Low-Stringency Single-Specific-Primer PCR as a tool for
detection of mutations in the \textit{matK} gene of \textit{Phaseolus vulgaris}
exposed to paranitrophenol

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\textbf{Abstract}

Low-stringency single specific primer polymerase chain reaction (LSSP-PCR) was assessed for its
suitability in detecting the genotoxic effect of paranitrophenol (PNP) in the dwarf bean (\textit{Phaseolus vulgaris}) exposed to different concentrations of PNP. DNA was extracted from both PNP-treated and non-treated shoots that was amplified by specific PCR, using universal primers of \textit{maturase K} chloroplast DNA. PCR products of approximately 776 bp were subsequently used as a template for LSSP-PCR analysis. We detected the genotoxic effect based on LSSP-PCR profiles of the DNA generated in PNP-treated over the non-treated control of bean shoots. A complex electrophoretic pattern consisting of many bands was obtained from control and treated samples. Surprisingly, DNA sequencing data revealed that the homology among the \textit{maturase} gene amplified from PNP-treated vs. non-treated samples of dwarf beans are comparable. These results showed that the use of LSSP-PCR analysis is not a proper tool to detect genotoxic effect in bean, at least in bean shoots that were exposed to PNP.

\textbf{Keywords}: Genotoxicity, LSSP-PCR, Paranitrophenol, \textit{Phaseolus vulgaris}, \textit{maturase K}.

\textbf{Özet}

Paranitrofenole maruz kalan \textit{Phaseolus vulgaris'}te \textit{matK} geni mutasyonlarının
tespitinde bir araç olarak Düşük Kesinlikte Tek-Özgün Primerli PCR kullanımı

Düşük kesinlikte tek özgün primer lipolimeraz zincir reaksiyonunun (LSSP-PCR), paranitrofenolün
(PNP) sebep olduğu genotoksik etki tespitindeki uygunluğu farklı konsantrasyonlarda PNP'ye maruz
birakılan bodur fasülyeye (\textit{Phaseolus vulgaris}) değerlendirildi. PNP ile muamele edilmiş veya
edilmiş olmayan filizlerden izole edilen DNA, \textit{maturaz K} kloroplast DNA'sının evrensel primerleri
kullanılarak özgün PCR ile çoğaltıldı. Hemen akabinde, 776 bç'lik PZR ürünler LSSP-PCR analizi
için kalıp DNA olarak kullanıldı. PNP ile işlenmiş fasülye filizinden elde edilen DNA üzerindeki
genotoksik etkilerin PNP ile işlenmiş ve işlenmiş olmayan kıyaslama LSSP-PCR profiline dayanarak
tespit ettik. Kontrol ve işlenmiş örneklerde birçok bantta oluşan karsılıklık elektroforetik motifler elde
edildi. Şu anda bir şekilde DNA düz analizi verileri PNP ile işlenmiş ve işlenmiş olmayan bodur fasülye
örneklendenden \textit{maturaz} geni homolojisinin karşılaştırılabilme özelliğini gösterdi. Bu sonuçlar,
LSSP-PCR analizinin fasülyeye, en azından PNP ile işlenmiş filizlerde, genotoksik etkinin tespitinde
uygun bir araç olmadığını gösterdi.

\textbf{Anahtar Kelimeler}: Genotoksisite, LSSP-PCR, Paranitrofenol, \textit{Phaseolus vulgaris}, \textit{maturaz K}. 

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Introduction

Maturase K (matK) is a chloroplast-encoded gene which is nested between the 5' and 3' exons of \textit{trnK}, tRNA-lysine (Sugita et al., 1985). Sequence analysis indicated that this region displayed homology to domain X of mitochondrial group II intron maturases (Sugita et al., 1985; Neuhaus and Link, 1987). Although maturase K gene (matK) contains many indels (insertions and deletions) throughout its reading frame, yet domain X lacks any of these indels (Hilu and Liang, 1997; Hilu and Alice, 1999; Hilu et al., 2003). Maturase K is the only gene found in the chloroplast genome of higher plants that contains this putative maturase domain X in its protein (Neuhaus and Link, 1987). Maturases are considered as splicing factors because of their ability to splice and fold group II introns. The coding region of \textit{matK} is generally located within intron of the chloroplast \textit{trnK} gene (Vogel et al., 1997). \textit{matK} is very useful in DNA barcoding to genetically identify plant families (Qiu et al., 1999; Li and Zhou, 2007).

Genotoxic compounds are those which cause damage to DNA. Para-nitrophenol is a synthetic chemical that is used to manufacture drugs, fungicides, insecticides (Yang et al., 2010). Pesticides, such as parathion and methyl parathion, are hydrolyzed and transformed to PNP; which in turn these pesticides are considered as the main source of PNP that is released to the environment (Kitagawa et al., 2004). \textit{In vitro} assay using CHO cell PNP was positive for chromosome aberration at levels of 100 µg/ml (Ohno et al., 2005), proving the hypothesis that PNP induces chromosomal aberrations.

The LSSP-PCR is a simple technique that permits detection of single or multiple mutations in gene-sized fragments (Pena et al., 1994). This sensitive and rapid method uses PCR amplification of a single oligonucleotide primer “driver” that is specific to one of the extremities of the fragment, under very low stringency conditions (Pena et al., 1994). In a sequence-dependent manner, the driver hybridizes both to the highly specific complementary extremity, and to the low specificity of multiple sites within the fragment. The reaction thus yields a large number of products that can be resolved by polyacrylamide gel electrophoresis to give rise to a multiband DNA fragment "signature" that reflects the underlying sequence. Changes as small as single base mutations can drastically alter the multiband pattern, which ultimately produce new signatures. LSSP-PCR has been broadly used for the detection of mutations in human genetic diseases (Pena et al., 1994), sequence variations in human mitochondrial DNA (Barreto et al., 1996) and for genetic typing of infectious agents such as papillomavirus (HPV; Villa et al., 1995), \textit{Trypanosoma cruzi} (Vago et al., 2006), \textit{Trypanosoma rangeli} (Marquez et al., 2007), and \textit{Leishmania infantum} (Alvarenga et al., 2012). The objective of this study was to describe the potential use of LSSP-PCR as a molecular biomarker to detect DNA mutation in \textit{maturase} K gene in dwarf bean tissues exposed to paranitrophenol.

Materials and methods

Plant growth and treatment conditions

The dwarf bean (\textit{Phaseolus vulgaris}) was used as the plant material in this study. The selected seeds were sterilized with 75% (v/v) ethanol for 2 min, followed by 20% (v/v) sodium hypochlorite for 10 min and were washed five times in sterile distilled water. Uniformly three plant seedlings were transferred to a Magenta box containing MS (Murashige and Skoog, 1962) liquid medium (control) or supplemented with different concentrations of PNP (20, 40, 80, 160, 320, and 640 µg/ml). PNP-treated seedlings were grown for 10 days in the growth chamber. Plant growth conditions was previously described (Enan, 2006).

DNA isolation

DNA was extracted from fresh plant shoots using DNeasy plant minikit (Qiagen, USA), following the instruction of the manufacturer. The final DNA concentration was determined by agarose gel electrophoresis against known standards (Invitrogen, USA).

Specific PCR amplification of \textit{matK} fragments

To eliminate any possibility of bacterial contamination due to the very low-
stringency conditions of the LSSP-PCR reaction, all experiments were carried out with extreme precautions. PCR reactions were performed with specific primers matK472F (5′-CCCRTYCATCTGGAAATCTTGGTTC-3′) and matK1248R (5′-GCTRTRATAATGAGAAAGATTTCTGC-3′) as described by Yu et al. (2011). Amplification of specific PCR products was carried out in a volume of 25 µl containing 30 ng of genomic DNA and a master mix containing 1.5 mM MgCl₂, 200 µM of each deoxynucleotide (dNPTs), 20 pmol of each primer, 1.0 U Taq DNA polymerase (Invitrogen-BRL), in 10 mM Tris–HCl [pH 8.0] and 50 mM KCl. After an initial denaturation step of 94 °C for 5 min, the specific PCR program consisted of 35 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. The last cycle consisted of an extension step at 72 °C for 5 min. The PCR products were run on ethidium bromide-stained gel and the bands corresponding to the specific fragment (876bp) generated by universal specific primers were purified using Purelink PCR purification Kit (Invitrogen, USA).

LSSP-PCR analysis
For the production of LSSP-PCR signatures, previously amplified matK fragments were purified used as a template in the LSSP-PCR (Pena et al., 1994). LSSP-PCR was also carried out in a 25µl volume containing 5ng of DNA template, 1.5 mM MgCl₂, 200 µM of the four deoxynucleotide triphosphates, 120 pmol of matK472F or matK1248R primer 4.0 U Taq DNA polymerase in 10 mM Tris–HCl [pH 8.0] and 50 mM KCl. After a denaturation step at 94 °C for 5 min the LSSP-PCR program consisted of 35 cycles of denaturation at 94 °C for 1 min, annealing at 30 °C for 1 min and extension at 72 °C for 1 min. Ten microliters of LSSP-PCR products were analyzed by electrophoresis on 8% (w/v) polyacrylamide gels followed by ethidium bromide. The similarity among the LSSP-PCR profiles of control and those obtained with the DNA of PNP-treated samples was analyzed accordingly.

DNA sequencing of PCR products
In order to determine the nucleotide sequence of the 776 fragments generated with universal specific primers, PCR products of control and PNP-treated samples were purified and sequenced by Source BioScience (Nottingham, UK) according to Sanger et al. (1977). The sequence was analyzed for homology with database sequences with Multiple Sequence Alignment by MultiAlin (Corpet, 1988).

Results
We used the LSSP-PCR method to detect mutations in matK gene of dwarf bean tissues. DNA was amplified (first step) using universal primers to produce 776 bp fragments containing the maturase K region (Figure 1).

Each fragment was isolated by electroelution and subjected to a second PCR amplification (second step) using a single primer annealed under low-stringency conditions. The generated profiles of the PCR products of each sample were resolved and analyzed by non-denaturing polyacrylamide gel. A complex pattern consisting of many bands was obtained which was different depending on the concentration of PNP. We showed the LSSP-PCR profiles of DNA obtained from PNP-treated or PNP-untreated samples amplified with either matK742 forward primer (Figure 2) or matK1248R reverse primer matK1248R primer (Figure 3).

Figure 1. Agarose gel electrophoresis of PCR amplification of matK fragment with 776 bp obtained in control and treated samples. Lane M: 100 bp DNA ladder; Lane C: untreated sample (control); lanes 1-6: plant samples treated with 20, 40, 80, 160, 320 and 640 µg/ml PNP, respectively.
Figure 2. Ethidium bromide-stained polyacrylamide gel electrophoresis showing gene signatures obtained by LSSP-PCR with \textit{mat}k742 forward primer. Lanes M: 100 bp DNA ladder; Lane C: untreated sample; lanes 1-6: plant samples treated with 20, 40, 80, 160, 320 and 640 µg/ml PNP, respectively.

Figure 3. Ethidium bromide-stained polyacrylamide gel showing gene signatures obtained by LSSP-PCR with \textit{mat}k1248 reverse primer. Lane M: 100 bp DNA ladder; Lane C: untreated sample; lanes 1-6: plant samples treated with 20, 40, 80, 160, 320 and 640 µg/ml PNP, respectively.

The LSSP-PCR profiles were unique for each treatment, suggesting that this technique may be applicable for the detection of genotoxic impact of environmental contaminants. The sequence of the \textit{maturase K} gene was deposited in Genbank (accession numbers JQ403111). We also determined whether these sequence identities showed similarities between the different samples treated with PNP (Figure 4). Our sequence alignment data obtained by MultiAlin indicated that the sequence identities of all treated samples shared 100% homology with sequences of untreated samples (Figure 4). This suggests that a mutation in the \textit{matK} gene as a hotspot gene is not induced by treatment with PNP.

Discussion
To our knowledge, this is the first study to employ LSSP-PCR for monitoring biological effects of pollution. Molecular biomarkers are effective early warning signals of adverse biological effects. The purpose of this study was to evaluate the performance of LSSP-PCR method in the detection of genotoxic effect of paranitrophenol (PNP) on dwarf beans (\textit{Phaseolus vulgaris}). In the past 25 years, numerous biomarkers have been developed with the objective to apply them for environmental biomonitoring (Sanchez and Porcher, 2009). Molecular marker techniques have provided new tools of detection of mutations in DNA in response to chemical pollution using DNA sequence and structure. The alterations in genomic DNA induced by genotoxic pollutants can be monitored using different biomarkers’ assays both at the biochemical and the molecular levels. In the past few years, several of techniques revealed that mutations in DNA could be generated and identified mostly by the polymerase chain reaction (PCR). Some of the examples of PCR assays were utilized to detect genotoxic effects of environmental pollutants arbitrary-primed PCR (AP-PCR; Welsh and McClelland, 1990) and randomly amplified polymorphic DNA (RAPD) (Williams \textit{et al}., 1990). One of the main advantages of using LSSP-PCR for studies related to genotoxicity is that the signatures were not unduly sensitive to the concentration of DNA template.
Figure 4. DNA sequences of the matK genes from untreated and PNP-treated plant samples aligned by using MultiAlin.
In the present study, genotoxic effect of PNP was performed using LSSP-PCR that can detect single or multiple mutations in gene-size DNA fragments. The chloroplast *matK* gene (*matK*) is one of the most variable coding genes of angiosperms, which has been suggested to be a “barcode” for land plants (Yu et al., 2011). Good reproducibility as a solution in the LSSP-PCR profiles using both forward and reverse primers was obtained. However, we observed that many of these bands are larger than the template. Our results confirm the data obtained from other studies that PCR products of the first few cycles may themselves act as primers in further rounds of amplification (Barreto et al., 1996). In the current study, the sequenced PCR products of *matK* fragment confirmed the results of the specific PCR (Figure 1). On the other hand, unexpectedly, the data of sequence alignment quite contradicts that of the LSSP-PCR signatures. Sequence alignment of all PNP-treated samples of dwarf bean with the untreated control samples indicated that there is no any nucleotide substitution in the *matK* sequence. In previous study, Oliveira et al. (2003) described that, very similar signatures were obtained with specific primers (G1 and G2) for identification of *Leptospira interrogans* serovars. Although the sequence data of the 285 bp fragments of the three serovars of *L. interrogans* indicated the presence of three nucleotide alterations in these fragments, they found that identical LSSP-PCR profiles were obtained for the three serovars with individual primers of G1 and G2. Barreto et al. (1996) reported that the variations observed in LSSP-PCR are attributed to several variables: (i) the number of cycles has a marked effect on the signature up to 35 cycles (ii) the ramping speed of thermocyclers (type of thermo cyclers) had marked effect on the LSSP-PCR signatures and (iii) changes in the annealing temperature a range between 25-35°C had no marked effect but Ta > 40°C showed a deterioration of the signature.

In conclusion, the chloroplast *matK* used in this study as a molecular biomarker gene to measure genotoxicity of PNP using LSSS-PCR, is not affected by PNP at DNA level but may be down regulated at transcriptional or post-transcriptional levels, which should be confirmed in further studies.

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