Protective Role of Calcium on DNA Methylation Caused Cadmium Stress in *Vicia Faba S*eedlings

Hatice Ogutcu, Esra Arslan, Guleray Agar, Medine Gulluce, Metin Turan, and Fikrettin Sahin

***Abstract*—** The aim of the present study was to evidence the possible antagonistic effect of calcium (Ca^{2+}) against cadmium (Cd^{2+}) induced DNA methylation changes by using CRED-RAs (Coupled Restriction Enzyme Digestion-Random Amplification)) in *Vicia faba* seedlings. The results showed that all doses of Cd^{2+} (10^{-3} M-10^{-5} M) caused DNA methylation changes. In addition, when different three concentrations of Ca^{2+} (10^{-2} , 10^{-4} , 10^{-6}M) were added together with 10^{-3} M, 10^{-4}M, 10^{-5} M doses of Cd^{2+}, DNA methylation changes decreased. The results of this experiment have clearly shown that Ca^{2+} could be used effectively to protect *Vicia faba* seedlings from the destructive effects of Cd^{2+}. Especially, the degree of antagonistic effect of Ca^{2+} against Cd^{2+} is probably related to its concentration ratio.

***Keywords*—** Cadmium, Calcium, CRED-RA, DNA methylation

I. Introduction

THE increase in the use of heavy metals resulted in the contamination of the environment. Heavy metals pose serious problems for the environment and human health because they are the pollutants that can be toxic and mutagenic forms. Cadmium (Cd^{2+}), which is among the most toxic of heavy metals originates from industrial and agricultures activities. The cytotoxic and mutagenic effects of Cd^{2+} have been demonstrated in different plant and animal species. Cd^{2+} was found to inhibit seed germination and root growth, decrease the mitotic index of cells, besides that it produced chlorophyll mutation, chromosomal aberration, DNA damage, important effects on protein metabolism and enzymes in plants [1]-[7]. Additionally, recent studies have showed that Cd^{2+} have effect on methylation of DNA [8], [9]. In recent years, several studies have been reported that toxic effect of Cd^{2+} may be reduced by calcium (Ca^{2+}) in different animal and plants [6], [7], [10]. However, the effect of Ca^{2+} on Cd^{2+} induced DNA methylation using CRED-RA has not yet been reported.

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Therefore, in this study, we aimed to investigate whether protective role of Ca^{2+} against Cd^{2+} which induces DNA methylation.

II. Materials And Methods

A. Sample Collection

Vicia faba seeds were obtained from the Department of Field Crops, Faculty of Agriculture, Ataturk University (Turkey). The seeds were surface-sterilized with 0.5 % NaOCl (sodium hypochlorite) solution for 10 min and then washed with sterile water three times.

Seeds were placed on two layers of filter paper moistened with 25 ml distilled water. 25 seeds were used in each petri dish. The dishes were kept at 25 ± 1°C under dark condition until primary roots were grown at 0.5-1 cm length. After, the *Vicia faba* seedlings were exposed to different concentrations of solution containing zero (control), 10^{-3} M, 10^{-4} M, 10^{-5} M of $CdCl_2$ and the joint concentration of $CdCl_2$ together with 10^{-2} M, 10^{-4} M, 10^{-6} M of $CaCl_2$ for 96 h.

Treated seedlings were grown in pots of a peat/soil mix (5 plants/pot) at 25±1°C with a 16 h photoperiod of 60 µmol photons/m^2s provided by white fluorescent lamp, at a relative humidity of 70–75%. Each treatment was replicated three times. Bulk leaves were randomly collected from ten plants for each treatment after emergence of leaf 3 (leaves numbered from base) and were stored at -80 °C.

B. DNA Extraction

Genomic DNA was extracted from powdered plant materials using a method described by Li and Quiros [11]. Approximately 1 g of tissue samples were ground to a fine powder in liquid nitrogen in 2ml eppendorf tubes. Next 1,000 µl of DNA in DNA extraction buffer (100 mM Tris-HCl (pH 8.0) 50 mM EDTA, (pH 8.0) 500 mM NaCl, 20% SDS and 10 mM 2-mercaptoethanol) was added and mixed well. The mixture was incubated at 65^0C in a water bath for 45 min with intermittent shaking at 5-min intervals. The mixture was centrifuged at 12.000 g for 15 min at 4^0C and the supernatant was transferred into a new 1.5ml tube mixed gently with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1). The DNA was precipitated by the addition of 0.6 volume of freezer cold isopropanol left at -20^0C for 10 min. The upper phase was discarded by centrifugation for 10 min at 12000 rpm and at -4^0C. The pellet was washed with 70% ethanol. The dried DNA was allowed to air dry before being

dissolved in 100 µl of TE buffer.

C. CRED-RAs Digestion and PCR

Genomic DNA sample from each treatment were separately digested with HpaII and MspI endonucleases (which cut the sequence 5´-C/CGG-3´with different sensitivity to cytosine methylation; MspI cuts if the inner C is methylated, whereas HpaII cannot cleave in the presence of methyl groups). After checking digestion on agarose gel, were prepared a PCR reaction cocktail.

CRED-RA PCR reaction was contained 25 ng digested DNA, 400 µM dNTP, 10 pmol primer, 2,5 mM $MgCl_2$, 1 U *Taq* DNA polymerase and 1X PCR buffer (10X) in a total volume of 20 µL. DNA amplification was carried out in a thermocycler programmed as follows: 1 cycle of 5 min at 95°C, 42 cycles of (1 min at 94°C, 1 min at 36°C and 2 min at 72°C), 1 cycles of 15 min at 72°C.

16 CRED-RA primers were tested with bulked DNA of control treatment (0 ppm fipronil). Only 8 primers amplified polymorphic amplicons and used in CRED-RA PCR reactions. These primers for CRED-RA analyses are; AGAGCCGTCA (OPY-7), CAGGCCCTTC (OPA-1), AATCGGGCTG (OPA-4), CAGCACCCAC (OPA-13), CAGAAGCGGA (OPW-4), CACAGCGACA (OPW-13), TGATGCGTG (OPW-11) and AGGCCCGATG (OPW-6).

D. Electrophoresis

The PCR products (20 µl) were mixed with 6x gel loading buffer (3 µl) and subjected to agarose (1.5% w/v) gel electrophoresis in 0.5x TBE (Tris-Borate- EDTA) buffer at 70 V for 150 min. Amplification products separated by gel electrophoresis were stained in ethidium bromide solution (2 µl Etbr/100ml of 1x TBE buffer) for 40 min. The amplified DNA products were detected using the Bio Doc Image Analysis System and analysed using the Uvi-soft analysis package (Cambridge Electronic Design Ltd, Cambridge, UK).

E. Data analysis

The average number of CRED-RA pattern polymorphisms (%) were calculated for each dose to realize CRED–RA analysis. To calculate the number of polymorphisms (%), the following formula was used 100x *a/n* where *a* is the average number of polymorphic bands detected in each treated sample, and *n is* the number of total bands in the control. Polymorphisms in CRED-RA profiles included disappearance of a normal band and appearance of a new band compared with the control. The average was calculated for each experimental group. To compare the sensitivity of each parameter, changes in these values were calculated as a percentage of their control (set to 100%).

III. Results And Discussion

In total, 15 oligonucleotide primers with % 60-70 GC content were used for CRED-RA analyzing and only eight gave specific and stable results. Compared with the PCR products obtained from the control DNA, Cd^{2+} treatments resulted changes of DNA methylation. These changes are characterized by variation loss of normal bands or appearance of new bands. Total DNA hyper-methylation increased progressively with increased Cd^{2+} concentration. The methylation value was 6.4 in control. The highest methylation value was 25.5 and the lowest was 12.1 in Cd^{2+} applications. However, the value was progressively decreased when Ca^{2+} and Cd^{2+} applications combined with together. In this case, the highest methylation value was 17.6 and the lowest was 9.7.

The toxic effect of Cd^{2+} is known on plants and animals. The effects of Cd^{2+} on the frequencies of total chromosomal aberration, mitotic index, DNA mutation have been reported in plants [1]-[6]. Furthermore, effects of Cd^{2+} genomic template instability have been showed by the RAPD analysis [7], [12]. In additional, epigenetic effects of Cd^{2+} have been detected by DNA methylation analysis [8], [9], [17]. These studies have showed that Cd^{2+} caused methylation and demethylation of DNA. Although the molecular responsible for methylation of DNA Cd^{2+} remains unclear [10] it has been suggested that Cd^{2+} can change methylation of DNA as DNA methyltransferase (MeTase) activity.

However, these effects of Cd^{2+} seen at higher levels changed after treatment with different concentrations of Ca^{2+}. These results showed that Ca^{2+} can reduce epigenetic effects of Cd^{2+} as mentioned above. Ca^{2+} is essential for many cellular functions Since Ca^{2+} and Cd^{2+} have similar binding sites, the mechanism of protective action of Ca^{2+} can be related to Ca^{2+} competitive inhibition, substitution or replacement of Cd^{2+} and also activation of Ca-ATPase. Thus, Ca^{2+} reduces this effect of Cd^{2+} on broad bean seedling. Other investigators have noted the similar protective effect of Ca^{2+} against toxic effects of Cd^{2+} [6], [7].Therefore, the protective role of Ca^{2+} against the changes in DNA methylation pattern caused Cd^{2+} may be connected to replacement of Cd^{2+} at these sites or specifically bound to DNA cytosine and change their methylation directly.

Consequently, Ca^{2+} can reduce the toxic effect of Cd^{2+} in plants. Similarly, Ca^{2+} may be increase resistance and yield by alleviate the negative on plants of Cd^{2+}.

IV. Conclusions

Heavy metal stress (Cd^{2+}) is one of the abiotic stress that cause biochemical, physiological and especially genetic and epigenetic changes. These epigenetic changes have been proved with CRED-RA technique in this study and also the results of this experiment have clearly shown Ca^{2+} that could be used effectively to protect *Vicia faba* seedlings from the destructive effects of Cd^{2+} stress.

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