).

With the BF + BR primers (Figure 1B), a higher variability was observed, up to 0.77 in distance matrix, with the most divergent profiles observed between biological replicate 1 exposed to 15 mg.L^{-1} Cd (1 15 Cd) and the same biological replicate exposed to both 15 mg.L^{-1} Cd +10 mg.L^{-1} Si (1 15 Cd 10 Si). A slightly lower but still significant distance (0.75) was observed between plants exposed to 15 mg.L^{-1} Cd and 10 mg.L^{-1} Si (2 15 Cd 10 Si, 3 15 Cd 10 Si) compared to plant treated with 15 mg.L^{-1} Cd (1 15 Cd). Identical profiles were found in several pairs, such as between plant in control conditions (3 control) and plant exposed to 15 mg.L^{-1} Cd and 10 mg.L^{-1} Si (1 15 Cd 10 Si). For the CF + CR primers (Figure 1C), the profiles were more monomorphic, but some difference remained. The greatest disimilarities (distance 0.43) was observed between plants exposed to 15 mg.L^{-1} Cd and 5 mg.L^{-1} Si (2 15 Cd 5 Si) and combinations involving plant exposed to 5 mg.L^{-1} Si (3 5 Si) and plant exposed to 10 mg.L^{-1} Si (1 10 Si). In addition, the profiles of plant under control conditions (1 control) differed the most from other profiles, except those of with 15 mg.L^{-1} concentration of Cd (1 15 Cd) and combination 15 mg.L^{-1} of Cd + 5 mg.L^{-1} Si (2 15 Cd 5 Si).The BF + BR primer combination revealed the highest variability, underscoring its sensitivity to subtle differences in experimental conditions.

CDDP technique is the second marker technique used in this work. In this technique, five primer combinations F1 + R1, R2, R2B, R3 and R3B were used. Compared to the PBA marker technique, we obtained more polymorphic profiles using the CDDP technique, especially for the primer combinations R2, R2B and R3 (Figure 2).

The first primer combination used in the analysis was F + R1 (Figure 2A). For this combination, we did not observe any variability between plants affected purely by Si alone. However, the combination of Si and Cd showed variability among plants. The control plant (control 1) showed a different profile to all plants used in the analysis except the plant treated with 15 mg.L^{-1} Cd (3 15 Cd). The other control plants (controls 2 and 3) differed in number and size of fragments relative to all plants affected only by Si and showed a variable profile relative to the other variants relative to plants with 15 mg.L^{-1} Cd (3 15 Cd),15 mg.L^{-1} Cd + 5 mg.L^{-1} Si (1 15 Cd 5 Si, 2 15 Cd 5 Si), and 15 mg.L^{-1} Cd (2 15 Cd). The overall difference between variants using this primer ranged from 0.00 (indicating identical profiles) to a maximum of 0.43. The use of the F + R2 (Figure 2B) primer combination gave us a much more variable profile among plant variants. The distance between variants ranged from 0.20 to 0.75. In this case, we can see that even among biological replicates, the biological variability of these plants was captured in each case. The greatest variability occurred between plants treated with 5 mg.L^{-1} Si (3 5 Si) and 15 mg.L^{-1} Cd + 10 mg.L^{-1} Si (3 15 Cd 10 Si), control plant (3 control) and plant treated with 15 mg.L^{-1} Cd with the combination of 15 mg.L^{-1} Cd +10 mg.L^{-1} Si (1 15 Cd Si), control (1 control) and 15 mg.L^{-1} Cd + 10 mg.L^{-1} Si (15 Cd 10 Si). Slightly more fragments, 167, were amplified using the F + R2B (Figure 2C) primer combination. Among plant variants, the highest variability was observed with among all primers used. The distance between plants ranged from 0.17 to 0.77. The higher variability (distance 0.77) occurred between the plants treated with 15 mg.L^{-1} Cd (2 15 Cd) and with 5 mg.L^{-1} Si (2 5 Si). Interestingly a distance of 0.77 also occurred between the plants with 10 mg.L^{-1} of Si and 5 mg.L^{-1} Si (1 5 Si), 10 mg.L^{-1} Si (1 10), 10 mg.L^{-1} Si (3 10 Si), and 10 mg.L^{-1} (1 10 Si). This implies quite a large variability among the Si affected plants. The distance between plants using the F + R3 (Figure 2D) primer combination ranges from 0.11 to 0.71. A distance of 0.70 occurred between plants treated with 15 mg.L^{-1} Cd + 10 mg.L^{-1} Si (1 15 Cd 10 Si) and 10 mg.L^{-1} Si (3 10 Si), and a distance of 0.71 occurred in the plants treated with 15 mg.L^{-1} Cd (3 15 Cd) and 5 mg.L^{-1} Si (1 5 Si). Several matching profiles were amplified by using the F + R3B (Figure 2E) primer combination, as evidenced by the 0.00 level distance in the heat map. However, a distance at the 0.67 level was observed between the control plant (1 control) and the plants treated only with Si with

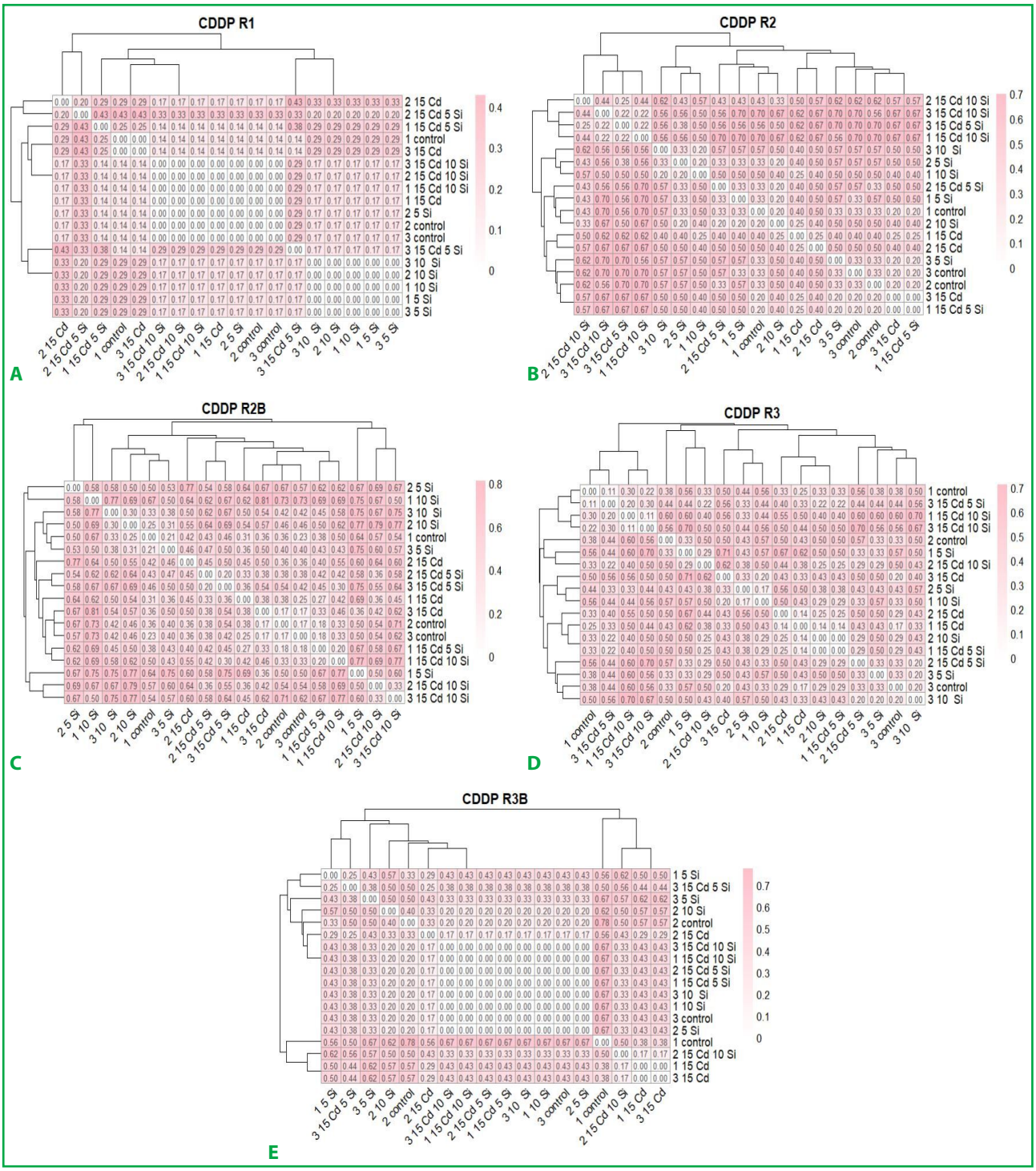

Figure 2 The heat maps shows the distances between treatments and gives an overview of the effect of Si in combination with Cd on plants using CDDP markers technique

Numbers 1,2,3 at the beginning means the replication of the variant, the numbers 5,10,15 means the concentration of Cd and Si, control means control samples, planted without treatment. On the top of heat maps are the names of primers which was used in analysis. Legen on the right side represents dissimilarity. The range is based on the dissimilarity matric. The distances range from 0.00 (identical profiles, highlighted in white) to 0.8 (the most divergent profiles, highlighted in pink)

different concentrations (2 5 Si, 1 10 Si, 3 10 Si, 3 5 Si) and plants teated both Cd + Si (1 15 Cd, 5 Si, 2 15 Cd, 5 Si, 1 15 Cd, 10 Si). Interestingly, the highest distance between control plants (1 control and 2 control) suggests that the two biological replicates of the control group differ from each other at the genomic level more than the other variants. These findings highlight the interplay between Cd and Si concentrations and their influence on genomic variability, as well as differences between biological replicates. This variability could be due to natural biological differences between the plants, even if they are under the same control conditions. CDDP DNA marker system has been used for polymorphism detection in several plants such as *Cicer arietinum* L. (Hajibarat et al., 2015), *Rosa rugosa* Thunb. (Jiang & Zang, 2018), *Ficus carica* L. (Haffar et al., 2022), *Pistacia vera* L. (Aouadi et al., 2019), *Triticum turgidum* L. (Seyedimoradi, Talebi, & Fayaz, 2016). These studies demonstrate that CDDP markers are useful for determining the diversity of different plant species. The CDDP marker system has been shown to be an effective tool for capturing changes in coding regions after seed treatment with cobalt ($Co(NO_3)_2$) in *Vicia faba*. However, different primer pairs were used in the study, namely for ABP1-1, which is complementary to a conserved region of plant-specific auxin-binding proteins, and for Myb2, for which a small number of polymorphic loci were found (Šiukšta et al., 2022). In spite of its advantages, this system also has disdvantages such as reproducibility problems have been noted for some primers, but the author does not specify which primers specifically (Collard & Mackill 2009). Over the decades, the development of DNA marker techniques has focused only on agriculturally important plants. The disadvantage is that if we want to study a plant about which we know very little from a molecular point of view, these techniques may not be 100% efficient. and there are some limitations (Amiteye, 2021). The negative impact of Cd at the genomic level has also been observed using other DNA marker techniques such as RAPD, it has also been investigated in the hyperaccumulating plant *Sedum alfredii* L. (Deng et al., 2007), however, when observing the impact of Si in association with Cd, monitoring at the transcriptomic level is more widely used (Kabir et al., 2016).

4 Conclusions

In this study, were used two DNA marker techniques to assess variability in *Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L. var. plainsman after Cd treatment/ Cd treatment with addition Si. These techniques used in the study created a distinct profiles, but both proved highly effective for detecting polymorphisms in heavy metal-exposed amaranth. The BR + BF primer combination generated the highest number of amplified fragments, reaching up to 169. In contrast, the CDDP marker technique with the R3B primer amplifiedthe fewest fragments. Primer combinations R2, R2B, and R3 produced more diverse profiles compared to others. Additionally, variations were observed between biological replicates. These results collectively show that the interaction between Si and Cd induces genomic variability that can be captured using specific primer combinations, with Si playing a crucial role in modulating the effects of Cd on plants. However, the different Si concentrations of 5 and 10 $mg.L^{-1}$ did not have a large effect on the variability when considering the comparison between these variants

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