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TREHALOSE-BASED ADDITIVE IMPROVED INTER-PRIMER BINDING SITE REACTIONS FOR DNA ISOLATED FROM RECALCITRANT PLANTS

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ABSTRACT

Trehalose-based (TBT-PAR) additive was tested in order to optimize PCR amplification for DNA isolated from recalcitrant plants. Retrotransposon-based inter-primer binding site reactions were significantly improved with TBT-PAR solution using genomic DNA isolated from flax (*Linum usitatissimum* L., genotypes *Kyivskyi*, *Bethune*) grown in radio-contaminated and non-radioactive remediated Chernobyl experimental fields. Additionally, similar improvements were observed using 19 recalcitrant genotypes of maize (*Zea mays* L.) and three genotypes of yacon (*Smallanthus sonchifolius,* Poepp. *et* Endl., genotypes *PER05*, *ECU45, BOL22*) grown in standard field conditions.

Keywords: TBT-PAR additive, recalcitrant plants, PCR, iPBS technique

INTRODUCTION

The extraction of nucleic acids (DNA, RNA) may be problematic due to the presence of specific components that can interfere with the extraction process. Mucilage and phenolics contained in the seed coat are good examples of hindering components **(Renouard *et al.*, 2012)**. Pre-requisite for downstream applications is DNA of good quality, purity and ~~with~~ optimal concentration. Therefore, the presence of high concentrations of polyphenols, polysaccharides, proteins and other secondary metabolites poses problem in several plant species in getting high quality DNA fit for PCR applications **(Demeke and Jenkins, 2010, Azmat *et al.*, 2012)**. Moreover, the growing number of DNA extraction protocols and methods for specific plant species aren't always simple and can't be reproduced for all plant species **(Porebski *et al.*, 1997).**
The extraction and purification of DNA is a general approach to DNA sequencing and gene isolation in plant systematics and biotechnology research, followed by amplification using PCR reactions **(Bellstedt *et al.*, 2010)**. The PCR amplification of DNA extracted from plants is sometimes difficult due to the presence of inhibitory compounds, and therefore, a reliable method to remove the effect of inhibitory compounds that contaminate DNA of various plant species was needed. Consequently, the addition of TBT-PAR solution during routine PCR applications is an effective method to improve amplification of DNA extracted from herbarium specimens or plants that are known to contain PCR contaminants **(Samarakoon *et al.*, 2013)**.
Effects of inhibitory compounds on PCR are different. Some contaminants will cause template inhibition and others will inhibit the enzymatic activity of the DNA polymerase **(Matheson *et al.*, 2010)**. Overall, it can be concluded that in target nucleic acid are present contaminants from different sources inhibiting the PCR amplification of DNA **(Wilson, 1997)**.
Wang *et al.* (1993) presented the view that the effort and time required for isolation of DNA is often the limiting step. At present, PCR reaction requires only a small amount of DNA template for successful amplification and the DNA can be use directly for PCR. The extraction of sufficient DNA can be possible in an appropriate buffer, but standard buffer should allow optimal DNA extraction while at the same time not inhibiting the PCR amplification reaction.
Numerous techniques of molecular markers were applied in the research of flax genetic resource evaluation, and in molecular marker development **(Deng *et al.*, 2010)**, for example, in molecular applications are often used the long terminal repeat (LTR) retrotransposons as markers **(Kalendar *et al.*, 2010)**. Generally, retrotransposons are ubiquitous, abundant and dispersed components of eukaryotic genomes and their activity simultaneously leads to plant genome diversification and provides a means of its detection **(Kalendar, 2011)**.
The aim of the presented study was to improve the amplification of DNA extracted from recalcitrant plants and minimized occurence of failures in profiles of PCR products. The effect of trehalose-based (TBT-PAR) additive in inter-primer binding site (iPBS) reactions of recalcitrant plants – Chernobyl flax, maize and yacon was tested.

MATERIAL AND METHODS

Plant material

Flax (*Linum usitatissimum* L.) was grown in the fields of Chernobyl area starting in 2007. Two genotypes of flax (*Kyivskyi*, *Bethune*) grown in the radio-contaminated and non-radioactive remediated experimental Chernobyl fields were used for analysis. Radio-contaminated experimental field is distant 5 km from CNPP (Chernobyl Nuclear Power Plant), near the village Chistogalovka with soil radioactivity 20650 ± 1050 Bq.kg^{-1} of ^{137}Cs, and 5180 ± 550 Bq.kg^{-1} of ^{90}Sr. Non-radioactive control field is located directly in remediated area of Chernobyl town and have soil radioactivity 1414 ± 71 Bq.kg^{-1} of ^{137}Cs and 550 ± 55 Bq.kg^{-1} of ^{90}Sr. Contents of aleurite (silt) and pelitic soil ranges from 20 to 30 % in both experimental fields. The soils contain 12 % clay, 2 % organic materia and are sod-podzolic with a loamy-sand texture, which is derived from sandy fluvio-glacial deposits. Soil in remediated non-radioactive field have pH of 6.6, soil in radio-contaminated field have pH of 5.6 and electric conductivity of soil is 0.20 dS.m^{-1}. The parts of soil particles (in total 22.9 %) have size from 0.1 to 0.2 mm.
Three tropical and subtropical genotypes (*PER05, ECU45, BOL22*) of yacon (*Smallanthus sonchifolius* (Poepp. *et* Endl.), and 19 recalcitrant genotypes (*MBS 847, MO 17, TO 234, A 654, Oh 43, DE 811, Tva 194-9, Tva 261-9, Tva 260-9 , Tva 53-9, Tva 56-9, Tva 19, Tva 202-9, Tva 201-9, Tva 99-9, TO 784Ft, TO784CMs, TO2M, FANAL8*) of maize (*Zea mays* L.) were used. The yacon samples were provided from the Institut of Tropics and Subtropics of the Czech university of Life Sciences and the maize samples were provided from ZEAINVENT Trnava s.r.o.

DNA isolation

Genomic DNA was isolated from flax (*Linum usitatissimum* L.) and maize (*Zea mays* L.) using the method described by **Rogers and Bendich (1994)**. For DNA isolation from yacon three total genomic DNA extraction methods - **Rogers and Bendich (1994)**, **Saghai-Maroof *et al.* (1984)**, **Friar (2005)** and one manufactured extraction kit - GeneJET™ Plant Genomic DNA Purification Mini Kit (ThermoScientific) were proved, because of high level of polyphenols content in the yacon leaves **Žiarovská *et al*. (2013)**.

Retrotransposon-based marker (iPBS) method

The protocol using the addition of TBT-PAR solution for flax DNA amplification was tested. The reaction mixture for iPBS technique contained Termo Scientific Dream *Taq* PCR Master Mix (2×); Dream *Taq* DNA polymerase supplied in 2× Dream *Taq* buffer; dATP, dCTP, dGTP and dTTP (0,4 mmol.dm^{-3} each) and 400 nmol.dm^{-3} of each iPBS primer. For better reproducibility of iPBS profiles 3 µl of 5× TBT-PAR solution in a total volume of 15 µl were added. The 5× TBT-PAR additive contained 750 mM trehalose, 1 mg/mL nonacetylated BSA, 1% Tween-20 and 8.5 mM Tris hydro-chloride, pH 8,0. During PCR reactions, TBT-PAR was used at 1× concentration **(Samarakoon *et al.*, 2013)**. To improve PCR conditions 1U of Dream *Taq* Polymerase was added.
The iPBS reactions of maize were performed in reaction mixture containing Termo Scientific Dream *Taq* buffer; 400 nmol.dm^{-3} primer; 0,75 nmol.dm^{-3} $MgCl_2$ and 3 µl of 5× TBT-PAR additive in a total volume of 15 µl. Yacon iPBS reactions were performed in a 15 µl reaction mixture with MyTaq™ Mix, 20 ng of DNA, 300 nmol.dm^{-3} of iPBS primers and TBT-PAR solution was added. The primers for iPBS reactions are characterized in the table 1.
Retrotransposon-based marker technique (iPBS) of flax was performed in MyCycler™ Bio-Rad under following conditions: 95 °C 4 min; 95 °C, 52 °C, 72 °C 34×; 72 ° 10 min. The iPBS protocol of maize was comprised of initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 52 °C for 45 sec, 72 °C for 2 min 38× and then ended by 72 °C for 10 min. Yacon iPBS amplification was performed in BIO-RAD C1000™ Thermal Cycler under following conditions of gradient of the annealing temperature: 95°C 4 min (95 °C 1 min; 52 - 62 °C 1 min; 72 °C 2 min) 35x; 72 °C 10 min.
Amplified fragments of flax and maize were electrophoretically separated in 1,5 % agarose gel and yacon fingerprints were segregated in 1,2 % agarose gel (Agarose Basic, AppliChem). Electrophoreograms were processed with documentation system G:Box in GeneSnap program - Product version: 7.09 (Syngene) and GeneTools - Product version: 4.01 (Syngene).

Table 1 Characterization of the primers used for iPBS fingerprinting of flax, maize and yacon

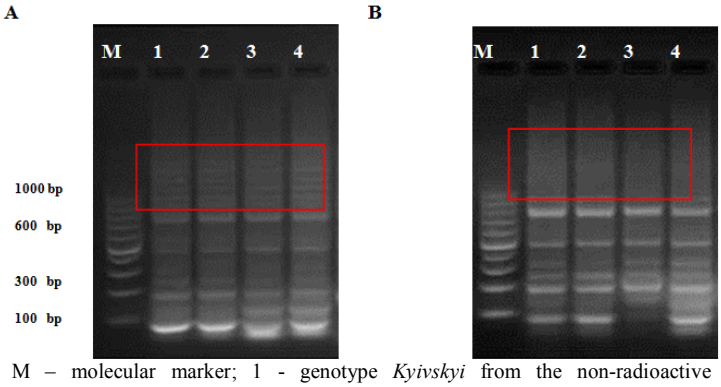
Primer name	Nucleotide sequence (5'⟶ 3')
1886***	attctcgtccgctgcgcccctaca
1879**	ccgagcggagtaaggagcattgtcc
1882*	tcgacttctcatgcatggcagcacc
2074*	gctctgatacca
2080*	cagacggcgcca

primers for iPBS amplication of flax DNA *
primer for iPBS amplification of maize DNA **
primer for iPBS amplification of yacon DNA ***

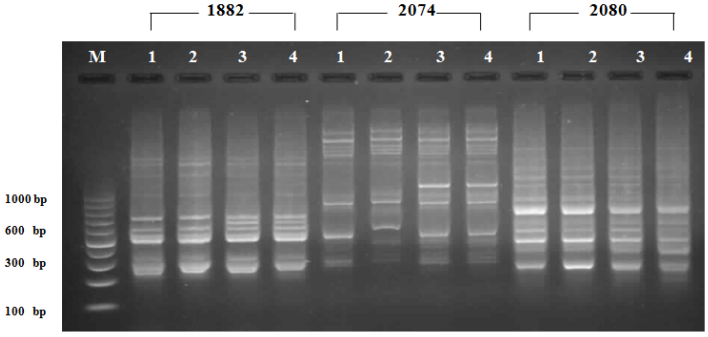
RESULTS AND DISCUSSION

Effect of TBT-PAR additive on iPBS amplification of flax (*Linum usitatissimum* L.), maize (*Zea mays* L.) and yacon (*Smallanthus sonchifolius*)

The aim of this study was to test efficiency of a PCR additive reagent (TBT-PAR) with trehalose, bovine serum albumin (BSA) and polysorbate-20. The results were better using TBT-PAR solution. The TBT-PAR additive was tested using DNA samples from flax (genotypes *Kyivskyi, Bethune*) grown in the Chernobyl area. Flax from two types of experimental fields, radio-contaminated and non-radioactive, was used in iPBS reactions. The iPBS reaction profiles were compared with and without the addition of TBT-PAR solution using the primer marked as 2080, and better results were observed using TBT-PAR solution in the experiments. The amplification of iPBS fragments with higher molecular weight was improved using TBT-PAR additive. In the experimental variants with TBT-PAR additive 5 amplified iPBS fragments with molecular weight over 1000 bp were detected. The amplified iPBS fragments with molecular weight from 100 to 1000 bp were similar in both types of PCR experiments – with and without TBT-PAR solution (figure 1). Overall, the TBT-PAR additive was applied in iPBS reactions with three types of primers – 1882, 2074, 2080 (figure 2).

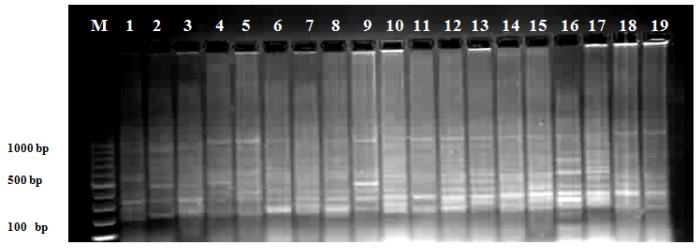


M – molecular marker; 1 - genotype *Kyivskyi* from the non-radioactive Chernobyl field, 2 – genotype *Kyivskyi* from the radio-contaminated Chernobyl field, 3 – genotype *Bethune* from the non-radioactive Chernobyl field, 4 – genotype *Bethune* from the radio-contaminated Chernobyl fiel
Picture A – the iPBS reactions of flax using TBT-PAR additive, picture B – the iPBS reactions of flax without TBT-PAR additive. The PCR products with higher molecular weight were better deducible on the picture A where the TBT-PAR additive was used.
Figure 1 Comparison of flax iPBS profiles using TBT-PAR (picture A) and without TBT-PAR additive (picture B); iPBS primer marked as 2080 was used



M – molecular marker; 1 - genotype *Kyivskyi* from the non-radioactive Chernobyl field, 2 – genotype *Kyivskyi* from the radio-contaminated Chernobyl field, 3 – genotype *Bethune* from the non-radioactive Chernobyl field, 4 – genotype *Bethune* from the radio-contaminated Chernobyl fiel
Figure 2 The iPBS marker profiles of Chernobyl flax (iPBS primers marked as 1882, 2074 and 2080).

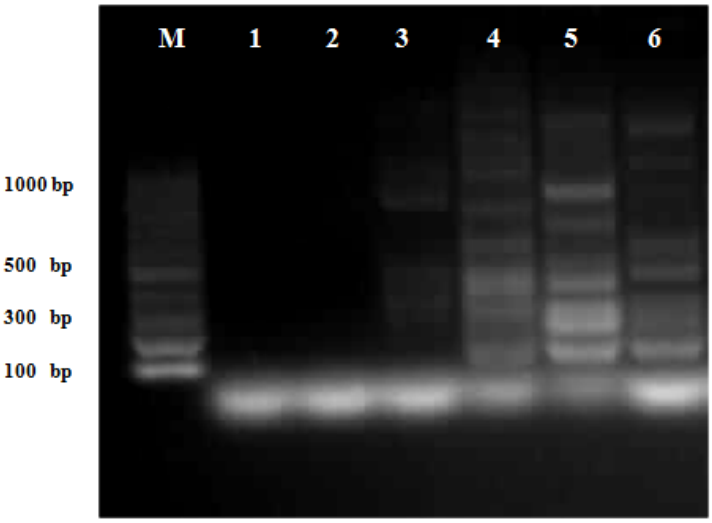
In total, DNA isolated from 19 recalcitrant genotypes of maize was tested. The maize DNA contaminated by inhibitory compounds wasn't amplified in subsequent iPBS reactions. Optimization of the reaction mixture components (buffer, $MgCl_2$, Dream *Taq* polymerase, concentrations of primers, DNA) and temperature profile of iPBS reactions wasn't sufficient for DNA amplification. Therefore, the TBT-PAR solution was tested in iPBS reactions. The TBT-PAR application enabled amplification of iPBS fragments and their readability from the agarose gel (figure 3). In many experiments inhibitory compounds such as polysaccharides and polyphenols contained in DNA samples may inhibit successful amplification. Application of TBT-PAR additive in the reaction mixture can be an effective solution.



M - molecular marker, 1 - genotype *MBS 847*, 2 - *MO 17*, 3 - *TO 234*, 4 - *A 654*, 5 - *Oh 43*, 6 - *DE 811*, 7 - Tva 194-9, 8 - Tva 261-9, 9 - *Tva 260-9* , 10 - *Tva 53-9*, 11 - *Tva 56-9*, 12 - *Tva 199-9*, 13 - *Tva 202-9*, 14 - *Tva 201-9*, 15 - *Tva 99-9*, 16 - *TO 784Ft*, 17 - *TO784CMs*, 18 - *TO2M*, 19 - *FANAL8*
Figure 3 The iPBS reactions profile of 19 recalcitrant genotypes of maize; iPBS primer marked as 1879 was used

Three yacon genotypes (*PER05, ECU45, BOL22*) in iPBS reactions with and without addition of TBT-PAR solution were tested. Amplification reactions in the experimental iPBS variants without TBT-PAR solution weren't sufficient.

When analysing of yacon samples, the effect of TBT-PAR was most visible (figure 4). **Žiarovská *et al.* (2013)** used iPBS method for yacon – *Smallanthus sonchifolius,* (Poepp. Et Endl.) germplasm evaluation. Because yacon is characterized by high level of polyphenols and the quality of extracted DNA is reduced **(Viehmanová, 2009)**, four DNA extraction methods were tested for the best results in the iPBS method. The formation of viscose pellet, and high level of contamination was observed in the DNA samples of yacon through the isolation process using **Rogers and Bendich (1994)** extraction protocol. **Friar's (2005)** extraction protocol and the GeneJET™ Plant Genomic DNA Purification Mini Kit were given the DNA suitable for PCR analyses, and no contamination was detected.



M - molecular marker, 1, 4 - genotype *PER05,* 2, 5 - genotype *ECU45*, 3, 6 - genotype *BOL22*

Figure 4 Comparison of iPBS reaction profiles of yacon TBT- PAR (samples 1-3) and using TBT-PAR additive (samples 4-6); iPBS primer marked as 1886 was used

Khanuja *et al.* (1999) observed that the presence of certain metabolites interferes with DNA extraction procedures and downstream reactions such as DNA amplification, restriction, and cloning. Therefore, the chemotypic heterogenity among plants may not allow optimal DNA yields. Diverse plants such as medicinal, aromatic and many other plants produce essential oils and secondary metabolites, for example alkaloids, flavonoids, phenols, gummy polysaccharides, terpenes and quinones. **Samarakoon *et al.* (2013)** described the method to remove the effect of PCR contaminants for recalcitrant plants.

Consequently, numerous procedures have been developed to reduce the levels of the inhibitory compounds, for example addition of polyvinylpyrrolidone for polyphenols, phenol for proteins, RNase for RNA, and NaCl for polysaccharides **(Bellstedt *et al.*, 2010)**. **Azmat *et al.* (2012)** used for DNA extraction from mature mango leaves a modified protocol based on the cetyltrimethylammonium bromide (CTAB) method. The DNA isolation methods may not be sufficient for many plants. **Belogrudova *et al.* (2005)** obtained a large amount of high quality DNA of *Liparis loeselii* using modified extraction method by **Frier (2005)**. *L. loeselii* has high content of phenols in leaves and it decreases the quality of isolated DNA. **Schori *et al.* (2013)** in their study used engineered DNA polymerase for improvement of PCR results for plastid DNA. The "KAPA3G" DNA Polymerase was derived from a previously engineered, more processive variant of *Taq* DNA polymerase and therefore, the KAPA3G Plant PCR Kit can be a very usefull tool for difficult taxa. Although the KAPA3G Plant PCR Kit did not always lead to high-quality sequence data, it effectively amplified DNA that failed to amplify with *Taq* polymerase.

CONCLUSION

In the presented study the potential of trehalose-based (TBT-PAR) additive to improve PCR amplification reactions was discussed. The TBT-PAR solution was tested using retrotransposon-based marker technique (iPBS) for Chernobyl flax (*Linum usitatissimum* L.), maize (*Zea mays* L.) and yacon (*Smallanthus sonchifolius*). It can be concluded that TBT-PAR additive is appropriate for application in the iPBS reactions. Efficiency of TBT-PAR solution was proved using the plants DNA with higher content of inhibitory compounds. Genomic DNA was isolated from flax and maize using the method described by **Rogers and Bendich (1994)**. The DNA from yacon was isolated using three extraction methods and one manufactured extraction kit **(Žiarovská *et* al., 2013)**. Numerous failures in iPBS profiles were removed after addition of TBT-PAR. In Chernobyl flax iPBS fragments with higher molecular weight were amplified, and improvements were also observed in the samples of maize and yacon.

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