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FOOD DYE AMARANTH-CAUSED RETROTRANSPOSON POLYMORPHISM AND SALICILIC ACID PROTECTION ON *Triticum aestivum* L.

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Abstract

In the present study, amaranth, which is a very important azo dye used in food, drug, paper, cosmetic and textile industries, was evaluated for its genotoxic potential by the plant model organism *Triticum aestivum* (wheat). The impacts of salicylic acid on genotoxic damage, and the alterations in DNA methylation due to amaranth stress in *Triticum aestivum* L. were also investigated. The marker techniques of Inter-retrotransposon amplified polymorphisms (IRAP) and Inter-Simple Sequence Repeats (ISSR) were used to identify genotoxic damage. Amaranth at four different doses (50, 100, 250, 500 mg/l) and salicylic acid at two different doses (0.50 mM and 0.75 mM) were used. Amaranth at all doses caused genotoxic damage and reduced Genomic template stability (GTS %). It was found that there was an increase in genomic stability due to doses of salicylic acid in combination with Amaranth.

Key words: amaranth, GTS, IRAP

Introduction. Amaranth, one of the colourant food dyes, is mostly preferred as a colourant in the soft drinks industry, as well as in many fruit flavoured beverages, and confectionery [1]. In addition to food industry, many industries such as cosmetics, textile and paper production commonly use amaranth [2].

Food additives may lead to toxic effects and also mutations and a mutagenic effect, which may be a potential source of danger for people. Because of its azo

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functional group and aromatic ring structure, amaranth is argued to be harmful to human health [3]. In the study conducted by Mpountoukas et al. in 2010 [2], it was indicated that amaranth may bind to DNA and lead to chromosomal abnormalities.

Salicylates are named as signalling agents produced by the plant only in case of need for activation of plant defensive genes under stress conditions. An increase occurs in the synthesis of allelochemical and defense proteins that ensure protection for the plant, as these signals become intense [4]. The protection of the plant against both pathogenic stresses and heavy metal stress is provided by SA. Heavy-metal-induced ROS (Reactive Oxygen Species) are largely unstable compounds and can be very damaging to the plant. Thus, SA is known to activate the antioxidant defense system and to be useful for the plant to protect itself against stress [5,6].

It is known that cytosine methylation is changed throughout the genome by environmental stresses. By utilizing epigenetic regulatory strategies like DNA methylation without changing the DNA sequence, plants quickly adapt to new conditions [7–9].

Through DNA demethylation, it is possible to activate transposon elements, which are an important part of plant genomes and inactivated by DNA methylation, by environmental factors [10]. Transcriptional and post-transcriptional epigenetic control appears to be crucial for the restriction of retrotransposition induced by environmental stress [11,12].

In the present study, the genotoxic effect of amaranth, which is carcinogenic to humans and animals, was tested on wheat. There is no study that investigates the retrotransposonal action of amaranth in plants and the protection effect of SA on DNA damage and retrotransposonal activity changes in plants.

Materials and methods. ***Plant materials and treatments.*** 0.5% sodium hypochlorite was used to sterilize *Triticum aestivum* L. seeds for 10 min, and then they were rinsed with sterile water. Until primary roots were grown at 0.5–1 cm length, the seeds were subjected to different concentrations of [0 (control), (50 mg/l, 100 mg/l, 250 mg/l, 500 mg/l)], and then they were grown in plastic pots filled with a soil mix under greenhouse conditions.

Then, by using salicylic acid solutions (0.50 mM and 0.75 mM) (three consecutive days), the leaves were sprayed at three-to-four-leaf stages. Furthermore, the untreated control group was sprayed with deionized distilled water. All leaves treated were collected after 7 days (totally 21-day-old seedlings) and were stored at 80 °C.

Isolation of genomic DNA. Genomic DNA isolation was performed using a previously described procedure [13], and after the isolation, the samples were stored at -20 °C until study.

The concentration of DNA was measured by NanoDrop spectrophotometer and performed for electrophoresis and 1% (w/v) agarose gel to see DNA quality.

IRAP amplification. Five LTR (Long terminal repeat) primers were used for IRAP reactions. Twenty microlitres reaction mixture containing 50 ng DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.25 µM dNTP, 1.5 U Taq DNA polymerase and 0.25 mM primer was used to perform PCR amplification. The profile of amplification consisted of one cycle at 95 °C, 5 min; followed by 42 cycles at 94 °C; 1 min, in diversified structure temperature for every one primer; 1 min, 72 °C; 2 min, next to last elongation 72 °C; 15 min.

ISSR amplification. ISSR amplifications were tested by twenty oligonucleotide primers, and eight of them were selected and used for the studies in the future. The PCR mixture (25 µL) was prepared with 40 ng of template DNA, 10× buffer, 200 Mm of each of the four dNTPs, 1 U of Taq DNA polymerase, 0.5 mM of primer and 1.5 mM $MgCl_2$. A thermal cycler programmed for an initial denaturation at the temperature of 94 °C for the period of 5 min followed by 35 cycles of 45 s at the temperature of 94 °C, 1 min at the annealing temperature and 1 min at the temperature of 72 °C, ends with a final extension stage of 7 min at the temperature of 72 °C was used to perform amplification.

Electrophoresis. 6X gel loading buffer was used to blend PCR products. Then, they were electrophoresed on 1.5% agarose gel for 2–3 h at 80 V in 0.5× TBE buffer. Ethidium bromide solution was used to stain the gel for 40 min, and the amplified DNA products were determined using the Bio Doc Image Analysis System with Uvi-soft analysis package.

Analysis. Appraised wielding the Total Lab TL120 computer software program was used to analyze the ISSR and IRAP structures. The genomic template stability (GTS, %) was calculated according to the following description: $\text{GTS} = 100 - (100 \times a/n)$, where a refers to the average number of polymorphic bands determined in every specimen treated, and n refers to the number of total bands in the control specimen. Polymorphisms in ISSR and IRAP profiles were revealed as the disappearance of a normal band and the appearance of a new band compared to the control. $100 \times a/n$ formula was used to calculate the polymorphism value %.

Results and discussion. ***IRAP analysis.*** In the present study, amaranth at four different concentrations (50, 100, 250 and 500 mM) and SA at two different concentrations (0.5 and 0.75 mg/L) were used to analyze the IRAP and ISSR. With regard to the IRAP analysis results, striking polymorphism was seen in the Amaranth stressed wheat samples. Based on these differences, while some primers led to alterations in several amplification products (5LTR1), more complicated patterns of gains or losses were provided by the others. Among seven primers used for the IRAP technique, five of them provided specific and stable DNA profiles in wheat genome.

A total of 167 bands were generated by the IRAP procedure. 3–7 bands were generated in untreated one (control) by each primer. In all treatments, the IRAP bands profile molecular size varied between 6 bp (SUKKULA) and 1212 bp

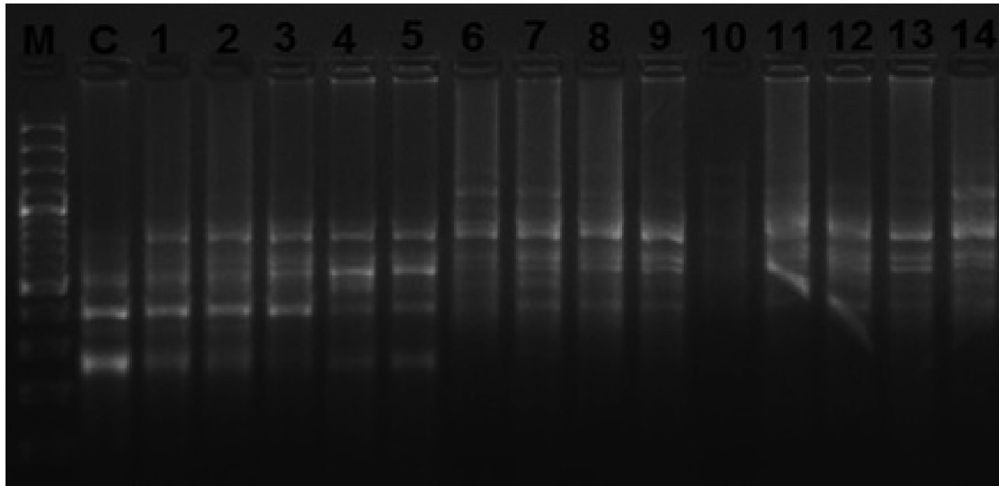

Fig. 1. IRAP banding pattern amplified with primer 3LTR-5 (M: Marker, C: Control, Salicylic acid 1 (0.5 mg/L), S. A2 (0.75 mg/L), Amaranth 1 (50 mg/L), Amaranth 1 S.A 1, Amaranth 1, S.A 2 Amaranth 2 (100 mg/L), Amaranth 2 S.A 1, Amaranth 2 S.A 2, Amaranth 3 (250 mg/L), Amaranth 3 S.A 1, Amaranth 3 S.A 2, Amaranth 4 (500 mg/L), Amaranth 4 S.A 1, Amaranth 4 S.A 2)

(LTR6150). There were significant differences in IRAP profiles between the amaranth and SA treated and untreated plants (Fig. 1). The increase in amaranth concentrations generally leads to a reduction in GTS rate (from 54.2 to 37.5%) in IRAP. While the highest GTS value (54.2%) was found in 50 mg/L amaranth treatment, the lowest GTS value (37.5%) was found in 500 mg/L amaranth treatment in the SA-untreated plants. Nevertheless, the treatment of SA led to increased GTS rate on amaranth treated plants (Table 1). 0.75 mg/L SA was found to be the best dose for the protection of the plants in food dye stress.

ISSR analysis. For ISSR, 74 bands were counted in the control as presented in Table 2. In addition, quite large molecular size scale 215 (UBC-824) to 1400 (UBC-825) bp was found for every primer. A total of 487 ISSR bands were produced by eight ISSR primers. Based on the ISSR analysis results, the highest and the lowest polymorphism (%) values were obtained at 500 mg/L amaranth (43.2%) and at 50 mg/L amaranth (36.5%) doses, respectively (Table 2). Along with the increase in the concentration of amaranth treatments, the GTS values tended to decrease. A GTS value of (63.5%) was observed in 50 mg/L amaranth, while a GTS value of (56.8%) was observed in 500 mg/L amaranth. Nevertheless, the treatment of SA led to increased GTS rate on amaranth treated plants.

In this study, genotoxic damage caused and retrotransposon polymorphism changes by amaranth and the healing effect of SA was examined. It is the first study conducted in this context.

It has been reported that food colours are well-known mutagenic and clastogenic agents; the genetic effects which can be observed as a function of dose and

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Molecular sizes (bp) of appeared/disappeared bands IRAP profiles of amaranth and amaranth-salicylic acid treated wheat seedlings and value of GTS and polymorphism (%)

Primers	Control	SA1	SA2	Am1	Am1 SA1	Am1 SA2	Am2	Am2 SA1	Am2 SA2	Am3	Am3 SA1	Am3 SA2	Am4	Am4 SA1	Am4 SA2
LTR 6149-5	4	−234	—	−40	+552	+442	−544	+552	+638	−64	−64	+552	−544	−544	+25
		+56			+48	+203	+48	−211	+552			+56	−226	+56	
		+20				+48		+64	−71				−71		
LTR6150	5	+772	+181	+1212	+824	+816	−833	+824	+322	−727	+1195	+278	+1212	−798	−833
				−789	+507	+560	−296	+304		−489	+798		−824	−498	−498
				−507	+225	+278				−225	−542		−313	+287	+216
				−243						+207	−260				
3LTR-5	5	+208	+201	+215	+248	+261	+281	+215	+241	−221	+248	−221	−254	+221	+221
		−89	−95	+230	+162	+175	+215			−155			−221		
													−215		
5LTR1	3	+387	+351	−366	−351	+365	−380	—	—	−365	+373	−380	−372	−323	+308
		+301	−286	−294	+35	+308	−293			−293	+301	+287	−293	+272	−222
			+64			+28				+35		+21	−13	+10	+14
SUKKULA	7	+387	—	−365	−351	−365	−380	—	—	−365	+372	−380	−372	+322	+308
		+301		−293	+35	+308	−293			−293	+301	+286	−293	−272	−222
						+28				−35		+21	−13	−6	−13
Polymorphism %		41.6	37.5	45.8	54.2	58.3	41.6	25	21	45.8	41.6	41.6	62.5	50	45.8
GTS Value %		58.4	62.5	54.2	45.8	41.7	58.4	75	79	54.2	58.4	58.4	37.5	50	54.2

Abbreviations: SA1: Salicylic acid (0.5 mg/L); SA2: Salicylic acid (0.75 mg/L); Am1: Amaranth (50 mgŁ); Am2: Amaranth (100 mgŁ); Am3: Amaranth (250 mgŁ); Am4: Amaranth (500 mgŁ);

T a b l e 2

Molecular sizes (bp) of appeared/disappeared bands ISSR profiles of amaranth and amaranth-salicylic acid treated wheat seedlings and value of GTS and polymorphism (%)

Primers	Control	SA1	SA2	Am1	Am1 SA1	Am1 SA2	Am2	Am2 SA1	Am2 SA2	Am3	Am3 SA1	Am3 SA2	Am4	Am4 SA1	Am4 SA2
UBC 807	8	+1083	+1100	+1083	+1074	+1074	−980	+1257	+1245	−653	+645	+645	−1223	607	+1000
		+637	+645	+645	+661	+990	−614	−969	+1000	−479	+513	+453	+1009	+460	−936
		+506	+506	−500	+500	+629	−486	+621	+629	+307	−405	+383	−872	−312	+592
		−329	−303	−312	−417	+513	−400	−486	−242	−312	−300	−637	−248	+460	
		−264			−316	−388	+257				−248		−506		
													+320		
UBC 816	6	+778	+778	+789	−755	+789	−1091	+1086	+767	−800	+1185	+767	−1076	−1076	+1063
		+509	−654	−641	−550	+571	−809	+789	+635	+635	−1070	+648	+767	+778	809
		−411	+529	−550	+469	−463	−635	+641	−581	−571	−800	−561	−622	+622	+622
		+369	−411	−408	+414	−411	−591	−581	−476	−457	+591	+416	−457	−550	−550
						−483	−483		+408			−411	+442	+408	
						−411							+406		
UBC 817	12	−825	+1154	−775	+1285	—	+1223	+1223	+1056	−1140	—	+1126	−1177	—	—
		+690	+1087	+690	+1238		−1162	+1154	−1000	−1113		+1087	−1126		
		+651	−1036	−637	+1169		−1113	−1113	−763	+1062		−1050	+1087		
		−568	+933	−581	+1081		−1075	+1062	+637	+666		−982	−1050		
		−511	+606	−488			+1015	+666	+587	−593		−900	+982		
		+466		−460			+933		−555			−775	−900		
		+292					−825					+644			
UBC 824	5	+718	−700	−709	−709	+689	−700	−700	—	−700	+718	−664	+578	+578	+612
		+436	+588	+588	+551	−568	+559	−568		+551	−568	+543	−517	−517	−517
		+282	+441		+431	+446	+222	+446		−389	+446	+446		+261	+405
			−286		−400						+282			+191	−250
											+215				

T a b l e 2

(Continued)

Primers	Control	SA1	SA2	Am1	Am1 SA1	Am1 SA2	Am2	Am2 SA1	Am2 SA2	Am3	Am3 SA1	Am3 SA2	Am4	Am4 SA1	Am4 SA2
UBC 825	9	+1312	—	+1306	—	+1354	—	−1280	−1280	−1280	+1293	+1217	+1250	+1400	+900
		+1250		+1209		−1280		+1226	+1209	+1100	+1107	+1133	+1171	+1368	+719
		+1217		−1107		+1133		+1142	−1107	+781	+873	−1092	+1079	+1250	
		+1142		+1061		+1021		+1061		+581	+681	+1031	+1031	+1171	
		−1092		−987		+610		+900		−408	+573	−860	+914	−1107	
		+987						−565			−376			+1061	
		−600									−262			−944	
UBC 856	15	+1027	+1137	−1115	+1122	+1130	—	−1115	+1137	+1122	+1144	—	+1130	+1122	+1107
		+561	+871	−900	+913	+913		+913	−926	+913	+385		−938	−885	+871
		+410		+746	−737	+719		−737	+709	−737	−385			+700	−689
				−521	+500	+506			+506	+506				−480	+473
					−395	−395				−400				−385	
UBC 840	8	+848	—	—	+708	+621	−954	+800	+848	−733	+646	+979	−715	+1157	+866
		+716			+590	+478	−581	+716	+560	−610	+525	−715	−513	+849	+645
		+581			−462		−500	−610	+537	+535		+537	−360	+700	+492
		+537					+537							−565	−400
UBC 855	11	−1325	−1325	−1170	+1278	+1278	+1292	—	+1300	—	−1325	+1192	−1325	+1331	+1319
		+1177	+1215	−944	+1149	−1177	−1170		−1170		+1200		−1215	+1192	−1170
		−1085	−1177		+1061	+1107	−1092		+1077				−958	+929	1092
		+958	+1107		−872	−914	−914		+929						+914
		−581	+929		+590	−600	−600								−700
			+609												+643
															+515
Polymorphism %		51.4	33.8	36.5	39.1	39.1	40.5	39.1	36.5	40.5	37.8	36.5	43.2	44.6	40.5
GTS Value %		48.6	66.2	63.5	60.9	60.9	59.5	60.9	63.5	59.5	62.2	63.5	56.8	55.4	59.5

For abbreviations see Table 1.

exposure period are base substitutions, sister chromatid exchanges, chromosomal aberrations and micronuclei, both in vivo and in vitro [14].

Studies have been conducted to test the genotoxic effect of amaranth in plants; mitotic index and micronucleus testing in *Allium cepa* L. root tip cells by Gomes et al. in 2013 [14] and micronucleus testing in *Vicia faba* by Zheng et al. in 2009 [15].

Random amplified polymorphic DNA (RAPD) was used as a marker for the determination of the genotoxic effect of synthetic dyes used as food additives [17].

The present study showed that amaranth caused DNA damage. These results are in agreement with the findings of previous studies on the effect of food dyes genotoxicity on different organisms [14,15,17].

Furthermore, it was demonstrated in this study that amaranth changed the LTR retrotransposon polymorphism and led to genomic template stability in wheat genome. LTR RTs (Long Terminal Repeat Retrotransposons) may pose a challenge for the integrity of the host genome due to their movement and potential to lead to mutagenic effects by epigenetic regulation. There are developed mechanisms of host genomes so that they can control the action and possible mutagenic effects of LTR RTs. Nevertheless, some LTR RTs escape from these defense mechanisms under stress conditions and continue to transpose [18]. All LTR RTs used in this study led to polymorphism in the wheat genome against stress.

One of the naturally synthesized plant hormones is SA, which has various roles in tolerance with both biotic and abiotic stress [19]. In general, SA has a role in the regulation of gene expression in the events like seed germination, seedling formation, cell growth, respiration, stomatal closure, senescence, creating responses to abiotic stresses, providing thermotolerance, flowering and fruit formation, and the resistance to pathogens and these roles are also executed by transcriptional regulation [4]. The treatment with SA is involved in the induction of antioxidant defense responses by promoting various antioxidant enzymes required for plant protection against water and salt stresses as well as other stress types [5,20]. In the present study, it was seen that stress induced the polymorphisms at the level of LTR and caused an increase in genomic instability and DNA damage compared to the control plants. Nevertheless, it was seen that there was a reduction in polymorphisms and an increase in genomic stability after the treatment with SA. This protective effect of SA is probably associated with its antioxidant and enzymatic activation properties.

In the literature review, there was no data on the effect of stress on LTR movements and the curative effect of SA. So, this study is original in this regard.

Conclusion. In summary, it should be concluded that amaranth stress readily induced genomic instability and LTR retrotransposon polymorphism. It was seen that the retrotransposon polymorphism was significantly reduced by the SA applied. Furthermore, it was concluded that the IRAP technique could be used

for the determination of the genotoxic effect of a food dye, because it identifies both DNA damage and retrotransposon movement.

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