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Evaluation of Nonanchored Inter Simple Sequence Repeat (ISSR) Marker to Detect DNA Damage in Common Bean (*Phaseolus vulgaris* L.) Exposed to Acrylamide

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ABSTRACT : Acrylamide is present as a contaminant in heated food products, predominantly from the precursor asparagine. Nonanchored inter simple sequence repeats (ISSRs) are arbitrary multiloci markers produced by PCR amplification with a microsatellite primer. In order to assess the feasibility of microsatellite primers as markers for DNA damage, the study was conducted on common bean (*Phaseolus vulgaris* L.) exposed to different concentrations of acrylamide. Polymorphisms were abundant among plant samples treated with acrylamide in comparison to control (untreated one) tested with 4- tri-nucleotide, 2 tetra-nucleotide, and 3- dinucelotide primers. The primer (CCG)4 was the best tested primer to generate polymorphism between the DNA of plants treated or not by acrylamide. Polymorphisms became evident as the presence and absence of DNA fragments in treated samples compared with the untreated one. The highest number of DNA variation on ISSR patterns was observed at the micromollar concentrations of acrylamide. Acrylamide was able to induce DNA damage in non concentration-dependent manner with effectiveness at micromollar concentrations. This study demonstrated that ISSR markers can be highly reliable for identification of DNA damage induced by acrylamide.

Keywords : Acrylamide, Genotoxicity, ISSR, *Phaseolus vulgaris*

INTRODUCTION

Acrylamide (AA) is a highly reactive and water-soluble polymer, which is commonly used in many application fields from wastewater treatment, adhesive development, to the intensive use in laboratory gels (Nordin et al. 2003). Recent studies reported the presence of acrylamide in heat treated food products. The formation of acrylamide is particularly associated with high temperature cooking process for certain carbohydrate-rich foods, especially when asparagine reacts with sugars (Mottram et al., 2002; Konings et al., 2003). Acrylamide can be oxidized to the more reactive glycidamide (GA) by cytochrome P450-depended monooxygenase (CYP2E1). GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests (Koyama et al., 2006). Microsatellites or simple sequence repeats (SSR) are short DNA sequence stretches consisting of motifs of one to six nucleotides that are tandemly repeated. The use of SSR as DNA markers is hampered by the requirement for sequence information from flanking regions, from which primers are designed for polymerase chain reaction (PCR) amplification (Weber and May, 1989). Discovery and characterization of a large number of SSRs is therefore time-consuming and expensive for many taxa. A developed modification of SSR-based marker systems, i.e. ISSR (intersimple sequence repeat) analysis, ISSRs is semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Amplification in the presence of nonanchored primers also has been called microsatellite-primed PCR, or MP-PCR, (Meyer et al., 1993). Such amplification does not require genome sequence information (Zietkiewicz et al., 1994; Tsumara

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et al., 1996; Nagaoka and Ogihara, 1997). Such markers have been used to assess genetic diversity in many plants like dentcorn and popcorn (Kantety et al., 1995), as well as to identify cultivars like chrysanthemum (Wolff et al., 1995). Plants are good indicators of cytogenetic and mutagenic effects. The major advantages of plants as monitoring tools are the following: (i) they are eukaryotes; (ii) they are easy to grow and resistant to environmental stress; (iii) they can be used for outdoor monitoring; (iv) there is a positive correlation with mammalian cytogenetic assays for mutagenesis; and (v) plants, like animals, are able to process complex pollutant molecules (Conte et al., 1998). The use of plant material appears to be particularly pertinent for the assessment of environmental health risks. Higher plants, sensitive to soil, water and air pollutants, have been widely used for the biomonitoring of DNA damage induced by genotoxic agents: they can metabolise promutagens and be useful in detecting genotoxic compounds in complex mixtures (Poli et al., 2003). The aim of the present study was to investigate whether arbitrarily ISSR could be suitable as reliable marker in assessing genotoxic activity of acrylamide in the common bean (*Phaseolus vulgaris* L.). In this report, an ISSR optimized protocol was developed for the analysis of plant genomic variations and its efficiency was demonstrated.

MATERIALS AND METHODS

Treatment and experimental design

Common bean (*Phaseolus vulgaris* L, chromosome lengths and a total genome size of 637 Mbp, 2n = 2x = 22) is the principal leguminosae used for human nourishment, mainly in Africa, where it represents an important source of protein. Bean seeds cultivar Giza 6 was obtained from certified germoplasm collection at field crop Research Institute, Agricultural Research Center (ARC), Giza Egypt. These seeds were surface-sterilized with 75% (v/v) ethanol for 5 min, followed by 20% (v/v) sodium hypochlorite for 10 min. Thereafter, the seeds were thoroughly rinsed with tap water and then soaked for 1 h in distilled water at 25℃. The seeds were germinated in a sterile glass jar with water-saturated cotton at 22℃ in a growth chamber with a 16 h day/8 h night photoperiod, with light intensity of μE m^{-2} s^{-1}, and left to grow until the roots reached 3-5 mm in length. Subsequently, five plant seedlings were transferred to a jar containing Murashige and Skoog (1962) medium supplemented with following defined concentrations of acrylamide (100, 200, 400 μM and 1.0, 2.0, 4.0 mM). These six concentrations of AA were selected on the basis of previous results (Puppel et al., 2005; Baum et al., 2005; Koyama et al., 2006). The exposure was continued during 14 days. Additional sets for negative control were separately maintained. After treatment, plants were utilized for chromosomal DNA extraction and ISSR-PCR analysis.

Electrophoresis of DNA and PCR products

DNA extractions were performed using Plant DNeasy minikit (Qiagen) following the instructions of the manufacturer. The DNA was resuspended in Tris/EDTA (10 mM Tris/HCl and 0.1 mM EDTA, pH 8.0). The quality and quantity of the extracted DNA were checked by measuring the UV absorption spectrum (Sambrook et al., 1989). For further confirmation, the DNA concentration and DNA quality of each sample was determined by comparison with known concentrations of Lambda phage DNA. Electrophoresis of PCR products was performed in 2.0% agarose (Bornet and Branchard, 2001), using a Tris-Borate-EDTA buffer system (1×TBE=90 mM Tris-base, 90 mM Boric acid and 2 mM EDTA). Amplified DNA was mixed with 1/5th volume of gel loading buffer (analytical grade water containing 25% ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol) with 15 μl of this solution loaded onto the agarose gel. A DNA molecular size marker M = 100 bp ladder (Promega, Madison, USA) was run for each agarose gel. DNA samples were subjected to electrophoresis at 100 V for 4.0 h, after which, the gels were stained in a 1×TBE solution containing ethidium bromide (0.5 μg/ml) for a period of not less than 40 min. Gels were photographed under UV illumination using a Pola-

roid camera (CU-5, Eastman Kodak, New York, NY).

PCR amplification with ISSR primers

Nine nonanchored oligonucleotide primers were used for ISSR as shown in Table 1. All the DNA amplifications have been done in triplicate. To optimize the reaction conditions, several PCR parameters were tested, including DNA concentration (10 values between 5 to 150 ng/reaction), more than two DNA concentrations were used for each sample to confirm reliability of ISSR method. Primer concentration (12 values between 10 to 500 pmol/reaction), annealing temperatures (12 values between 40 to 62℃), and number of cycles (8 values between 15 to 40). Reactions without DNA were used as negative controls. The optimum annealing temperature was determined for each primer from a minimum of 12 temperatures. Amplifications were carried out in a thermocycler (Genius, Techne). Selected amplification conditions were 5 min initial denaturation step at 95℃, followed by 27 cycles of 1 min at 95℃, 1 min (specific annealing temperature), and 2 min at 72℃. The reactions were completed by a final extension step of 5 min at 72℃. The optimal temperatures were chosen for each primer according to the highest number of well separated bands observed for each pattern , these values were 50℃ for (GACA)4 primer, 44℃ for (CAA)5 primer, 52℃ for (CAG)5 primer, and 62℃ for (CCG)4 primer. These temperatures were respectively 2, 4, 2, and 14℃ higher than the Wallace temperature.

Data collection

Amplification with each primer was repeated three times and clearly resolvable and reproducible fragments were considered for analysis. Each fragment was treated as a unit character and was scored as 1 (present) or 0 (absent) for the plant samples exposed to different concentrations of acrylamide and control (unexposed). To estimate the genomic alteration: the number of altered band (loss and gain) in each treatment compared with the control was scored.

RESULTS

Optimization of ISSR

In order to assess the potential of ISSR as reliable method for detection of genotoxic effect of acrylamide, DNA was isolated from 5 plants exposed to different concentrations of acrylamide and from control plant. DNA of these samples was subjected to amplification by ISSR PCR using nine nonanchored primers (Table 1). Several parameters that could affect pattern quality and reproducibility of ISSR profiles were investigated. First, the effect of annealing temperatures was analyzed. These were estimated based on the Wallace rule for oligonucleotide hybridization. More than 10 temperatures were tested for each primer. Using low stringency (Ta lower than Tm) and high stringency (Ta higher than Tm), clear and reproducible bands were observed until the PCR reaction was

Table 1. List of ISSR primer sequences, calculated Tm based on Wallace formula, and number of amplified fragments.

Primer	Sequence (5'-3')	Length (bp)	Tm (℃)	Ta (℃)	No. of selected fragments
Mp1	CAA CAA CAA CAA CAA	15	40	44	6
Mp2	CAGCAGCAGCAGCAG	15	50	52	11
Mp3	GATAGATAGATAGATA	16	40	-	No products
Mp4	GACAGACAGACAGACA	16	48	50	6
Mp5	CTCTCTCTCT CTCTCT	16	48	-	Smeared
Mp6	GAGAGAGAGAGAGAGA	16	48	-	Smeared
Mp7	GTT GTT GTT GTT GTT	15	40	-	No products
Mp8	CA CA CA CA CA CA CA CA	16	48	-	Smeared
Mp9	CCG CCG CCG CCG	12	48	62	13

inhibited by high stringency. Temperatures were chosen for each primer that maximized the pattern information (maximum amplification and good resolution). Figure 1 presents influence of the annealing temperature upon pattern quality amplified with (CAG)5 from 40-62℃. Optimal conditions were selected, using the primer (CAG)5, based on the reproducibility and scorability of the PCR products. The best patterns were amplified in the presence of 15 ng genomic DNA (Fig. 2A), 100 pmol of primer (Fig. 2B), and 27 PCR cycles (Fig. 2C).

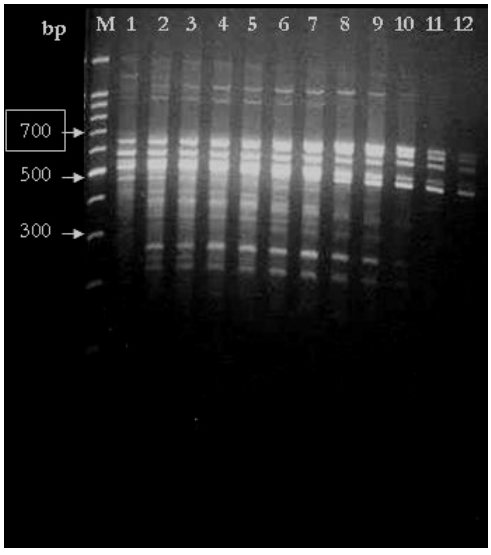

Fig. 1. Effect of annealing temperature on ISSR patterns using (CAG)5 primers, Lane M: 100 bp DNA ladder (promega); Lanes 1-12 different annealing temperatures 40℃, 42℃, 44℃, 46℃, 48℃, 50℃, 52℃, 54℃, 56℃, 58℃, 60℃, 62℃; respectively.

Evaluation of ISSR to detect DNA damage in common bean exposed to acryl amide

The PCR assay was repeated three times for each sample from control and exposed plants to ensure reproducibility. Changes that recurred in the three assays were taken into account in the determination of the genomic alterations. The results indicated that the primers used could be categorized into three groups: The first group includes primers that did not produce any PCR products, these primers are (GTT)5 and (GATA)4. The second group includes one tetra nucleotide primer (GACA)4, and 3-trinucleotide primers (CAA)5, (CAG)5, and (CCG)4 that are reproducible and produce polymorphic DNA fingerprint. The third group includes 3-dinucleotide primers (CT)8, (CA)8, and (GA)8, that produced smear. Figure 3

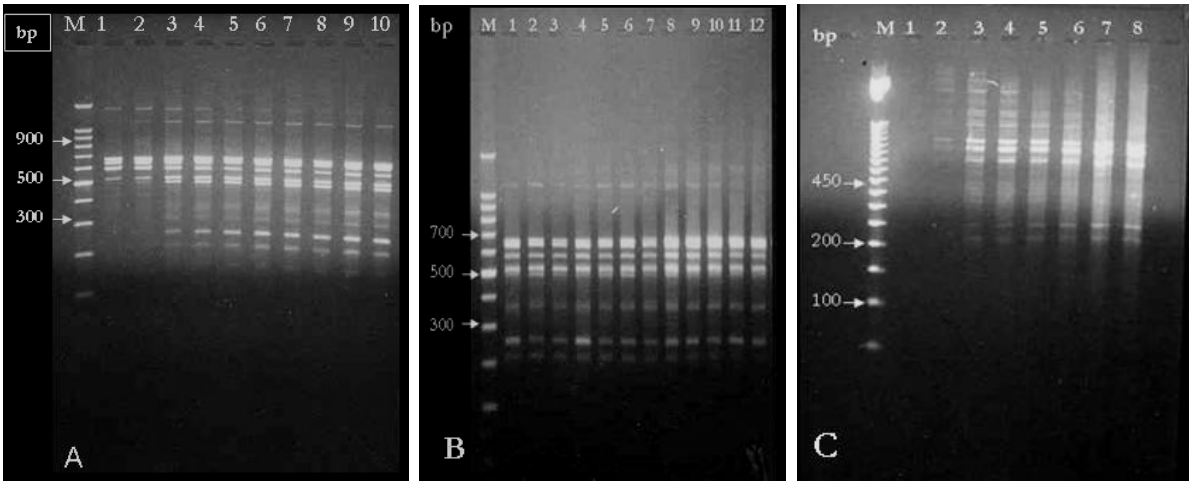

Fig. 2. Effect of PCR parameter variations on nonanchored ISSR fingerprints using the (CAG)5 primer at the optimal annealing temperature: **(A)** variation of DNA concentrations; Lanes 1-10: represent DNA quantity 5 ng,10 ng,15 ng, 20 ng, 25 ng, 30 ng, 50 ng, 100 ng, 130 ng, 150 ng; respectively, **(B)** variation of the primer concentration; Lanes 1-12: represent primer concentrations 10 pM, 20 pM, 50 pM, 100 pM, 150 pM, 200 pM, 250 pM, 300 pM, 350 pM, 400 pM, 450 pM, 500 pM; respectively, **(C)** variation of the number of PCR cycles; Lanes 1-8: represent different numbers of cycles, 15, 20, 25, 27, 30, 35, 40, 45 cycles respectively. Lane **M:** represent 100 bp DNA ladder in gel (A) and gel (B). Lane **M** in Gel (C) represents 50 bp DNA ladder.

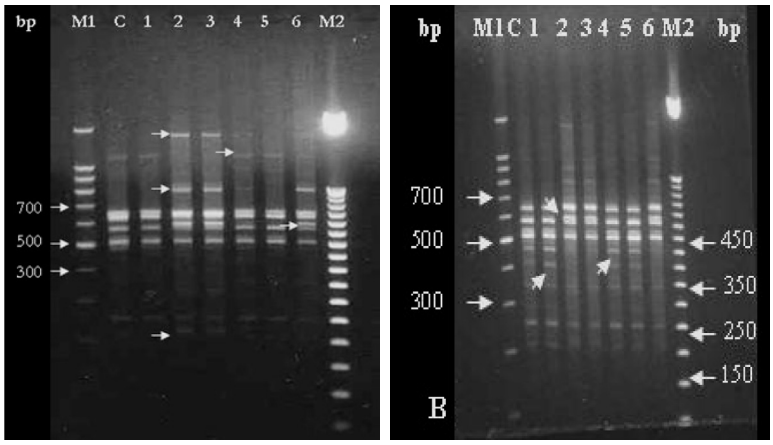

Fig. 3. ISSR amplification pattern derived from common bean plants exposed to different concentrations of acrylamide. Polymorphism generated by ISSR primer (CAG)5 (A) and primer (CCG)4 (B). The arrows indicate the bands showing polymorphism. Lanes 1-6: represent different concentrations of acryalmide; 100 μM, 200 μM, 400 μM, 1.0 mM, 2.0 mM, 4.0 mM; respectively. Lane C: represents control plant sample. Lane M1: 100 bp DNA Ladder; Lane M2: 50 bp DNA ladder (Promega).

Table 2. Summary of the gains and losses in ISSR bands from exposed common bean compared with control. Changes of polymorphic bands in ISSR profiles related to the tested concentration of acrylamide (AA).

concentration of acrylamide	a	b	a+b
100 μM	1	1	2
200 μM	11	2	13
400 μM	10	3	13
Total	**22**	**7**	**29**
1.0 mM	7	0	7
2.0 mM	2	0	2
4.0 mM	9	1	10
Total	**18**	**1**	**19**

Note: ***a*** indicates appearance of new bands, ***b*** disappearance of normal bands, ***a+b*** denotes polymorphic bands

shows results obtained for amplification of plant samples exposed to different concentrations of acryalmide. ISSR primer (CCG)4 amplified the maximum number of bands which was 13 bands (Table 1). While ISSR primers (CAA)5 and (GACA)4 amplified the minimum number of bands which was 6 bands (Table 1). Genomic changes were detected as gains or losses in the pattern of amplified bands. In the case of band gain, at the lowest concentrations, 22 new bands were amplified. In the same trend at the highest concentration 18 bands were amplified. Similarly, in the case of band loss, at the lowest concentration 7 bands were disappeared and at the highest concentration, only 1 band was disappeared (Table 2).

DISCUSSION

A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. Each marker technique has its own advantages and disadvantages. RAPD markers (Williams et al., 1990) are very quick and easy to develop (because of the arbitrary sequence of the primers) but lack reproducibility (Hansen et al., 1998; Virk et al., 2000). Microsatellites (Tautz, 1989) are specific and highly polymorphous but they require knowledge of the genomic sequence to design specific primers (Karp et al., 1997). Inter simple sequence repeat (ISSR) has been available; such amplification does

not require genome sequence information (Zietkiewicz et al., 1994).

The aim of this study was to evaluate the potential of the ISSR assay to qualitatively detect DNA damage induced in common bean exposed to different concentrations of acrylamide range from 100 μM to 4 mM for 14 days. In the beginning of this study, the effect of various PCR parameters has been used to test the specificity and reproducibility of nonanchored ISSR amplifications for a genotoxicity study. In order to maximize reproducibility of the ISSR fingerprints, the PCR-mixture parameters for the (CAG)5 primer were optimized, except for the annealing temperature, and then used these parameters for the other primers without adjustment. In this investigation ISSR-PCR reactions were carried out using annealing temperature higher than melting temperatures to avoid nonspecific amplification, significant changes were observed in banding patterns when the annealing temperature changed. Pharmawati et al. (2004) reported that band patterns were slightly changed at different annealing temperatures. In contrast (Sanchez de la Hoz et al., 1996; Lakshmanan et al., 2007) have been used low annealing temperatures for ISSR amplifications with different primers. Variation in primer concentration is one of the main sources of RAPD pattern variations (Hansen et al., 1998; Virk et al., 2000). In the present study ISSR patterns were slightly different with variation of concentrations of primer. Intense and homogeneous banding was observed between 100 and 500 pmole per reaction. In previous study Bornet and Branchard (2001) reported that no modification of ISSR patterns was observed when primer concentrations are changed and smears were noticed with 500 pmol per reaction.

In this investigation the ISSR patterns show slight modification when DNA concentrations are changed. A complete fingerprint was observed with 15 ng of DNA with 27 PCR cycles. Similarly, Ammiraju et al. (2001) observed that 15 ng of template DNA with 45 cycles of PCR gave clear banding patterns with many primers. In the same trend, Bornet and Branchard (2001) observed that the best patterns were amplified in the presence of 12 ng genomic DNA with 27 PCR cycles. By contrast Nagaoka and Ogihara (1997) used much less DNA (5 ng) in their analysis. In this investigation, 3-dinucleotide nonanachored primers (CT)8, (CA)8, and (GA)8 produced smear. No significant improvements were made for these primers even when adjusting annealing temperature as low as 30℃ and changing amplification cycles. Similarly Gupta and Varshney (2000) obtained smears and Nagaoka and Ogihara (1997) also have a similar problem in the case of wheat with (TA)n-rich primer anchored at 3'-end. By contrast, Ammiraju et al. (2001) and Bornet and Branchard (2001) have amplified high numbers of polymorphic bands by nonanchored and anchored dinucleotide primers. In this study nine ISSR primers were tested for their ability to mentor DNA damage in plant treated with acrylamide. Only four out of nine primers tested gave clear polymorphism for their ability to mentor genomic damage. In the present study, DNA damage induced by acryalmide was reflected by changes in fingerprinting patterns generated by ISSR; genomic alterations were detected as gains and losses in bands when compared with matched normal DNA. The DNA damage in the 5 exposed plant samples were characterized by the loss of 8 ISSR amplicons and gain of 40 ISSR amplicons. DNA adducts are the main reasons that influence the ISSR banding patterns of plant samples exposed to acrylamide. Atienzar et al. (2002) concluded that DNA damage and mutations are the main factors that influence RAPD pattern variation between benzo(a)pyrene exposed and non-exposed individuals. The results of the present study confirm that acrylamide acts in a non-concentration dependent way, even if more variations were observed with all the lowest concentrations (μM) that with all the highest ones (mM). In unrelated studies, similarly Besaratinia and Pfeifer (2003) reported that in vitro treatment of big blue mouse with acrylamide at millimolar concentrations induced DNA adducts but treatment with acrylamide at micromolar concentrations increased the frequency of mutations up to twofold. By contrast Puppel et al. (2005) observed that DNA strand breaks in V79 and CaCO-2 cells of rat was detected at the highest concentration of acrylamide (6 mM, 24 h).

CONCLUSION

This is the first report of the applicability of ISSR-PCR for assessing DNA damage in higher plants exposed to acrylamide and it could be concluded that ISSR are very useful and pertinent tools to detect DNA damage in plant exposed to chemicals.

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