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ACTA BIOLOGICA CRACOVIENSIA Series Botanica 63/1: 31–41, 2021
10.24425/abcsb.2021.136698

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$LaCl_3$ Induces Genomic DNA Instability and Increases DNA Methylation Levels in Wheat Roots

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Received December 19, 2020; revision accepted April 11, 2021

Accumulation of $LaCl_3$, a well-known Ca^{2+}-channel blocker, can inhibit plant growth. However, the current understanding of its effects on gene expression is limited. In this paper, different concentrations of $LaCl_3$ (0, 0.5, 1.0, 1.5, 2.0 mM) were used to treat germinated wheat (*Triticum aestivum* L.) seeds for 24 h. The degree of root growth inhibition gradually increased with increasing $LaCl_3$ concentration. qRT-PCR analysis revealed that the expression of several key genes related to the cell cycle process, such as *pcna*, *mcm2*, *rdr* and *cyclin B*, were significantly down-regulated. Further analysis of genomic DNA instability using Random Amplified Polymorphic DNA (RAPD) and methylation levels by Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) analysis indicated a significant increase in genomic DNA polymorphisms and methylation levels. The results of this study verified that the reasons why $LaCl_3$ treatment can inhibit the growth of wheat roots are as follows: interference in the normal progression of the cell cycle, induction of genomic DNA instability and increase in DNA methylation levels.

Key words: cell cycle, Ca^{2+}-channel blocker, RAPD, CRED-RA, *Triticum aestivum*

INTRODUCTION

For growth plants can uptake mineral elements from soil. Depending on the requirement of a nutrient element to produce optimum plant growth, the nutrient is referred to as either macronutrient and micronutrient or metals (Ca, Cu, Fe, K, Mg, Mn, Mo, Ni, Zn) and non-metals (N, S, P, B, Cl) (Pandey, 2015; Singh et al., 2016). Some of them, including Cu, Fe, Mn, Mo, Ni and Zn, as well as Co and Cr, are essential for plant metabolism in trace amounts. They are essential heavy metals (HMs). However, excessive amounts of these elements can become harmful to organisms. Other HMs such as Pb, Cd, Hg, and As, do not have any beneficial effect on organisms and are thus regarded as the "main threats" since they are very harmful to both plants and animals (Arif et al., 2016; Rascio and Navari-Izzo, 2011).

Essential and non-essential heavy metals generally produce common toxic effects on plants, such as low biomass accumulation, chlorosis, inhibition of growth and photosynthesis, altered water balance and nutrient assimilation, and senescence, which ultimately cause plant death (Anjum et al., 2015; Oves et al., 2016).

Calcium is an essential plant nutrient and the constituent of cell wall, middle lamella, enzyme cofactor, etc. (Pandey, 2015). As a second messenger, Ca^{2+} plays an important role in many physiological processes in plants such as apoptosis (Levine et al., 1996), cellular signaling (Kudla et al., 2010; Seybold et al., 2014), cell ion balance (Klima et al., 2018), cell cycle (Hepler, 2005; Humeau et al., 2018), transcription regulation (Galon et al., 2010), etc.

Plant growth, development and differentiation depend on the coordination of the cell cycle (Inze

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PL ISSN 1898-0295

and Veylder, 2006). The eukaryotic cell cycle is traditionally divided into four sequential phases: G1, S, G2, and M. G1, S, and G2 together are called interphase (Alberts et al., 2014). This progress is coordinated by cell-cycle regulators that drive chromosome duplication, chromosome segregation, and cytokinesis. The accuracy of this process is controlled by cyclin-dependent kinases (CDK) and regulatory cyclins as well as checkpoint proteins that delay cell-cycle progression to detect errors and preserve genomic integrity (Mills et al., 2018). There are three major regulatory transitions in most eukaryotic cells, including the G1/S or restriction checkpoint, the G2/M or DNA replication checkpoint, and the metaphase/anaphase or spindle apparatus checkpoint (Wenzel and Singh, 2018).

In the early stage of cell cycle, cyclin D (CYCD) binds to cyclin-dependent kinase A (CDKA) to form a complex (Boniotti and Gutierrez, 2001). The complex is activated by CDK activating kinase (CAK) and CDK-dependent kinase inhibitor (CKI) or KIP-related proteins (KRPs) (Godinez-Palma et al., 2017). RBR (retinoblastoma protein-related, RBR) is phosphorylated by the activated complex, which weakens the binding to E2F, and the released E2F promotes the expression of the gene required for G1 to switch to S phase (Desvoyes et al., 2013). After entering the S phase, CYCA forms a complex with CDKA and binds to the cyclin-dependent kinase subunit and CYCB, thereby activating CDKB and entering M phase. Eventually, cyclins are hydrolyzed by the anaphase-promoting complex pathway and exit the cell division phase, and one cell cycle is completed (Barford, 2011).

In addition to CDKs and CYCs, many other substances are involved in regulating cell cycle process, including proliferating cell nuclear antigen (PCNA) (Strzalka and Ziemienowicz, 2011), mini-chromosome maintenance protein complex (MCM) (Barez et al., 2014) and ribonucleotide reductase (RNR) (Torrents, 2014). They all play important roles in G1/S transition.

Due to the important roles of Ca^{2+}, the blocking of Ca^{2+} channel will lead to a series of adverse consequences, including cytoplasmic extravasation, blocked cell wall synthesis, and inhibition of cell division in meristematic tissues (Hepler, 2005).

The purpose of this work is to explore whether the Ca^{2+}-channel blocker $LaCl_3$ affects the normal progression of the cell cycle and increases genomic DNA polymorphism and instability, as well as DNA methylation levels.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L.) seeds, CB017-A, provided by Beijing Academy of Agriculture and Forestry Science, Beijing, China, were germinated in petri dishes. Then, randomly selected seeds with uniform germination were grown in different concentrations of $LaCl_3$ (0.5, 1.0, 1.5 and 2.0 mM) solutions. Zero mM of $LaCl_3$ treatment was used as control. All of the samples were cultivated in an incubator at 22°C for 24 h in darkness.

The lengths of roots in each $LaCl_3$ concentration treatment were recorded. To estimate the mitotic index, the root tips were excised and fixed in methanol : glacial acetic acid (3:1; v/v) for 24 h at 4°C. After washing 3 times with distilled water, the root tips were further dissociated with a hydrolysis buffer (95% ethanol: concentrated hydrochloric acid = 1:1; v/v) at room temperature for 5 min, and then the root tips were macerated in a drop of methanol : glacial acetic acid (1:1; v/v). The macerated tissue was cut up with a razor blade, 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 the number of cells in mitosis to the total number of the counted cells (ca. 2000 cells) (Zhang et al., 2016).

0.2 g of wheat roots was ground to a fine powder in liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA). The genomic DNA was then removed and the reverse transcription reaction was carried out using the PrimeScript® RTReagent kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. Specific primers for the genes used in the qRT-PCR experiments were designed according to previous studies and online Primer3Plus (http://www.primer3plus.com/), and all primer sequences are listed in Table 1. Transcript levels were quantified using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with the intercalating dye SYBR-green. *Actin 2* was used as the reference gene. The reactions were amplified for 2 min at 95°C, followed by 39 cycles of 95°C for 5 s, 60°C for 30 s, 95°C 5 s, 65°C 5 s. All reactions were performed in triplicate. The relative expression level was analyzed using the $2^{-\triangle\triangle Ct}$ method.

Approximately 100 mg of wheat roots were ground in liquid nitrogen and genomic DNA (gDNA) was extracted using a PlantGen DNA kit (ComWin Biotech, China) according to the manufacturer's recommendations.

TABLE 1. Primers used in qRT-PCR.

Number	Name	Sequence
1	*Actin 2*-FP	5'-GCTGGATTCTGGTGATGGTGTGAG-3'
2	*Actin 2*-RP	5'-CAATGAGAGATGGCTGGAAGAGGAC-3'
3	*pcna*-FP	5'-CACCAAGGAGGGTGTCAAGT-3'
4	*pcna*-RP	5'-GATCTTGGGGTGCCAGATAA-3'
5	*cyclin B*-FP	5'-CCATTATTGATCGGTTCATGCGA-3'
6	*cyclin B*-RP	5'-CTAGTGCAGAATTCAGCTGTGGTA-3'
7	*rdr*-FP	5'-TTCCCCATCCGGTTCCCGCA-3'
8	*rdr*-RP	5'-TGAGCCCGCGCTTCTTGAGC-3'
9	*Mcm 2*-FP	5'-ACGACGGCGCCACCGTTATC-3'
10	*Mcm 2*-RP	5'-TTGCGATGAAGCGCCGGACT-3'

For RAPD analysis, forty random primers (Table S1) were used (Zhang et al., 2016). All of the primers were synthesized by Sangon Biotech (Shanghai, China). The PCR mixture was as follows: 1× PCR buffer (with Mg^{2+}), 0.25 mM of a dNTP mix, 10 pmol of each random primer, 1U of *Taq* polymerase, and 10 ng of gDNA template, all in a total volume of 20 μL. The amplification protocol was as follows: pre-denaturation at 94°C for 5 min; followed by 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis in 1× TAE buffer at 100V for 40 min. The RAPD profiles were visualized under a UV transilluminator. A super DNA marker (ComWin Biotech, China) was used as a standard marker.

To analyze the results, the RAPD bands were scored as '1' for the presence of bands and '0' for the absence of bands. Polymorphic bands were divided into four types: a – appearance of extra bands;, b – disappearance of normal bands, c – increase in band intensity, and d – decrease in band intensity (Ercan, 2015). Genomic template stability (GTS) is calculated as follows: GTS (%) = (1-v/n) × 100%, where v is the total number of polymorphic bands ('a' and 'b'), and 'n' is the total number of bands in the control sample. To calculate the value of polymorphism %, the value of 100 * a/n was used (Erturk et al., 2014).

Approximately 1 μg of gDNA was digested with 1 μL *Hpa* II (New England Biolabs, China) for 2 h at 37°C, and another 1 μg of gDNA was digested with 1 μL *Msp* I (New Zealand Biolabs, China) for 2 h at 37°C. DNA was purified with a DNA purification kit (ComWin Biotech, China) according to the manufacturer's instructions. The PCR analysis, including primers and product analysis, was the same as used for the RAPD profiles. For CRED-RA analysis, the average values of polymorphism (%) were calculated, and the value of 100*a/n was used as the polymorphic rate (%) (a is the average number of polymorphic bands detected in each treated sample, and n is the number of total bands in the control sample).

Data for all three replicates experiments were presented as the mean ± standard error of the mean (SEM). Student's *t* test or ANOVA and Tukey's multiple comparisons tests were used to analyze between-group comparisons, and the significance was assigned at $p < 0.05$. Images were appropriately processed by Photoshop CS5 (Adobe Systems, USA). The GraphPad Prism 5 was used for data and graphing analyses.

RESULTS

The lengths of the five groups of wheat roots treated with 0, 0.5, 1.0, 1.5 and 2.0 mM $LaCl_3$ were measured separately and the results showed that wheat root length decreased gradually along with the increasing of La^{3+}concentrations (Fig. 1). Compared to the control group (CK, 0 mM), statistical analysis indicated that the mean of root length decreased to 82.58% (0.5 mM), 65.37% (1.0 mM), 55.93% (1.5 mM), and 50.05% (2.0 mM). The difference in the root growth rate between CK and 1.5 or 2.0 mM $LaCl_3$ treatment was significant

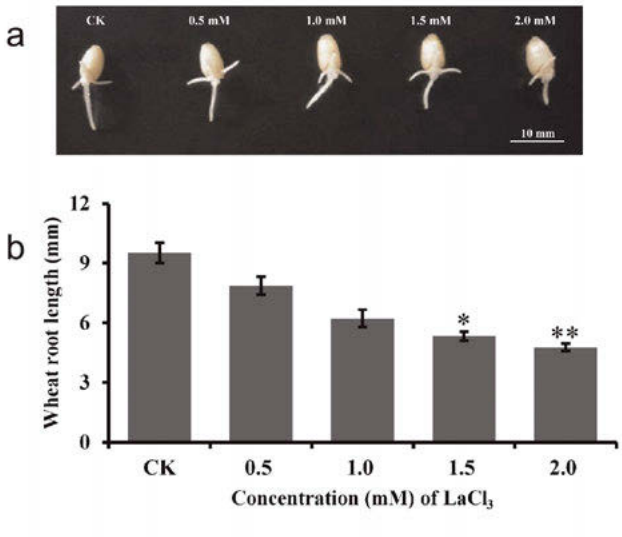

Fig.1. Growth status of wheat root in CK (0 mM $LaCl_3$) and four $LaCl_3$ treatment groups (0.5, 1.0, 1.5, 2.0 mM). (**a**) growth phenotype of wheat root in CK and four $LaCl_3$ treatments (**b**) statistical results of the root length in CK and four $LaCl_3$ treatments. Scale bar = 10 mm

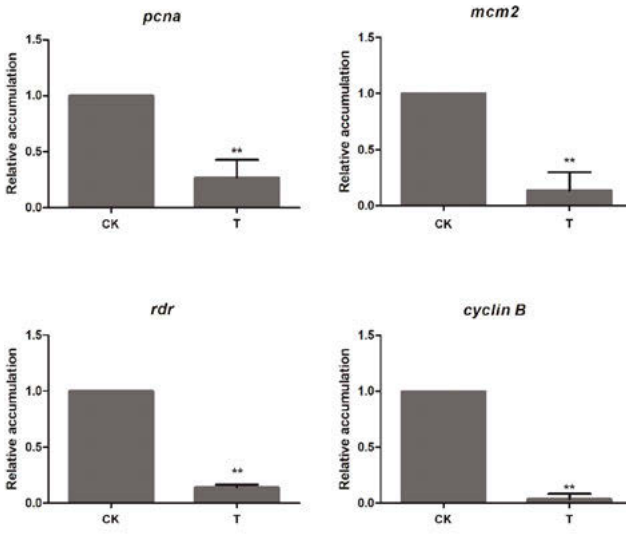

Fig. 3. Relative expression of four cell cycle-related genes in 1.5 mM $LaCl_3$-treated wheat root tips. qRT--PCR experiments and data analysis were performed in three biological replicates. CK - 0 mM $LaCl_3$, T - 1.5 mM $LaCl_3$.
* indicates significant difference ($p < 0.05$),
** indicates highly significant difference ($p < 0.01$).

($p < 0.05$) and extremely significant ($p < 0.01$), respectively.

Moreover, statistical analysis of the mitotic index showed that it decreased from 9.85% of CK to 8.40% (0.5 mM), 7.27% (1.0 mM), 5.13% (1.5 mM), and 4.08% (2.0 mM), respectively (Fig. 2).

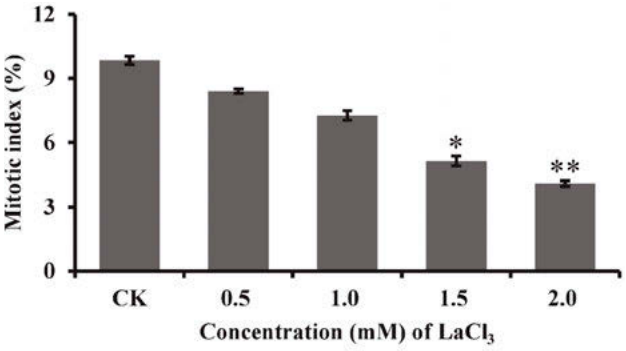

Fig. 2. The mitotic index in CK (0 mM $LaCl_3$) and four $LaCl_3$ treatment groups (0.5, 1.0, 1.5, 2.0 mM).

According to the data, we found that $LaCl_3$ concentration > 1.5 mM significantly inhibited the growth of wheat roots and the mitotic index, and its inhibition was positively correlated with $LaCl_3$ concentrations.

To further confirm that $LaCl_3$ did affect the normal progression of the cell cycle, the expression levels of 4 cell cycle-associated gene markers were detected using qRT-PCR (Fig. 3). Among them, *pcna, mcm 2* and *rdr* are considered to be important cell cycle marker genes regulating G1/S conversion, and *cyclin B* plays an important role in G2 phase. The results showed that the expression of all of the test genes decreased sharply in the 1.5 mM $LaCl_3$-treated sample compared to the control sample. We speculate that $LaCl_3$, as an effective Ca^{2+} channel blocker (Ma et al., 2018), interferes with the normal transcription of cell cycle-related genes by blocking Ca^{2+} signaling, affecting the transition of G1/S or G2/M, thereby impeding the normal progression of the entire cell cycle (Machaca, 2010). This may be the reason for the significant inhibition of wheat root length after $LaCl_3$ treatment.

Plant growth always requires precise expression of gDNA, while environmental stress induces gDNA damage or polymorphism and increases DNA methylation level (Erturk et al., 2014; Nardemir et al., 2015; Zhang et al., 2016). RAPD analysis was performed to verify the genetic effects of $LaCl_3$ treatment on wheat gDNA.

In the 40 random primers tested, we found a number of polymorphic bands in 29 primers, and all polymorphic bands (new appeared bands, disappeared bands, increase in band intensity and decrease in band intensity) were counted and analyzed (Table 2).

TABLE 2. Statistical data of RAPD results detected with 29 primers in $LaCl_3$ treatments compared with the control samples.

Primer	La^{3+} concentration (mM)																
	0	0.5				1				1.5				2.0			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
OPO-05	7	0	0	0	2	1	1	2	2	0	0	1	1	0	0	1	1
OPA-05	6	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	1
S-32	5	2	0	1	0	2	0	1	0	2	0	1	0	2	0	1	0
OBD-08	3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
OPJ-01	9	0	0	0	0	0	2	0	1	1	0	2	0	1	0	1	0
S-127	11	0	1	0	3	1	0	3	1	1	0	3	1	0	0	3	2
OPK-08	6	1	0	1	1	1	0	1	1	1	1	2	0	1	1	2	0
OPK-12	7	0	0	0	0	0	0	2	1	0	0	2	1	0	0	2	1
OPF-15	6	0	0	2	0	0	0	4	0	1	0	3	0	0	0	0	2
GLD-07	7	0	0	0	0	0	0	3	0	0	0	3	0	0	0	3	0
S-156	4	0	0	1	0	2	2	1	1	2	0	2	1	1	0	1	0
AZ-02	3	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0
S-443	4	0	0	0	2	0	0	0	0	0	0	1	1	0	0	0	2
AZ -03	7	0	2	0	0	0	1	0	0	1	1	1	0	2	3	1	2
AZ-01	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AZ-14	5	0	0	0	0	0	3	0	1	0	3	0	1	0	2	0	1
OPB-11	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OPK-02	4	1	1	0	0	2	0	0	0	2	0	2	0	2	0	3	0
S-98	5	1	0	0	0	1	0	2	0	2	0	2	0	2	0	2	0
OPH-04	6	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	0
S-132	6	2	0	0	0	2	0	0	0	4	0	2	0	3	0	2	0
AZ-13	7	3	1	1	1	3	1	1	1	3	1	1	0	3	1	3	0
GLA-17	8	0	1	1	1	0	1	1	1	0	1	3	2	0	1	3	0
S-130	6	0	1	0	0	0	0	1	0	0	0	2	0	1	0	3	0
OPK-15	5	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
OPB-01	6	0	0	1	0	1	0	1	0	1	0	2	1	1	0	2	2
OPH-05	4	1	0	1	0	1	0	1	0	2	0	1	0	2	0	3	0
AZ-05	6	0	0	0	0	1	0	1	0	1	0	1	1	1	0	1	2
AZ-07	5	0	0	1	0	0	0	1	0	1	0	1	0	1	0	1	0
Total	165	13	7	11	12	20	11	27	12	27	7	40	13	28	8	40	17
Average	5.69	5.91				6.01				6.38				6.38			
v=a+b	0	20				31				34				36			
m=c+d	0	23				39				53				57			
Polymorphism %		12.12				18.79				20.60				21.82			
GTS (%)	100	87.88				81.21				79.40				78.18			

a – new appeared bands, b – disappeared bands, c – increase in band intensity, d – decrease in band intensity; v = a + b, m = c + d. GTS (%) = (1-v/n) × 100%.

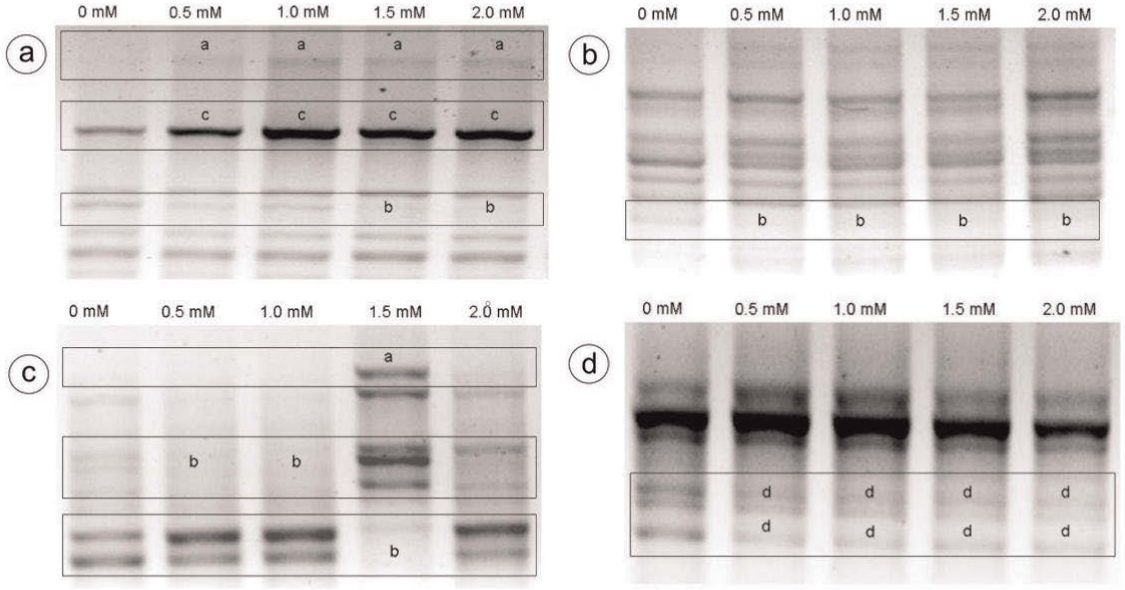

Fig. 4. RAPD profiles of genomic DNA of wheat roots treated for 24h with different concentrations of $LaCl_3$ (0, 0.5, 1.0, 1.5, 2.0 mM). (**a**) Amplified with primers: OPK-08 (**b**) Amplified with primers AZ-13 (**c**) Amplified with primers: GLA-17 (**d**) Amplified with primers: AZ-05. In figures, 'a' – new appeared bands, 'b' – disappeared bands, 'c' – increase in band intensity, 'd' – decrease in band intensity.

The new appeared bands, the disappeared bands, the increase in band intensity and decrease in band intensity in four treatment groups were shown in Fig. 4.

A total of 165 bands was obtained in the control, a maximum of 11 bands were amplified using primer S-127 and a minimum of two fragments with primer OPB-11. The average number of fragments detected was 5.69, 5.91, 6.01, 6.38, and 6.38 in the control (0 mM) and four treatment groups (0.5, 1.0, 1.5 and 2.0 mM), respectively.

According to previous studies, these polymorphic bands may be associated with genotoxic factor-induced DNA damage, point mutations or complex chromosomal rearrangements (Atienzar and Jha, 2006). Extra bands and an increase in band intensity could be ascribed to changes in DNA conformation (Erturk et al., 2014).

The statistical result of the number of bands that have changed in the four treatments was analyzed (Table 2): (1) the number of variable bands was 20, 31, 34, and 36, in 0.5, 1.0, 1.5 and 2.0 mM treatment; (2) the GTS (%) was 87.88% (0.5 mM), 81.21% (1.0 mM), 79.4% (1.5 mM), and 78.18% (2.0 mM); (3) the polymorphism (%) was 12.12% (0.5 mM), 18.79% (1.0 mM), 20.60% (1.5 mM) and 21.82% (2.0 mM). Moreover, the number of increase in bands intensity and decrease in bands intensity was 23, 39, 53, and 57 in 0.5, 1.0, 1.5 and 2.0 mM treatment, respectively. It is clear that there is a strong positive correlation between gDNA instability and $LaCl_3$ concentration, suggesting that the structure of gDNA in wheat roots may be altered or impaired by $LaCl_3$.

A change in DNA methylation patterns is one of the major causes of epigenetic variations (Jones and Sung, 2014). To detect the variety of the degree of DNA methylation, CRED-RA technique was performed.

Based on the presence or absence of a band, methylation patterns could be divided into four classes (Table 3): Class I, no bands using both enzymes, which means either an internal cytosine methylation occurs in a single strand or no methylation takes place; Class II, no bands using *Hpa* II, but bands occur using *Msp* I, which means external cytosine methylation occurs in a single strand; Class III, no bands using *Msp* I, but bands are generated using *Hpa* II, which indicates that internal cytosine methylations involve double DNA strands; and Class IV, bands are observed using both enzymes, which indicates that external cytosine methylations in double strands occur (Portis et al., 2004).

After enzyme digestion and PCR amplification, 28 of the 40 random primers generated specific and stable bands (Table 4). The number of bands

TABLE 3. Methylation sensitivity and restriction pattern of isoschizomers (Portis et al., 2004).

Types	Methylation status	Enzyme activity and bands visualized by PCR		Description
		***Hpa* II**	***Msp* I**	
Class I	CCGG; C<u>C</u>GG GGCC; GGCC	Active; No band	Active; No band	Demethylation; Inner cytosine in a single strand was methylated
Class II	<u>C</u>CGG GGCC	Active; No band	Inactive; With band	Outer cytosine in a single strand was methylated
Class III	C<u>C</u>GG GG<u>C</u>C	Inactive; With band	Active; No band	Inner cytosines in double strands was methylated
Class IV	<u>C</u>CGG GGC<u>C</u>	Inactive; With band	Inactive; With band	Outer cytosines in double strands was methylated

Underlined cytosine is methylated.

TABLE 4. CRED-RA profile variance of total number of bands, polymorphic bands and polymorphism (%) amplified with 28 primers in $LaCl_3$ treated samples compared with the control samples.

Primers	The number of bands				Polymorphic bands		Polymorphism (%)	
	Control group		Treatment group					
	***Hpa* II**	***Msp* I**	***Hpa* II**	***Msp* I**	***Hpa* II**	***Msp* I**	***Hpa* II**	***Msp* I**
OPO-05	5	5	3	5	2	0	66.67%	0.00%
OPA-05	3	4	3	3	0	1	0.00%	33.33%
AZ-09	5	4	6	3	0	1	0.00%	33.33%
OPA-10	6	6	3	6	1	0	33.33%	0.00%
S-126	10	10	8	7	2	3	25.00%	42.86%
OPJ-01	3	4	3	4	2	0	66.67%	0.00%
S-127	5	5	5	5	2	0	40.00%	0.00%
OPK-08	5	5	5	4	0	1	0.00%	25.00%
OPK-12	3	2	3	2	2	2	66.67%	100.00%
OPF-15	3	3	3	1	0	2	0.00%	200.00%
GLD-07	2	3	3	3	1	0	33.33%	0.00%
S-156	3	4	4	2	1	3	25.00%	150.00%
AZ-03	4	4	5	4	2	1	40.00%	25.00%
AZ-01	7	6	6	6	1	2	16.67%	33.33%
P-16	3	5	3	5	0	0	0.00%	0.00%
AZ-14	3	3	4	3	4	0	100.00%	0.00%
OPB-11	3	2	3	2	0	2	0.00%	100.00%
P-17	4	4	3	3	1	1	33.33%	33.33%
S-98	5	6	7	6	1	1	14.29%	16.67%
OPH-04	1	1	4	4	3	0	75.00%	0.00%
AZ-13	1	1	2	2	1	1	50.00%	50.00%
GLA-17	9	6	10	6	1	0	10.00%	0.00%
S-130	4	8	3	8	1	0	33.33%	0.00%
OPK-15	8	11	9	10	1	1	11.11%	10.00%

TABLE 4. cont.

Primers	The number of bands				Polymorphic bands		Polymorphism (%)	
	Control group		Treatment group					
	Hpa II	*Msp* I	*Hpa* II	*Msp* I	*Hpa* II	*Msp* I	*Hpa* II	*Msp* I
GLH-16	5	3	6	3	1	0	16.67%	0.00%
S-34	2	2	3	2	1	0	33.33%	0.00%
OPH-05	4	5	5	5	1	0	20.00%	0.00%
AZ-05	5	6	5	5	0	1	0.00%	20.00%
Average	4.32	4.57	4.54	4.25	1.14	0.82	25.20%	19.33%

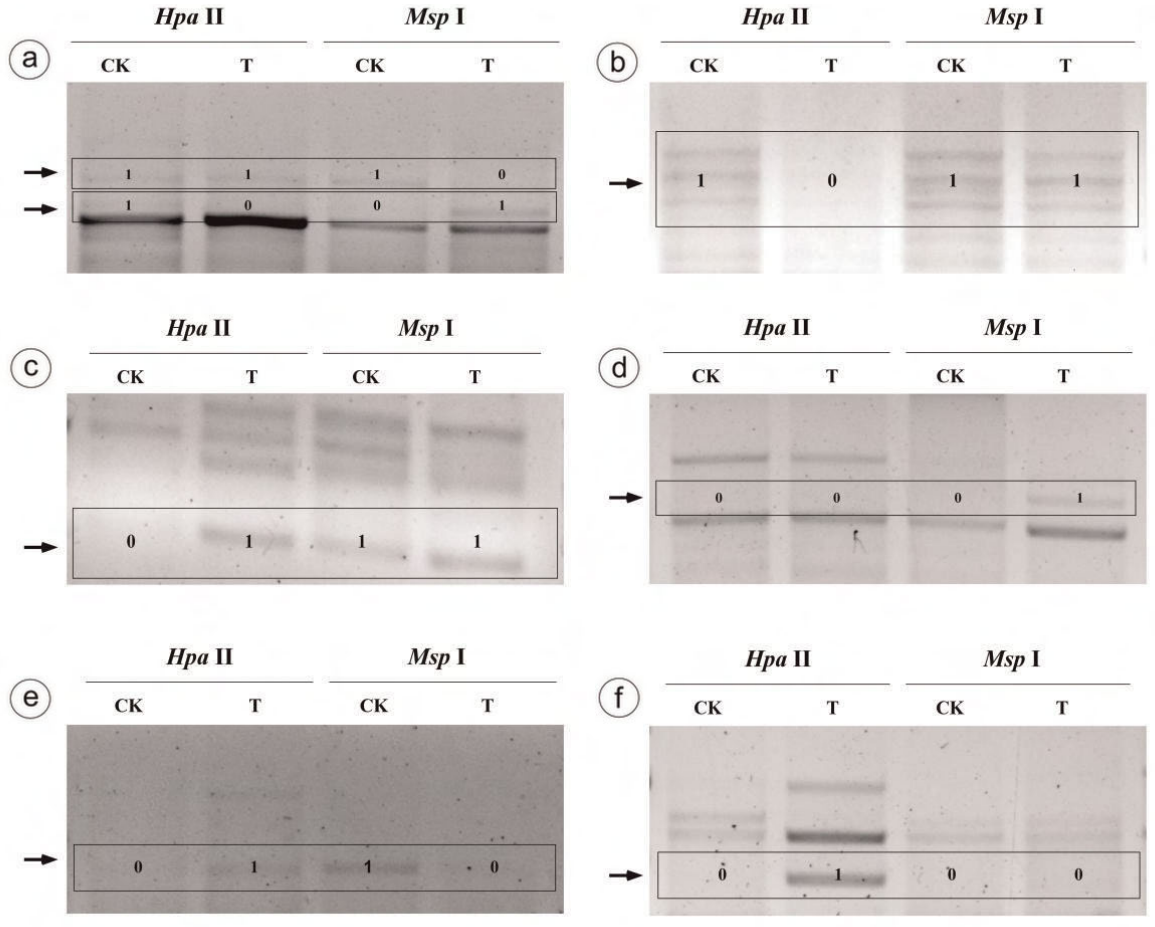

Fig. 5. CRED-RA profiles of genomic DNA of wheat roots treated for 24 h in CK and 1.5 mM of $LaCl_3$. Seven types were shown in different primers: (**a**) Type 1 and 2 in primer AZ-01 (**b**) Type 3 in primer OPA-10 (**c**) Type 4 in primer S-127 (**d**) Type 5 in primer OPB-11 (**e**) Type 6 in primer OPJ-01 (**f**) Type 7 in primer P-16. Black arrows and boxes indicate the site of corresponding band. '1' signifies presence of a band, '0' signifies absence of a band. CK - 0 mM $LaCl_3$, T - 1.5 mM $LaCl_3$.

using *Hpa* II was 4.32 in the control and 4.54 in treatment groups, and the average number of bands using *Msp* I was 4.57 in the control and 4.25 in the treatment groups. The average of polymorphic bands was 1.14 using *Hpa* II and 0.82 with *Msp* I. *Hpa* II polymorphic rate ranged from 0 to 100%, and *Msp* I polymorphic rate ranged from 0 to 200%, the average polymorphic rate was 25.20% with *Hpa* II and 19.33% with *Msp* I.

In this study, seven different types of bands were detected (Fig. 5). For example, Type 1 and 3, DNA in the control group could be digested by neither *Hpa* II nor *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 3. *Msp* I digested DNA in bands Type 1 but not in Type 3. For Types 5 and 7, DNA in the control group can be digested with *Hpa* II and *Msp* I. In turn, the pattern of *Hpa* II and *Msp* I action is different in the treated samples. *Hpa* II can digest DNA in the type 5 band, but cannot digest DNA in the type 7 band. *Msp* I does not digest DNA in the type 5 band, but can digest DNA in the type 7 band. The possible explanation of this phenomenon is that treatment with $LaCl_3$ led to demethylation of one of the DNA strands or transfer of methyl group from the outer to the inner cytosine (Table 3, Table 5). Further analysis showed that the degree of genomic DNA methylation in the treated group was significantly increased, compared with the control group. These findings indicate that $LaCl_3$ increases DNA methylation level, thereby affecting gene expression and inhibiting wheat root growth.

DISCUSSION

Blockage of Ca^{2+} channel by $LaCl_3$ has impact on plant growth, seed germination, the process of photosynthesis, resistance to environmental stresses, HMs toxicity, etc. (Hassan and El-Shafey, 2019). However, whether this blockage has any effects on plant DNA structure and modifications is unclear so far.

Therefore, in this study germinated wheat seeds were treated with $LaCl_3$. It was shown that wheat root growth was inhibited along with decrease in the mitotic index. In particular, the G1/S transition of the cell cycle was impeded. Under the circumstances, RAPD analysis was performed and it was revealed that $LaCl_3$ treatment could induce the changes of DNA conformation, point mutations or DNA damage. As a result, DNA cannot be duplicated properly, and the cell cycle cannot pass through the G1/S transition. Furthermore, CRED-RA examination indicated that the level of DNA methylation increased sharply. Since DNA methylation mediates gene silencing (Niederhuth and Schmitz, 2017), it means that gene expression levels can be reduced after $LaCl_3$ treatment.

Our results demonstrated clearly that $LaCl_3$ treatment can obstruct the cell cycle process, cause changes in DNA structure and configuration, and increase the DNA methylation level. This might be the main reason why wheat root growth is inhibited.

CONCLUSIONS

1) After $LaCl_3$ treatment, the transcription levels of several key genes involved in the wheat root tip cell cycle were significantly reduced, the cell cycle could

TABLE 5. Statistics of band types in the control and 1.5 mM $LaCl_3$ treatment using CRED-RA analysis.

Band type	Control group		Treatment group		Number	Results	
	***Hpa* II**	***Msp* I**	***Hpa* II**	***Msp* I**		**Control group**	**Treatment group**
Type 1	1	1	1	0	2	Class IV	Class III
Type 2	1	0	0	1	1	Class III	Class II
Type 3	1	1	0	1	9	Class IV	Class II
Type 4	0	1	1	1	2	Class II	Class IV
Type 5	0	0	0	1	4	Class I	Class III
Type 6	0	1	1	0	1	Class II	Class III
Type 7	0	0	1	0	11	Class I	Class III

'1' signifies presence of a band; '0' signifies absence of a band.

not proceed normally, and root growth was significantly inhibited.

2) The RAPD and CRED-RA analysis showed that the stability of gDNA was significantly decreased and the methylation level was significantly increased in wheat root tip cells after $LaCl_3$ treatment.

AUTHORS' CONTRIBUTIONS

FZ designed the experiment. XL and KM conducted the research and analyzed the data. XL and FZ wrote the manuscript. We declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (NSFC, grant No. 30971453) to FZ. We are grateful to LetPub (www.letpub.com) for providing linguistic assistance during the preparation of this manuscript.

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