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ORIGINAL RESEARCH PAPER

EGTA, a calcium chelator, affects cell cycle and increases DNA methylation in root tips of *Triticum aestivum* L.

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Abstract
In this study, when germinated *Triticum aestivum* L. seeds were treated with 0, 2, 4 and 6 mM ethyl glycol tetraacetic acid (EGTA), root growth was suppressed and the mitotic index decreased. These inhibitory effects were positively correlated with EGTA concentration. RT-PCR analysis revealed that the expression of several gene markers related to the G1/S transition of the cell cycle were significantly down-regulated. Confocal microscopy of Fluo-3/AM-stained roots showed chelation of nearly all of the Ca^{2+} within the root meristematic regions. Both random amplified polymorphic DNA (RAPD) and coupled restriction enzyme digestion-random amplification (CRED-RA) techniques showed significant increases in the levels of genomic DNA polymorphisms and degree of DNA methylation. The study provides information concerning the impact of Ca^{2+} chelator, EGTA, on the growth, expression of cell cycle transition marker genes, and changes in DNA structure and methylation in the wheat roots.

Keywords
Ca^{2+} deprivation; EGTA; genomic DNA damage; DNA methylation; RAPD; CRED-RA; *Triticum aestivum* L.

Introduction

Plant growth, development, and differentiation rely on coordination of the cell cycle which can be divided into four consecutive phases: G1, S, G2, and M. To ensure the faithful duplication and passage of genetic information during cell division, the transitions between different phases of the cell cycle are precisely coordinated and controlled by the cyclin-dependent kinases (CDKs) [1]. Cyclin D-CDK4/6 complexes phosphorylate the retinoblastoma protein (pRB) to dissociate it from E2F family of transcription factors, which is a crucial step in the coordination of the G1/S transition [2,3].

The pRB is the product of the retinoblastoma gene (*RB*) which was first identified based on its mutation in a rare malignancy of the eye, and later on as a tumor suppressor gene [4]. It has been shown that the pRB protein blocks S-phase entry and cell growth, making it responsible for a major G1-phase checkpoint [5]. During early G1 phase, the pRB protein is hypophosphorylated and binds strongly to E2F transcription factors, thus preventing transcription of E2F target genes. As the G1 progresses, pRB is sequentially hyperphosphorylated and inactivated: initially by early/mid-G1 CDKs (CDK6 and CDK4), and subsequently by late G1 CDKs (CDK2). Hyperphosphorylation of pRB releases E2F, which transactivates target genes that promote S-phase entry [6]. Other proteins engaged in G1/S transition include minichromosome

maintenance protein complex (MCM), proliferating cell nuclear antigen (PCNA), and ribonucleotide reductase (RNR).

The minichromosome maintenance complex subunits (MCM2–7) are members of the AAA^+ superfamily of ATPases, and thus use energy derived from cycles of ATP binding and hydrolysis to move or reorganize bound substrates [7]. MCM's have been shown to be tightly bound to chromatin during the second half of G1 phase and to subsequently be displaced from replicating chromatin during S phase [8]. Dimitrova et al. gave direct evidences that MCM2 was a component of the mammalian early G1 phase pre-replication complex [9].

Proliferating cell nuclear antigen (PCNA) is an evolutionarily well-conserved protein found in all eukaryotic species as well as in Archaea. It was first shown to act as a processivity factor of DNA polymerase δ, which is required for DNA synthesis during replication [10].

One of the key processes in the G1/S transition of the cell cycle is the biosynthesis of deoxyribonucleotides. RNR is an essential enzyme for de novo synthesis of deoxyribonucleotides. It catalyzes the reduction of the four ribonucleotide diphosphates to their corresponding deoxyribonucleotides and maintains a highly regulated and balanced pool of DNA precursors during chromosome replication. The structure of RNR is composed of two identical large subunits and two identical small subunits which RDR (ribonucleotide reductase small subunit) belongs to one of them [11,12].

All in all, MCM2, PCNA, and RDR are crucial for G1/S transition of cell cycle.

The most important events during cell cycle are DNA replication and the segregation of replicated chromosomes into the two daughter cells, which are identical to the mother cell. In plants, many internal or external factors can affect cell cycle process to interfere with normal developmental processes. An important factor influencing plant development is Ca^{2+}. As a second messenger, Ca^{2+} participates in nearly all aspects of plant development, including cell wall formation, cellular membrane permeability, cell ionic equilibrium and osmotic regulation, polar cell growth, cell division and proliferation [13], nuclear gene transcription [14], cell differentiation [15], and apoptosis [16].

There are many defects associated with low levels of Ca^{2+} or Ca^{2+} deprivation in plants, such as poor root growth and development, necrosis and curling of young leaves, blossom end rot, bitter pit, fruit cracking, poor fruit storage [13,17], and inhibition of photosynthesis, nitrogen metabolism, and plant growth [18]. However, the underlying mechanisms of these Ca^{2+} influences are not entirely clear [13].

As stated, Ca^{2+} is involved in the regulation of cell cycle processes, but to date, the mechanisms involved are not fully understood. In this study, germinated wheat seeds were treated with different concentrations of EGTA, an effective Ca^{2+} chelator [19,20]. It was shown that the growth of the roots was suppressed and the mitotic index decreased. Results from RT-PCR revealed that the G1/S transition of cell cycle was disturbed. Fluo-3/AM staining confirmed Ca^{2+} deprivation within the treated root meristems. Damage to genomic DNA and increased DNA methylation were demonstrated through RAPD and CRED-RA analyses. To the best of our knowledge, it is the first study explaining the molecular and epigenetic mechanisms of Ca^{2+} deprivation-dependent inhibition of plant growth.

Material and methods

Plant material and reagents

Wheat seeds (CB037-A) were provided by Beijing Academy of Agricultural and Forestry Sciences, Beijing, China.

Seed germination and treatment

The seeds were pre-treated with distilled water for 2 h and then placed on moistened filter papers in 10-cm-diameter Petri dishes for germination at 25°C in darkness.

When the radicles emerged from the seed coat to a length of 1 mm, germinated seeds were treated with different concentrations of EGTA (Solarbio, Beijing, China, #E8050) (2, 4, and 6 mM in distilled water) in darkness at 25°C for 24 h. An additional control treatment was administered using only distilled water.

Statistical analysis of EGTA effects on root length and mitotic index

The lengths of roots in each EGTA concentration treatment were recorded. For estimation of mitotic index, the root tips were excised and fixed in Carnoy's fixation (100% ethanol : 100% glacial acetic acid = 3:1; v/v) at 4°C for 24 h. After a complete washing with distilled water, the root tips were dissociated with hydrolysis buffer (95% ethanol : concentrated hydrochloric acid = 1:1; v/v) at room temperature for 5 min. After a thorough wash with distilled water, the root tissues were macerated in a drop of methanol : glacial acetic acid (1:1; v/v). The macerated tissue was cut up with a razor blade, and placed in a drop of modified carbol fuchsin on a slide and squashed between the slide and a coverslip. The mitotic index was calculated as the ratio of number of cells in mitosis to the total number of counted cells (ca. 2000 cells).

RNA extraction and RT-PCR analysis

Isolation of RNA from 0.2 g of wheat root tips ground to a fine powder in liquid nitrogen followed the TRIzol (Invitrogen, Carlsbad, CA, USA, #15596-026) method. Total purified RNA was reversely transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo, USA, K1066). Both the sequence of primers used and RT-PCR reaction performed in the experiment were according to Pena et al. [3] and Ni et al. [21] and are listed in Tab. 1. The PCR products were separated by electrophoresis through a 1.5% agarose gel and were visualized under a UV transilluminator. The *Actin2* gene was amplified as the internal standard.

Fluo-3/AM staining

Intracellular free Ca^{2+} within root tips in control and 4 mM EGTA treated samples was detected using the fluorescent dye Fluo-3 AM (Beyotime, Shanghai, China, #S1056), which is cut into Fluo-3 by an intracellular esterase after crossing the cell membrane. The Fluo-3 can specifically combine with the Ca^{2+} and gives a strong fluorescence

Tab. 1 RT-PCR reaction system and amplification procedure [3].

Genes	Forward and reverse primers	PCR conditions
mcm2+	5′-ACGACGGCGCCACCGTTATC-3′	95°C 5 min; 35 cycles; 95°C 1 min; 58°C 30 s; 72°C 45 s; 72°C 1 min
mcm2–	5′-TTGCGATGAAGCGCCGGACT-3′	
pcna+	5′-CACCAAGGAGGGTGTCAAGT-3′	95°C 5 min; 35 cycles; 95°C 1 min; 58°C 30 s; 72°C 45 s; 72°C 1 min
pcna–	5′-GATCTTGGGGTGCCAGATAA-3′	
rdr+	5′-TTCCCCATCCGGTTCCCGCA-3′	95°C 5 min; 35 cycles; 95°C 1 min; 53°C 30 s; 72°C 45 s; 72°C 1 min
rdr–	5′-TGAGCCCGCGCTTCTTGAGC-3′	
Actin2+	5′-GCTGGATTCTGGTGATGGTGTGAG-3′	95°C 5 min; 35 cycles; 95°C 1 min; 60°C 1 min; 72°C 1 min; 72°C 10 min
Actin2–	5′-CAATGAGAGATGGCTGGAAGAGGAC-3′	

mcm2 – minichromosomal maintenance factor; *pcna* – proliferating cell nuclear antigen; *rdr* – ribonucleotide reductase small subunit.

[22]. The roots were collected and rinsed twice with 0.2 mM $CaCl_2$ and stained with 20 μmol/L Fluo-3/AM for 2 h at 4°C. Next, the samples were washed twice with 0.2 mM $CaCl_2$ and incubated for another 2 h at 20°C. Intracellular free Ca^{2+} in roots was visualized using a Zeiss LSM 5 LIVE with an excitation wavelength of 488 nm and an emission wavelength of 525 nm [23].

Extraction of genomic DNA

Genomic DNA (gDNA) was extracted from 0.1 g of wheat root tips ground in liquid nitrogen, using a PlantGen DNA Kit (ComWin Biotech, Beijing, China, #CW0553) according to the manufacturer's protocol.

RAPD analysis

Damage to gDNA in EGTA treated samples was assessed through RAPD profiles using forty random primers (see Tab. S1) [24–27] synthesized by Sangon Biotech (Shanghai, China).

The PCR mixture was as follows: 1× PCR buffer (with Mg^{2+}), 0.25 mM dNTP mix, 10 pmol random primer, 1 U Taq polymerase, 15 ng gDNA templates in a total volume of 20 μL. The amplification protocol began with a 5 min pre-denature step at 94°C, followed by 45 cycles of 1 min for denaturing at 94°C, 1 min for annealing at 36°C, and 2 min for extension at 72°C. After 45 cycles, a further 10 min step at 72°C was performed to allow completion of primer extension [26,28]. Amplification products were separated through gel electrophoresis in a 1.5% agarose gel in 1× TAE buffer with 100 V for 1 h. RAPD profiles of gDNA were visualized under a UV transilluminator. Super DNA Marker (ComWin Biotech, Beijing, China, #CW2583) was used as standard marker.

CRED-RA analysis

Digestion of 1 μg of gDNA was performed with 1 μL *Hpa*II (New England Biolabs, Beijing, China, #R0171) or 1 μL *Msp*I (New England Biolabs, Beijing, China, #R0106) at 37°C for 2 h, respectively. DNA was purified using a DNA Purification Kit (ComWin Biotech, Beijing, China, #CW2301) according to the manufacturer's instructions. The PCR analysis, including primers and product analysis, was the same as used for the RAPD profiles.

Statistical analysis

All of the experiments were repeated at least three times. Data were presented as mean ± standard error of the mean (*SEM*). Statistical comparisons among EGTA treatments and controls were made using a Student's *T*-test or ANOVA and Tukey multiple comparisons test and significance was assigned at $p < 0.05$. Images were appropriately processed using Photoshop CS5 (Adobe Systems, San Jose, CA, USA). GraphPad Prism 5 (Graphpad Software, San Diego, USA) was used for data and graphing analysis.

Results

The effects of EGTA on root growth and mitosis in root tip meristem of *T. aestivum* L.

Root length of germinated wheat seeds treated with 0, 2, 4, or 6 mM of EGTA tended to decrease gradually with increasing EGTA concentrations (Fig. 1a). Statistical analysis indicated that the means of root length decreased to 67%, 58%, 48% compared

with the control group (Fig. 1b), and those of the mitotic index decreased from 7% of the control to 5%, 3%, and 2%, respectively (Fig. 1c).

The data demonstrate that EGTA inhibits roots growth and cell division and that the inhibitory effects are positively correlated with its concentrations.

The effects of EGTA on Ca^{2+} distribution in root tip meristem of *T. aestivum* L.

To visualize the distribution of Ca^{2+} in roots after EGTA treatment, the control and 4 mM EGTA-treated roots were loaded with 20 μmol/L of Fluo-3/AM, a Ca^{2+}-specific indicator [22]. In confocal images of the control group (0 mM of EGTA) strong fluorescence signals, primarily distributed within the meristematic regions, and

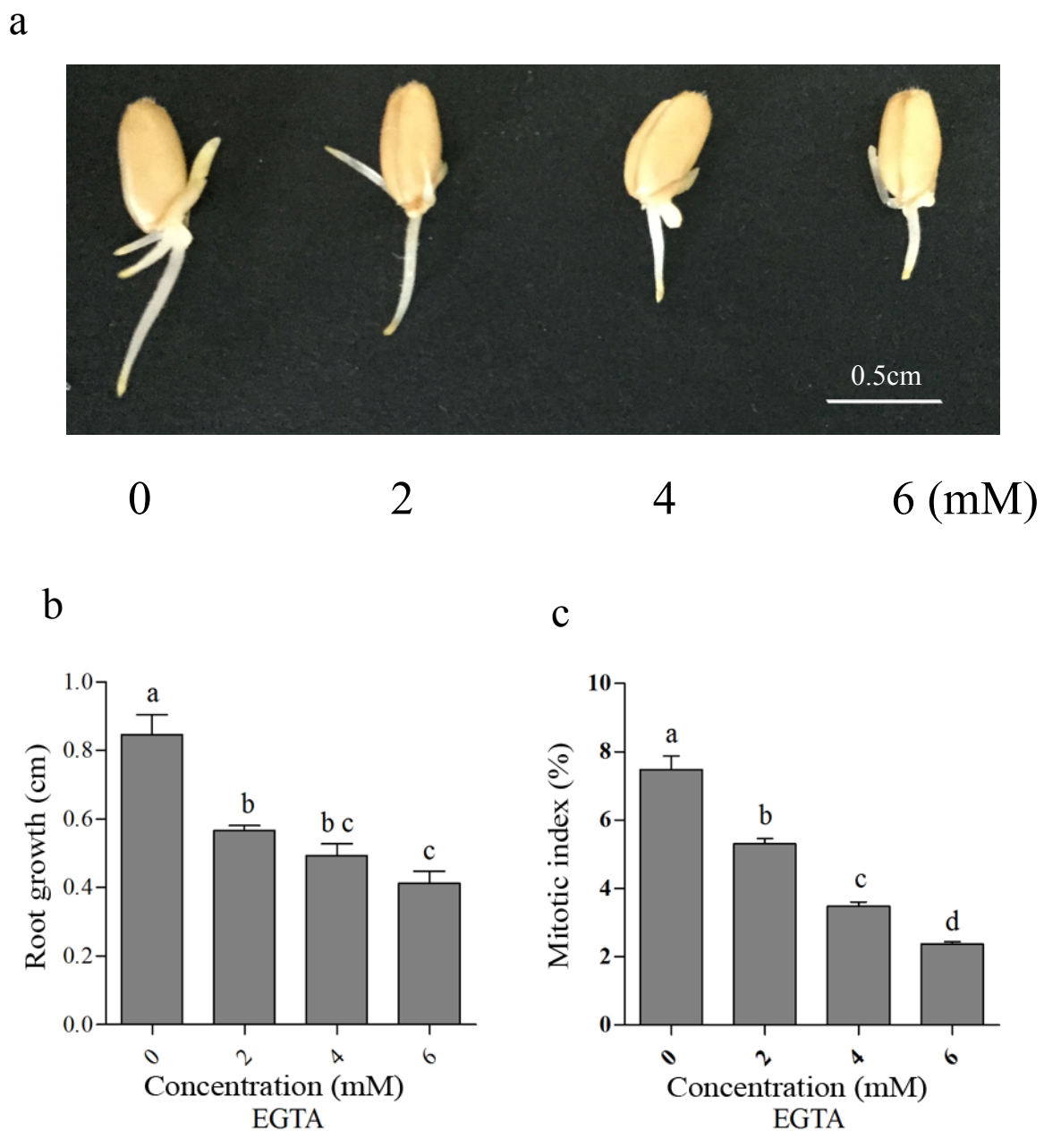

Fig. 1 Effects of different concentrations of EGTA (0, 2, 4, 6 mM) on root growth and mitotic index after 24-h treatment. **a** Micrograph showing grown roots of germinated seeds treated with the four tested EGTA concentrations. Scale bar: 0.5 cm. **b** Mean root growth and (**c**) mitotic index after EGTA treatment. Different letters demarcate significant differences between treatment concentrations after analyzing using GraphPad Prism 5.

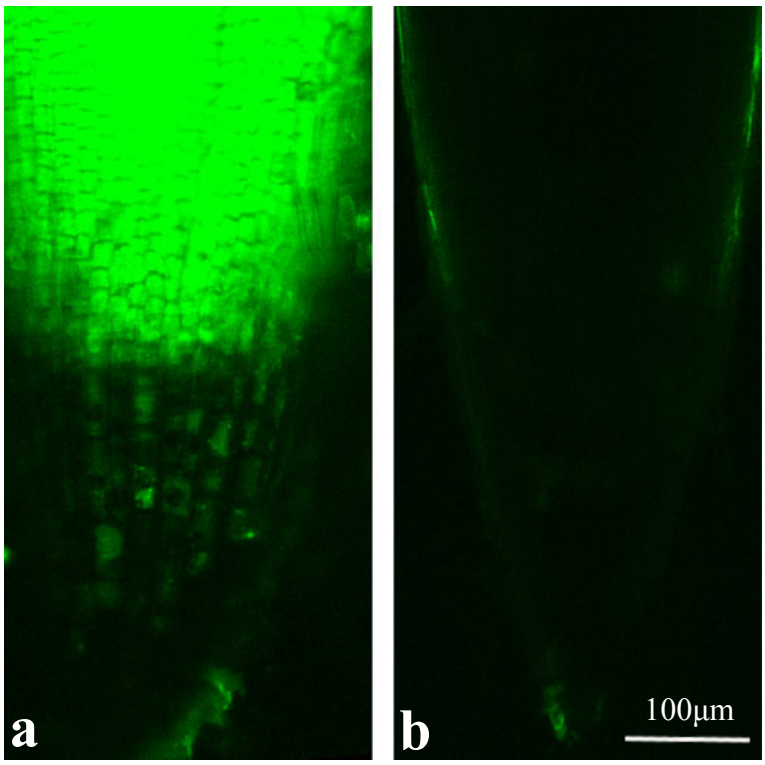

Fig. 2 Confocal images of wheat roots incubated in Fluo-3/AM ester solution after treated with distilled water (control; **a**) or 4 mM EGTA (**b**). Scale bar: 100 μm.

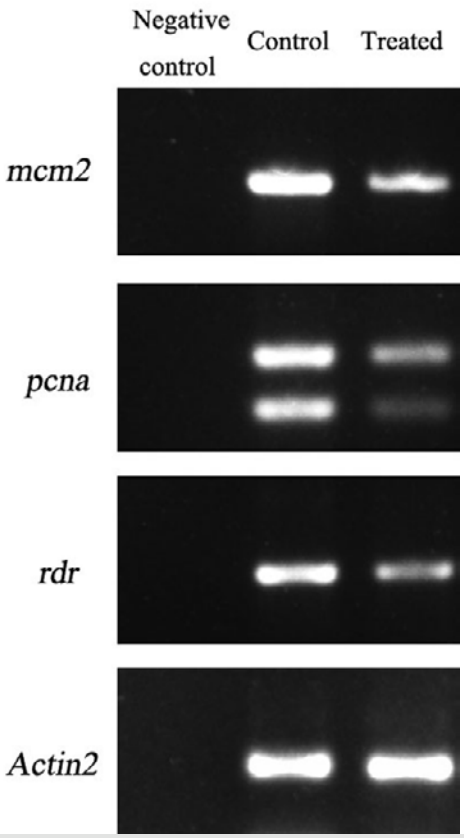

Fig. 3 RT-PCR bands for the genes *mcm2*, *pcna*, and *rdr* from control and 4 mM EGTA-treated root samples. These genes are the E2F transcriptional targets. *Actin2* was used as the internal standard gene.

scattered signals within the root caps were observed (Fig. 2a). In EGTA treated roots, fluorescence labeling was absent in the meristematic regions, and a few signals appeared on the outer edges and the root caps (Fig. 2b).

These results strongly suggest that Ca^{2+} was primarily localized within the root meristematic cells of control roots, and that EGTA effectively chelated meristem Ca^{2+}.

RT-PCR analysis

To determine which phase of the cell cycle was affected by EGTA treatment, gene expression levels of minichromosome maintenance complex subunits 2 (*mcm2*), proliferating cell nuclear antigen (*pcna*) and ribonucleotide reductase small subunit (*rdr*) were quantified using RT-PCR. Expression of all three examined genes decreased sharply in the 4 mM EGTA-treated samples compared with the control samples (Fig. 3). As *mcm2*, *pcna*, and *rdr* are potential E2F transcriptional targets and are regarded as cell-cycle gene markers important in regulation of the G1/S transition [3,7–12], the downregulation of these marker genes implies that EGTA treatment interferes with G1/S transition of the cell cycle.

RAPD analysis

The RAPD analysis was performed to verify the effects of EGTA treatment on wheat gDNA. Of the 40 random primers tested (Tab. S1), 31 primers generated specific and polymorphic bands which were selected for the analysis of genetic diversity.

As shown in Tab. 2 and Tab. 3, a total of 242 bands were obtained from the control. Of them, a maximum of 12 bands was amplified by primer OPB-11 and OPK-02, while only three were amplified by GLH-16. The average number of bands was 8 and the product sizes ranged from 100 bp to 5 kb.

With the EGTA treatment, polymorphic bands emerged and could be divided into four types: (a) appearance of extra bands; (b) disappearance of normal bands; (c) increased band intensity, and (d) decreased band intensity [29] (Tab. 2 and Tab. 3, Fig. 4a–d). Compared with the 242 bands obtained from the control samples, 262 polymorphic bands (Tab. 3, a+b+c+d) were generated in EGTA-treated samples. The polymorphic value for the samples treated with 2, 4, and 6 mM of EGTA was 30%, 35%, and 43%, respectively. These results showed a strong positive correlation between the concentrations of EGTA and the increase in polymorphisms (Tab. 2 and Tab. 3).

On the other hand, the effect of each EGTA treatment on polymorphisms differed among the 31 primers. For example, 11 new bands appeared for S32 ("a" in Tab. 2) and four bands disappeared for OPO-05 ("b" in Tab. 2). With the use of OPH-04, changes in the intensity of 18 bands were detected – 15 showed increase in

Tab. 2 RAPD profile primer band count for control wheat seeds and counts of primer bands that newly appeared (a) and disappeared (b) with 2, 4, and 6 mM EGTA treatments.

Number of primer	EGTA concentration (mM)						
	0	2		4		6	
		a	b	a	b	a	b
A.z-09	8	0	0	0	1	0	0
OPK-08	8	0	0	0	0	0	1
OPO-05	9	1	1	0	2	0	1
S32	4	3	0	4	0	4	0
S126	7	0	0	0	1	0	1
OPB-01	11	0	0	0	0	0	1
OPB-04	9	2	0	2	0	3	0
OPB-11	12	0	0	0	0	0	2
OPF-15	7	0	0	0	0	0	1
OPK-02	12	0	0	0	0	0	0
OPK-12	9	0	0	0	0	0	0
OPK-15	11	0	0	0	0	0	0
A.z-1	11	1	0	0	0	0	0
A.z-5	10	2	0	2	0	2	0
A.z-6	9	1	0	0	0	0	1
A.z-7	7	1	0	1	0	1	0
A.z-14	10	0	0	0	0	0	0
S34	4	1	0	2	0	2	0
S98	7	0	0	0	0	0	0
S129	8	2	0	2	0	2	0
S130	5	0	0	0	0	0	0
S132	8	1	0	1	0	1	0
S156	5	1	0	1	0	1	0
S443	8	1	0	1	0	1	0
S461	6	0	0	0	0	0	0
OPH-04	6	1	0	1	0	1	0
GLA-17	6	1	1	3	1	1	0
GLD-07	4	0	0	0	0	0	0
GLH-16	3	0	0	0	0	0	0
P16	8	0	0	0	0	0	1
P17	10	0	0	0	0	0	1
Total bands	242	19	2	20	5	19	10
a+b		21		25		29	

the intensity, while 3 exhibited decrease ("c" in Tab. 3). Also, use of primer S126 led to decrease in the intensity of five bands ("d" in Tab. 3).

In general, EGTA treatment led to increase in the appearance of new bands and enhancement of bands intensity.

CRED-RA analysis

Effects of the 4 mM EGTA treatment on the degree of DNA methylation were assessed using the CRED-RA technique, which is based on the isoschizomer pair restriction enzymes *Hpa*II and *Msp*I. Both enzymes recognize the 5′-CCGG-3′ sequence and digest DNA when none of the cytosines are methylated. However, the ability of each member of this isoschizomer pair to cut DNA depends on cytosine methylation conditions specific to each enzyme. If one or both cytosines are methylated in a single strand (hemi-methylation), but not both DNA strands, *Hpa*II does not cut the strand, whereas *Msp*I does not cut if the outer cytosine is methylated in one or both strands [28,30–33]. Therefore, the amplification bands of the enzyme-digested products can show both the presence of DNA methylation and types of DNA methylation occurring [28,30–33].

According to the presence or absence of specific bands, methylation patterns can confidently be divided into four types (Tab. 4) [30,33]: Class I is identified by absence of bands for both enzymes, and is seen when either no methylation occurred or only inner methylation in a single strand occurred; Class II is identified by the absence of the *Hpa*II band and presence of the *Msp*I band, and occurs when the outer cysteine of a single strand is methylated; Class III is identified by the absence of the *Msp*I band and presence of the *Hpa*II band, and signifies methylation of the inner cytosine of both strands; Class IV is identified through the appearance of bands for both enzymes, and occurs when both DNA strands are methylated at the outer cytosines (Tab. 4) [30,33].

After digestion with restriction enzymes *Hpa*II and *Msp*I, the 40 random primers for RAPD analysis (Tab. S1) were also used here for PCR amplification, with 11 of the primers producing specific and stable bands (Tab. 5). In total, 249 bands were identified, of which 125 bands were *Hpa*II-specific and 124 bands were *Msp*I-specific. *Hpa*II polymorphism ranged from 0 to 67% and *Msp*I polymorphism ranged from 0 to 200%, respectively (Tab. 5).

Here, eight different types of bands, named band Type 1–8, were distinguished after amplification (Fig. 5a–f, Tab. 6) and each represented a specific DNA methylation status.

Tab. 2 Continued

a+b – total count of bands that disappeared (a) or appeared (b) with EGTA treatments.

Tab. 3 RAPD profile primer band count for control wheat seeds and counts of primer bands that increased (c) and decreased (d) in intensity with 2, 4, and 6 mM EGTA treatments.

Number of primer	EGTA concentration (mM)						
	0	2		4		6	
		c	d	c	d	c	d
A.z-09	8	2	0	1	2	2	0
OPK-08	8	0	0	0	0	0	1
OPO-05	9	4	1	0	0	0	2
S32	4	0	1	0	1	0	2
S126	7	0	1	0	1	0	3
OPB-01	11	0	1	0	1	0	1
OPB-04	9	3	0	2	0	3	0
OPB-11	12	0	0	0	0	0	2
OPF-15	7	0	0	4	0	0	2
OPK-02	12	1	1	1	1	1	2
OPK-12	9	1	0	0	1	6	0
OPK-15	11	2	0	2	0	2	0
A.z-1	11	3	0	1	0	0	3
A.z-5	10	2	0	2	0	2	0
A.z-6	9	1	0	1	0	1	1
A.z-7	7	3	0	3	0	3	0
A.z-14	10	1	0	1	0	1	0
S34	4	1	0	1	0	1	0
S98	7	2	0	2	0	2	0
S129	8	3	0	3	0	3	0
S130	5	0	1	3	0	4	0
S132	8	2	0	2	0	2	0
S156	5	1	0	1	0	2	0
S443	8	3	0	3	0	2	1
S461	6	0	0	0	0	0	2
OPH-04	6	5	1	5	1	5	1
GLA-17	6	0	1	5	1	1	1
GLD-07	4	1	0	1	0	1	0

As for Type 1, 2, and 8, the DNA in the control group could not be digested by both *Hpa*II and *Msp*I. In turn, in the treated samples the mode of *Hpa*II and *Msp*I action was different. *Hpa*II did not digest DNA in the band Type 1 but did in bands Type 2 and 8. *Msp*I digested DNA in bands Type 1 and 2 but not in Type 8. The possible explanation of this phenomenon is that treatment with EGTA led to demethylation of one of the DNA strands or transfer of methyl group from outer to the inner cytosine (Tab. 4).

The results showed a 19% decrease in levels of DNA methylation in bands Type 2, 3, and 8 for treatment relative to control samples. Although there was no change in the levels of DNA methylation in bands Type 1 and 6, the methylated sites must have been altered (19%). For band Type 4, the methylated sites were altered or the methylation levels were increased by 5% in treatment samples relative to control samples. Treatment with EGTA increased DNA methylation levels in bands Type 5 and 7 by 57%.

Overall, these results showed a significant increase in the degree of DNA methylation in 4 mM EGTA-treated root tip meristems of *T. aestivum* L. relative to control samples.

Discussion

In eukaryotes, control of the G1 to S-phase transition is a key step in cell cycle regulation because cells typically become committed to divide after they have replicated their DNA [34]. In addition to two cell cycle kinase complexes, CDK4/6-Cyclin D and CDK2-Cyclin E, a number of transcription factors, proteins, enzymes, etc. are involved in regulating this transition. The minichromosome maintenance complex subunits 2 (MCM2), proliferating cell nuclear antigen (PCNA) and ribonucleotide reductase small subunit (RDR) have been considered as marks of the G1/S transition [3,7–12].

Ca^{2+} is an essential element and plays vital roles in the normal structure, constitution, and functions of living organisms. However, Ca^{2+} deficiency is very common in nature and has many negative or even lethal effects on the cells of organisms. Ca^{2+} deficiency in plants can severely affect their growth, development, and metabolism [13,17]. Studies conducted on animal cells have shown extracellular Ca^{2+} deprivation to block the initiation of DNA synthesis and proliferative development of cells in late G1 phase, effectively preventing cells from entering S phase [35–38]. Nevertheless, the molecular and epigenetic mechanisms of Ca^{2+} deficiency effects on cell cycle regulation are unclear so far.

Tab. 3 Continued

Number of primer	EGTA concentration (mM)						
	0	2		4		6	
		c	d	c	d	c	d
GLH-16	3	0	1	0	2	2	0
P16	8	0	0	0	1	0	3
P17	10	1	1	2	1	2	1
Total bands	242	42	10	46	13	48	28
c+d		52		59		76	
a+b+c+d		73		84		105	

c+d – total count of bands that increased (c) or decreased (d) in intensity with EGTA treatments; a+b+c+d – total count of bands that varied form control bands.

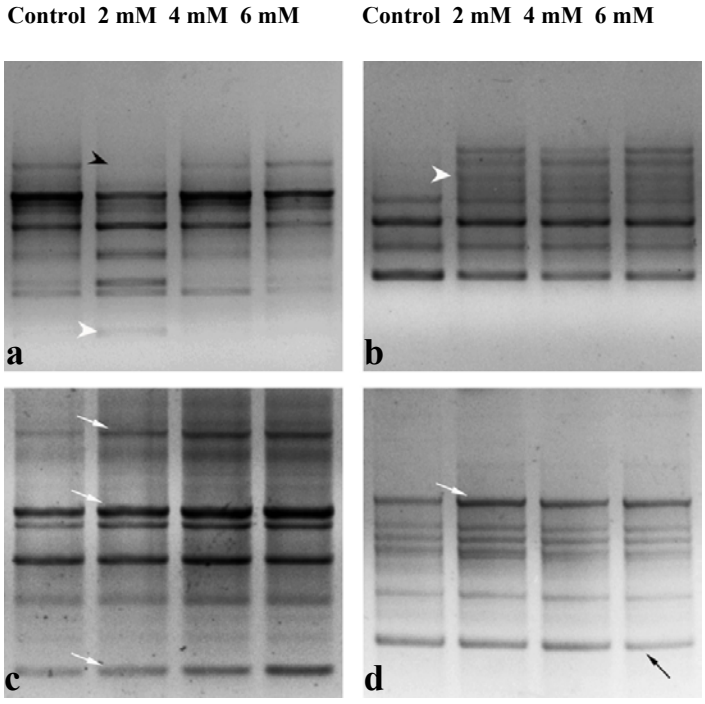

Fig. 4 RAPD bands of genomic DNA from root tips of wheat seeds treated with 0, 2, 4, or 6 mM EGTA for 24 h. Amplification was performed with the primers: (**a**) OPO-05, (**b**) S-32, (**c**) OPB-04, and (**d**) A.z-6. White arrow heads denote appearance of band not present in the control lane; black arrow heads point to bands that disappeared with EGTA treatment; fine white arrows point to bands that increased in intensity with EGTA treatment relative to controls; fine black arrows point to bands that decreased in intensity with EGTA treatment relative to controls.

The random amplified polymorphic DNA (RAPD) was developed in 1990 by Williams et al. [39] and Welsh and McClelland [40]. This assay employs PCR amplification of random fragments of genomic DNA with short primers of arbitrary nucleotide sequences under low-temperature annealing conditions [41].

The popularity of the RAPD assay has grown because it is simple, efficient, and does not require sequence information [42]. It has been widely used to assess genetic diversity within species [43,44], relationships among populations [43], identify cultivars [45], evaluate and characterize germplasm [46], and for marker-assisted selection [47] and breeding [24]. More recently, the RAPD assay has proven to be useful in investigating DNA damage and mutations within organisms under stress, toxicity, or irritation, etc. [24–26,28,48,49].

Coupled restriction enzyme digestion-random amplification (CRED-RA) is a method used to detect DNA methylation which is one of the main causes of epigenetic variations [28]. This technique allows amplification of a DNA fragment containing a cut site of a methylation sensitive restriction enzyme, only when the cytosine residue within the site is methylated and protected from digestion [50]. Thus, presence of a PCR band reveals methylated site(s). Unmethylated site(s) lead to absence of the amplicon (that would be present in uncut DNA) due to digestion by the enzyme [50].

In this paper, germinated wheat seeds were treated with EGTA, a specific Ca^{2+} chelator [19,20]. Confocal microscopy showed that nearly all of the Ca^{2+} in wheat root tip meristems was chelated by EGTA. At the same time, the root growth was inhibited and the mitotic index was decreased. These inhibitory effects were positively correlated with the EGTA treatment concentrations. Therefore, our results provide strong evidence that Ca^{2+} deprivation causes impaired growth and reductions to the mitotic index in EGTA-treated roots.

In order to explore the molecular mechanisms of the observed EGTA action, further studies on the expression of cell cycle-related gene markers, changes to genomic DNA structures and sequences, modification of DNA methylation, were conducted.

RT-PCR examination indicated that the transcriptional levels of all three investigated gene markers (*mcm2*, *pcna*, and *rdr*) related to the G1/S transition of the cell cycle were downregulated in the treated group compared to the control group. These results suggest that Ca^{2+} deprivation disturb the G1/S transition of the cell cycle, and then inhibition of normal DNA replication. Our results are constant with previous studies in animal cells [35–38,51].

Signs of genomic DNA polymorphisms, such as the appearance of extra bands, disappearance of normal bands, increase or decrease in band intensity, were detected

Tab. 4 Methylation sensitivity and restriction pattern of isoschizomers [30,33].

Types	Methylation status	Digestibility of enzymes restriction pattern	
		*Hpa*II	*Msp*I
Class I	CCGG C<u>C</u>GG	Active	Active
	GGCC GGCC		
Class II	<u>C</u>CGG	Active	Inactive
	GGCC		
Class III	C<u>C</u>GG	Inactive	Active
	GG<u>C</u>C		
Class IV	<u>C</u>CGG	Inactive	Inactive
	GGC<u>C</u>		

Underlined cytosine is methylated.

using the RAPD technique. The percent of polymorphic bands increased with increases in the EGTA treatment concentration. Ercan [29] and Atienzar et al. [52–55] suggested that the loss of normal bands may be related to the DNA damage, point mutations, or complex chromosomal rearrangements; while appearance of new bands and an increase in band intensity could be ascribed to changes in DNA conformation. The RAPD results from this study suggest that EGTA treatments induced changes to DNA structures.

In this study, the CRED-RA technique was used to assess the degree of gDNA methylation in wheat treated with 4 mM EGTA. Eight different types of bands (Tab. 6), corresponding to eight different types of DNA methylation, were identified. These bands showed the degree of DNA methylation to significantly increase compared with EGTA treated group to the control group (Tab. 4).

In conclusion, our results state clearly that Ca^{2+} in root tip meristematic cells of wheat can be chelated effectively by EGTA. We also showed that Ca^{2+} deprivation can induce changes to DNA structures and increases the levels of DNA methylation. Under the experimental conditions tested here, Ca^{2+} deprivation disrupted the cell cycle in G1 phase and prevented DNA replication, resulting in inhibition of root growth.

Tab. 5 CRED-RA profile counts of the total number of primer bands and the number of polymorphic primer bands, and the percent of polymorphisms from wheat seeds treated with 0 mM (control group) and 4 mM EGTA (treatment group).

Primers	Total number of bands				Polymorphic bands		Polymorphism (%)	
	control group		treatment group					
	*Hpa*II (a1)	*Msp*I (b1)	*Hpa*II (a2)	*Msp*I (b2)	*Hpa*II (a)	*Msp*I (b)	*Hpa*II	*Msp*I
OPD-08	10	7	9	8	1	1	11.11	12.50
OPK-08	7	6	7	8	0	2	0.00	25.00
OPO-05	10	11	11	11	1	0	9.09	0.00
S126	6	9	7	9	1	0	14.29	0.00
S127	10	10	8	7	2	3	25.00	42.86
OPB-01	3	4	3	4	2	0	66.67	0.00
OPB-11	5	5	5	5	2	0	40.00	0.00
OPK-15	3	2	3	2	2	2	66.67	100.00
Az-3	3	3	3	1	0	2	0.00	200.00
Az-5	2	3	3	3	1	0	33.33	0.00
P16	2	3	5	3	3	0	60.00	0.00
Total	61	63	64	61	15	10		
a1+a2	125							
b1+b2		124						

a1+a2 – total bands digested with *Hpa*II; b1+b2 – total bands digested with *Msp*I.

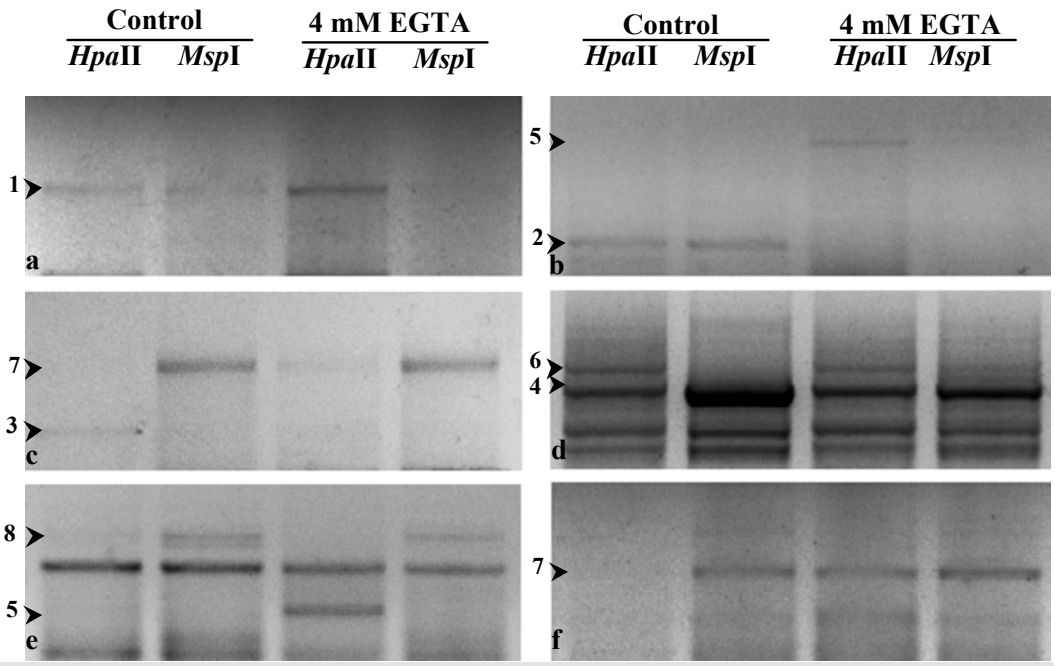

Fig. 5 CRED-RA bands of genomic DNA from root tips of wheat seeds treated with 0 (controls) or 4 mM EGTA for 24 h. Amplification was performed with the (**a**) A.z-3, (**b**) OPK-15, (**c**) OPB-11, (**d**) OPK-08, (**e**) OPB-01, (**f**) A.z-5. Numbers 1–8 represent different band types.

Tab. 6 Occurrences of CRED-RA band types in samples from control groups and 4 mM EGTA-treated groups.

Band types	Control group		Treatment group		Number	Results	
	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I		control group	treatment group
Type 1	1	1	1	0	1	Class IV	Class III
Type 2	1	1	0	0	1	Class IV	Class I
Type 3	1	0	0	0	2	Class III	Class I
Type 4	0	0	0	1	1	Class I	Class II
Type 5	0	0	1	0	4	Class I	Class III
Type 6	1	0	1	1	3	Class III	Class IV
Type 7	0	1	1	1	8	Class II	Class IV
Type 8	1	1	0	1	1	Class IV	Class II

"1" – presence of a band; "0" – absence of a band ("Control group" and "Treatment group" columns).

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Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3502/0:

Tab. S1 Primers used in RAPD analysis.

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