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Humic acids protective activity against manganese induced LTR (long terminal repeat) retrotransposon polymorphism and genomic instability effects in *Zea mays*

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ABSTRACT

Long terminal repeat (LTR) retrotransposon polymorphism and genomic instability are considered to be one of the most important rearranging mechanisms under environmental stress. Triggering of this knowledge, we aimed to elucidate protective effect of humic acid (HA) on genomic instability and LTR retrotransposon polymorphism in *Zea mays* seeds subjected to manganese stress. REMAP (Retrotransposon-microsatellite Amplified Polymorphism) and IRAP (Inter-Retrotransposon Amplified Polymorphism) were used to define the GTS (Genomic Template Stability) levels and retrotransposon polymorphism. The results showed that all concentration used Mn led to an increase in retrotransposon polymorphism and DNA damage a reduction GTS rate showing the DNA damage. However, the treatments of humic acid (10%) together with Mn resulted as decreasing DNA damage and retrotransposon polymorphism and also increasing GTS. It can be suggested that HA applications removes the negative effects of Mn on retrotransposon polymorphism and GTS, when considering the research results.

1. Introduction

Manganese (Mn), one of the heavy metals, is a micronutrient element and indispensable in many biological activities of proteins and enzymes associated with physiological processes such as photosynthesis, respiration and synthesis of proteins and carbohydrates in plant (Lima et al., 2008). Although Mn at certain concentrations is essential for plants, it can still be toxic to organism if present at extreme concentrations. In recent years, it has been many research to expose Mn toxic effects on plant metabolisms, for example, Shi and Zhu (2008) explain as decrease of nutrient absorption, Wang et al. (2013) state as inhibition of chlorophyll biosynthesis and reduction of photosynthetic rate, Suresh et al. (1987) inform as interaction with water balance of the plant by inhibiting stomatal opening. At the same time, Mn is known as mutagenic/genotoxic compound, genotoxic activity of Mn has been showed by different mutagenic assay (Doroftei et al., 2010; Erturk et al., 2012a, 2012b). Authors reported that genotoxicity of Mn can be arise from the generation of reactive oxygen species (ROS) such as $^1O_2$,. O_2^-, $OH^\cdot$ and H_2O_2 (Li et al., 2010). ROS has wide-ranging damages to proteins, nucleic acids, and lipids (Bai et al., 2003; Erturk et al., 2015). Oxidative damage on DNA causes effects such as base modifications like alkylation, methylation and oxidation, single- and double-strand breakage, and cross-linkage to proteins. At the same time, plants have improved several antioxidant defense mechanisms to fight against heavy metal stress (Apel and Hirt, 2004; Madsen-Bouterse et al., 2010). In additional, in recent years, several studies have been reported that epigenetic mechanism influence gene expression directly or indirectly for plants adaptation under stress (Lu et al., 2007; Cheng et al., 2012). These epigenetic mechanisms can specify as DNA methylation, histone modifications, expression of non-coding RNAs, and regulation of gene expression (Erturk et al., 2015). DNA hyper or hypomethylation is correlated with gene expression, cell differentiation, and phylogenetic development associated with numerous biological processes such as transcriptional silencing of genes and transposable elements (TEs) inactivation (Xue-Lin et al., 2009). Complex eukaryotic genome have huge amount not active TEs and a few of them are transpositionally active. Some researchers have demonstrated that the retrotransposons activity increased under different stress conditions (Hirochika et al., 1996; Takeda et al., 1999; Grandbastien, 2004; Picault et al., 2009).

TEs activity can be responsible for the molecular evolution of plant resistance genes and evolution (Kumar and Bennetzen, 2000; Gbadegesin et al., 2008; Hayashi and Yoshida, 2009). Some author suggest that TEs activity can be associated with signal transduction pathways related to plant defense responses, on account of their promoter all contain *cis*-regulatory elements and their promoter elements are alike to plant protection genes (Pouteau et al., 1994; Grandbastien

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et al., 1997; Takeda et al., 1999). TEs can be resistance effects of stress. But, active transposons will be cause mutation, variations in genome size, genomic instability. In addition, several studies have been shown that HA can be to modify toxic reactions of heavy metal in plant (Tsiridis et al., 2006; Haghighi et al., 2010; Farouk et al., 2011; Tang et al., 2014).

HA are described as anticlastogenic, antitoxic action and antimutagenic (Ferrara et al., 2000, 2004, 2006; Marova et al., 2011). There are a few research claim that HA may have adverse toxicity just like cadmium, nickel, aluminum, crom and zinc (Cakmak et al., 1989; Gichner et al., 2008; Buyukkeskin et al., 2014). Under the stress conditions, HA have a positive effect on plant growth through epigenetic mechanism. Antimutagenic and growth-stimulating effects of HA alter inorganic constituents like nitrogen, potassium and micronutrients. Yildirim et al. (2014) have reported that HA have protective activity against dicamba induced genotoxicity and DNA methylation in *Phaseolus vulgaris L.* However no information is reported the retrotransposonal activity of Mn in plant and whether the protective role of HA on retrotransposonal activity changes and DNA mutation in plants. Therefore, in this study, we aimed investigated insertion polymorphism of retroelements and genomic instability in *Zea mays* seedlings subjected to Mn stress and whether HA has any role on these polymorphism.

2. Material and methods

Approximately 20–25 sterile seeds of maize (*Zea mays*) were planted onto plastic pots. 10% Humic acid obtained from lignite coal using method described by Francisco Martin (Martin, 1975). Plants instantly planting were exposed to different concentrations of $MnSO_4.H_2O$ (20, 40 and 60 mM) and the combination 1500 ppm (0.075 g) HA together with $MnSO_4.H_2O$ (20, 40 and 60 mM) and untreated plant. The seedlings were grown in normal growth conditions trought two weeks. The pots were kept at 25 ± 1 °C in 16/8 h light (intensity of 65 μmol m^{-2} s^{-1}) /dark photoperiod. Afterwards 14 days of treatment, plants were collected for each treatment and were stored at.

−80 °C. Genomic DNA was extracted from the seedlings using the method described by Sigmaz et al. (2015) with minor changes and stored at −20 °C for further use. The quality and concentration of the DNA were measured using a spectrophotometer and electrophoresis in a 0.8% (*w*/*v*) agarose gel. Five primers (Stowaway, Sukkula, BARE 1(0), N-57(*Nikita*), *Nikita*-E2647) were used in IRAP PCR reactions (Table 1) PCR amplifications were carried out in Corbatt Mastercycler Gradient Authorized Thermal Cycle in a total volume of 20 μl, containing 50 ng genomic DNA, 1× PCR buffer (without $MgCl_2$) 0.25 μM dNTP, 0.25 mM primer, 2.5 mM $MgCl_2$ and 1.5 U *Taq* DNA polymerase (BioLab M0267S).The amplification profile composed of an initial denaturation at 95 °C for 5 min, followed by 42 cycles at 94 °C for 1 min, in different annealing temperature for each primer for 1 min, 72 °C for 2 min, penultimate step 15 min at 72 °C and a final extension of 10 min at 4 °C. PCR products were separated by electrophoresis using 2% agarose gel in 0.5 × TBE buffer with constant voltage of 60 V for 180 min. Gels stained with Etd-Br visualized under UV light and photographed using gel visualization system.

The primers IRAP Stowaway, Sukkula, BARE1(0), *Nikita*-E2647 were combined with ISSR primer 8081/or 8082 that gives the best amplification in *Zea mays*. REMAP-PCR reactions were carried out with eight LTR-ISSR primer combinations (Table 1).

REMAP amplifications were performed in a total volume of 20 μl, containing 50 ng genomic DNA, 1× Taq buffer (without $MgCl_2$) 0.25 μM dNTP, 0.25 mM LTR primer, 0.25 mM ISSR primer, 2.5 mM $MgCl_2$ and 1.5 U *Taq* DNA polymerase (BioLabs M0267S).The amplification electrophoresis and visualized profiles are same with IRAP. IRAP and REMAP patterns were evaluated using the Total Lab TL120 computer software. Genomic Template Stability (GTS, %) was calculated as follows: $GTS = 100\text{-}(100\times a/n)$, 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 (Sigmaz et al. (2015)) Polymorphisms in IRAP and REMAP 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%) (Liu et al., 2005).

3. Results

Five primers out of ten for IRAP amplification (Stowaway, Sukkula, BARE 1(0), N-57(*Nikita*), *Nikita*-E2647) were used. Four retrotransposon primers (Stowaway, Sukkula, BARE1(0), *Nikita*-E2647) combined with ISSR primers (ISSR 8081or/8082) for REMAP amplification resulted in specific and stable DNA profiles *in Z. mays* genome. The IRAP and REMAP profiles showed significant differences between treated (Mn and/or Mn-HA treatments) and untreated plant. These changes are characterized by variation in band intensity, loss of normal bands or appearance of new bands. For all the primers used in the study, polymorphic bands (loss and/or gain of bands) were detected in Mn and Mn-HA treated plants compared with control. The molecular size of bands for IRAP and REMAP profiles range from 310 bp (BARE1(0)- 40 mM Mn + 1500 ppm HA) to 12,400 bp (*Stowaway* 20 mM Mn) and from 128 bp (*Nikita*-E2647 + ISSR 8081, 60 mM Mn) to 10,400 bp (*Nikita*-E2647+ ISSR 8082, 40 mM Mn) in treated plant, respectively (Table 1).

In IRAP, results showed that all doses of the treated Mn caused an increase (from 22.7% to 39.60%) in rate of the retrotransposon-induced polymorphism. Moreover, polymorphism was reduced in dose of humic acid adminisreated with manganese. Changes in REMAP profiles were also measured as GTS (which is a qualitative measurement reflecting the changes in REMAP patterns) in relation to the pattern showed in the control plants. In REMAP, generally increased Mn concentration resulted in decreasing GTS value (from 74.1% to 58.0%). On the contrary, these effects of Mn-induced retrotransposition polymorphism were decreased after treatment of HA. HA treated along with manganese were increased GTS value (Table 1).

In addition to all these results, the higher level of manganese (60 mM) has toxic effects according to both IRAP and REMAP results. And HA has played an important role in reduced of these toxic effects.

4. Discussion

Even, Mn is an essential micronutrient for plant growth; extreme concentrations of Mn can exhibit toxic effects on organism (Millaleo et al., 2010). In the present study, Retrotransposonal polymorphism, a decrease in GTS and an increase in DNA damage, were observed in *Z. mays* treated with Mn (Table 1).These GTS decreased and DNA damage increased might result from Mn-caused the generation of reactive oxygen species (ROS) such as $^1O_2$,. O_2^-,·OH and H_2O_2 (Li et al., 2010). Oxidative damage on DNA causes effects like base modifications, like alkylation, methylation and oxidation, single and double strand breakage and cross-linkage to proteins. As result of these modifications, transcription and translation are affected.

Previous studies found that Mn affects inhibition transcription and translation by DNA damage in plant and animal organisms (Nicosia et al., 2014; Zhu et al., 2014; Sheshadri et al., 2015). In additional, our study showed LTR retrotransposon polymorphism, induced Stowaway, Sukkula, BARE 1(0), N-57(*Nikita*), *Nikita*-E2647 in *Z. mays* seeds exposed to Mn stresses. Plants have been developed a set of biodefence to cope with these sources of damage by differential expression of several genes under the different stresses. One of the molecular mechanisms is retrotransposons activity. Retrotransposons are mostly quiescent, but under stress conditions force them to be active. Many studies, there are report that the transcriptional levels of retrotransposons increase under different stress in plant (Hirochika et al., 1996; Takeda et al., 1999; Grandbastien, 2004; Picault et al., 2009). Grandbastien et al.

Table 1
Molecular sizes (bp) of appeared/disappeared bands REMAP and IRAP profiles of manganase and Mn-humic acid treated *Zea mays* seedlings and value of GTS and polymorphism (%).

REMAP Primers (5′ → 3′)	+/-	C	MANGANESE			MANGANESE + HUMIC ACID			IRAP Primers (5′ → 3′)	+/−	C	MANGANESE			MANGANESE + HUMIC ACID		
			20 mM	40 mM	60 mM	20 mM + 1500 ppm HA	40 mM + 1500 ppm HA	40 mM + 1500 ppm HA				20 mM	40 mM	60 mM	20 mM + 1500 ppm HA	40 mM + 1500 ppm HA	40 mM + 1500 ppm HA
Nikita-E2647 + 8081 (*ACCCCT CTAGGCGACATCC* + *GAGA GAGAGAGAGAGAC*)	+	**15**	-	351;303	410;128	703;	729;370; 355	374;	***Stowaway* (*CTTATA TTTAGGAACGGAGGGA GT*)**	+	**11**	12,400;	11,200;	12,000;	12,800;	12,800;	11,600;
	-		935; 306	935;193; 184	935;306; 193;184	-	-	935;658; 193					931;				
Stowaway + 8081 (*CTTATATT TAGGAACGGAGGGAGT* + *GAGAGAGAGAGAGAGAC*)	+	**13**	682	839;670; 302	1113; 831;	951;	747;	974;		−		10,400	508;	10,400; 1349	−	10,400	725;
	-		1200	-	1200	-	297;	-	**N57-(*Nikita*) (CGCA TTTGTTCAAGCCTAAA CC)**	+	**7**	1275;	1314;	1286; 1276;	1295;	1290; 1276;	1295; 1276
Nikita-E2647 + 8082 (*ACCCCT CTAGGCGACATCC* + *CTCTCTCT CTCTCTCTCTG*)	+	**8**	10,000;	10,800;	1200;	-	-	-					1276; 1013	1074; 846			
	-		10,400; 1211	10,400; 1211	10,400; 1211; 921	10,400; 1211	10,400; 1211;	10,400;		−		691	−		−	-	691
Bare 1(0) + 8082 (*CTAGGGCA TAATTCCAACA CTCTCTCT CTCTCTCTCTG*)	+	**8**	-	-	-	-	-	-	**Sukkula (GATAGGGT CGCATCTTGGGC GTGAC)**	+	**12**	−	441	447	−	-	-
	−		1074; 859;	1074;859;	1074; 859	1074	1074	1074									
Sukkula + 8082 (GATAGGGT CGCATCTTGGGCGTGAC + CTCTCTCTCTCTCTCTCTG)	+	**10**	-	1155;	371;853	853	655	660		−		8461; 1363	8461; 1363;	8461; 1363; 854;	8461;	8461; 1363	8461; 1363;
	-		281;592; 719	1086; 769; 719	1086; 7149; 444;	1086;	1086; 719;	1086; 719;	***Nikita*-E2647 (ACCC CTCTAGGCGACATCC)**	+	**10**	1371;	1156; 894;	882;	984;	1336;	1336;
Sukkula + 8081 (GATAGGGT CGCATCTTGGGCGTGAC + *GAGAGAGAGAGAGAGAC*)	+	**10**	626;	-	337;	341	300	-									
	-		517; 249	800; 517; 249; 200	800; 295; 200	800	800	800; 200; 185									
Stowaway + 8082 (*CTTATATT TAGGAACGGAGGGAGT* + CTCTCTCTCTCTCTCTCTG)	+	**7**	1342	1801; 1141; 927	1801; 1517; 1191; 700	-	-	1517;		-		1419;	1091	1091; 496	−	1261	1261
	−		1414;	−	1414;	-	-	1414;	**BARE1(0) (*CTAGGG CATAATTCCAACA*)**	+	**10**	-	-	368	-	-	310
Bare 1(0) + 8081 (*CTAGGGCA TAATTCCAACA* + *GAGAGAGA GAGAGAGAGAC*)	+	**11**	-	-	-	-	-	-		-		1206; 1178; 1069	1206; 1178; 1069	1206; 1178; 1069;681	1206; 1178;	1206; 1178	1206; 1178
	-		669;575; 429	669;575; 528;429	669;575; 528;429	508;429	528; 429;	575; 528 429									
GTS %			**74.1**	**62.2**	**58.0**	**83.7**	**82.3**	**79.8**				**77.3**	**68.9**	**60.4**	**87.6**	**79.3**	**74.4**
Polymorphism %			**25.9**	**37.8**	**42.0**	**16.3**	**17.6**	**20.1**				**22.7**	**31.1**	**39.6**	**12.4**	**20.7**	**25.5**

(1997) demonstrated that the expression of *Tnt1* and *Tto1* retrotransposons increased in tobacco plant exposed abiotic stresses such as $CuCl_2$ and salt stress. Some researchers suggest that the primary reason for sequence similarity on defense genes' motifs involved transcriptional activations is retrotransposons activity in plants (Bichler and Herrmann, 1990; Pastuglia et al., 1997; Gutterson and Reuber, 2004; Ross and Shen, 2006). Tuteja (2007) expresses the retrotransposons activity changes the membrane receptors, ion channels, receptor-like kinases (RLK) and histidine kinase (HK) receiving first stress signals or intracellular Ca^{+2}, inositol phosphatases (INSP), ROS and abscisic acid (ABA), including the production of secondary signaling molecules. Our results indicated that Mn played an effective role in epigenetically changes. However, these effects of Mn on cells altered and decreased by different concentration of HA treatment. The protective effect of humic acids against many heavy metals and pesticide toxicity such as cadmium, zinc, aluminum, mercury, maleic hydrazide and dicamba have also been studied in plant and animal (De Marco et al., 1999; Ferrara et al., 2000, 2004; Hammock et al., 2003; Koukal et al., 2003; Voets et al., 2004; Buyukkeskin et al., 2014; Yildirim et al., 2014). The primary reason of HA protective effects may be associated with the presence and role of HA in glutathione (GSH) biosynthesis (Vaughan et al., 1985; Concheri et al., 1994; De Marco et al., 1999). Some researchers have been reported that humic acids (HAs) can be anticlastogenic and can expose antitoxic action and antimutagenic activity (Marova et al., 2011). Humic acid may be role in coordinated action of non-enzymatic antioxidants and ROS detoxificaiton enzymes. Todorava et al. reported that it was founded protective role of application of humic acid on triticale seeding subjected to UVB stress through a cordinate section of nonenzymatic antioxidants and ROS detoxification enzymes. In present study, all concentration of HA against Mn toxicity performed as a protective role. HA protective effects are probably related to its antioxidant actions and also its enzymatic activation system actions. The contribution effect of HA may improve the plant resistance against heavy metals toxicity. Our research findings suggest that the HA could serve the purpose as an antimutagenic activity when need against action to chemical mutagens in the plant.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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