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Short Communication

INSERTION OF A REAMPLIFICATION ROUND INTO THE ISSR-PCR PROTOCOL GIVES NEW FLAX FINGERPRINTING PATTERNS.

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Abstract: We expanded the basic ISSR-PCR protocol by an additional PCR reamplification round in order to detect whether increased PCR productivity would give new bands in ISSR patterns. We found that the reamplification step had a prominent impact on the quality of the inter-simple-sequence repeat (ISSR) PCR patterns of flax, depending on the particular primer used for PCR amplification. We could clearly distinguish between two types of reamplification effect. Most ISSR primers (16 out of 21) gave no reamplification effect as usual, but five primers (23.8%) provided a new ISSR fingerprinting pattern after the 2nd reamplification round, leaving the previous 1st round pattern completely blank. Therefore, we recommend the expansion of a basic ISSR-PCR protocol for another reamplification round in order to mine out full the fingerprinting potential from ISSR-PCR method.

Key words: Genetic Markers; Germplasm Identification; Inter-microsatellite Repeats; ISSR-PCR; *Linum usitatissimum* L.; PCR Reamplification

INTRODUCTION

Oligonucleotide primers of ISSR-PCR are usually designed by random playing with the core sequence motifs of microsatellites; there is only one generally accepted rule: the adjunction of either the 5' or 3' flanking anchor. These anchors ensure a higher resolution and better reproducibility of the bands in ISSR patterns [1], which is desirable for automated computer-assisted interpretation of ISSR patterns.
In this paper, we report on the prominent effect given by a PCR reamplification step inserted into the standard ISSR-PCR protocol [1, 2], when the ISSR primers of a particular sequence are designed.

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MATERIALS AND METHODS

Plant material

Four sample accessions of *Linum usitatissimum* L. (cv.VENIKA, cv.WIKO, cv.BILTSTAR, and cv.ARIANE) were selected for this study from the germplasm collection of AGRITEC Research Breeding Services Ltd., Šumperk, the Czech Republic.

DNA isolation

DNA samples were extracted according to a modified version of the protocol of [2] using 3-day old seedlings germinated in the dark (22ºC), because we found that in ethiolated tissue, the amount of PCR inhibitors contaminating isolated DNA is substantially reduced. Leaf tissue (30 mg) was first homogenized under liquid nitrogen with a mortar and pestle in the presence of 200 mg glass powder and 30 mg polyvinyl pyrrolidone, then further thoroughly ground in a 1 mL extraction buffer (3mL 1M Tris-HCl pH7.5; 0.75 mL 0.5M Na_2EDTA; 3 mL 5M NaCl, 0.75 mL 10% SDS; optionally 30µL 2-mercaptoethanol may be added; water to 15 mL final volume) pre-warmed to 60ºC. In the next step, the homogenate was incubated in a 1.5 mL Eppendorf tube for 1 hour at 65ºC. After spinning for 20 minutes at maximum speed in a table centrifuge (12,000 g), the supernatant (ca. 700 µL) was transferred into a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed by repeated inversion of the tube. After this, centrifugation at maximum speed for 20 minutes was used to completely clear out the top aqueous phase. This upper phase was then transferred into a new tube. Added cold isopropanol precipitated the DNA during overnight incubation at -20ºC. The DNA precipitate was then collected by a brief (1-3 minute) spinning at maximum speed; the resulting pellet was washed with 600 µL 70% ethanol, dried for 5 minutes in air (over-drying would prevent full redissolution of the DNA precipitate) and resuspended in 80µL 0.01M TE buffer. A subsequent incubation for 1 hour at 37ºC in the presence of 5 µL RNase A (Sigma, USA; stock solution 10 mg/mL) eliminated RNA from the sample. The DNA purity was strongly increased by the second precipitation with 10 µL 5M ammonium acetate and 200 µL absolute ethanol overnight at -20ºC. After brief spinning at maximum speed, the precipitate was collected, washed with 70% ethanol, briefly dried and resuspended in 100 µL TE buffer. After a spectrophotometric determination of the DNA concentration, it was used in a ISSR-PCR reaction.

ISSR-PCR protocol

The PCR reaction mixture (25 µL volume) contained 2.5 µL 10x PCR buffer, 4 mM $MgCl_2$, 2.5 mM dNTP mix, 0.2 µM ISSR primer, 20 ng template DNA (or, in the 2nd reamplification round, 1 µL PCR reaction product from the 1st round) and 1U TaKaRa polymerase (TaKaRa Shuzo, Japan). PCR thermocycling was carried out on a Perkin-Elmer 480 thermal cycler using the following

thermoprofile: the initial pre-denaturation cycle at 94ºC for 3 minutes was followed by 35 cycles of 94ºC for 60 seconds; the annealing temperatures in the 1st round and the 2nd reamplifying round were 55ºC, and 60ºC, respectively. The cycles were finished by one cycle of 72ºC for 3 minutes. The sequences of 21 ISSR primers (Generi Biotech, Czech Republic) used are listed in Tab. 1.
The ISSR patterns displayed on ethidium bromide-stained agarose gels were scanned into TIFF format images on a UVP GDS 5000 system (UVP Ltd., Cambridge, England). The detection system was optimized to maximum sensitivity.
All the DNA samples were isolated, PCR amplified, and analyzed in two independent repetitions. Both ISSR-PCR reaction products from the 1st and 2nd (reamplification) PCR round were always separated jointly on one gel.

Tab. 1. The sequences and reamplification effects of individual ISSR primers.

Number	Name	Sequence	Reamplification effect
1	3PCT4	5'-VRV $[CT]_6$	NO
2	3PCT5	5'-VRV $[TG]_6$	NO
3	3PCT6	5'-VSS $[GATA]_6$	YES
4	PCT6	5'-$[GATA]_6$	YES
5	3PCT1S	5'-YHY $[GA]_6$	NO
6	3PCTanS	5'-SSS $[GA]_6$	NO
7	3PCTanW	5'-WWW $[GA]_6$	NO
8	PCT1an3W	5'-$[GA]_{15}$ WWT	NO
9	PCT1an3S	5'-$[GA]_{15}$ SSG	NO
10	PCT2an3W	5'-$[CT]_{15}$ WWT	YES
11	PCT2an3S	5'-$[CT]_{15}$ SSG	NO
12	PCT1noan	5'-$[GA]_{15}$	NO
13	PCT2noan	5'-$[CT]_{15}$	NO
14	PCTAT	5'-N7 $[AT]_{10}$	NO
15	PCTAA	5'-N7$[A]_{20}$	NO
16	TETRA1	5'-KKB NVS S $[ATCT]_6$	YES
17	TETRA2	5'-KKB NVS S $[GGCT]_6$	NO
18	TETRA3	5'-KKB NVS S $[CTTT]_6$	NO
19	TETRA4	5'-KKB NVS S $[GACA]_6$	NO
20	TETRA5	5'-KKB NVS S $[CTAT]_6$	YES
21	HEPTA1	5'-KKB NVS S $[CCC\ T\ AAA]_4$	NO

RESULTS AND DISCUSSION

We studied the effect of the insertion of a reamplification round into ISSR-PCR in order to inspect whether enhanced PCR productivity would give new bands

on ISSR patterns, as had already been reported earlier [3]. In addition, any single-stranded DNA species from the 1st round reaction mixture are reportedly reduced after a reamplification round, which should be useful in avoiding misinterpretation of band polymorphism [4]. When the reamplification step is included in the PCR protocol, we may presume three possible effects on the ISSR patterns: a) no increase in the level/amount/concentration of PCR products, due to a partial inhibition of the PCR reaction by an excessively high concentration of the 1st-round PCR products used in the 2nd round as DNA template [5]; b) a smear of reaction products instead of distinct bands [3]; or c) a desirable increase in PCR product concentration, *i.e.* a higher intensity of bands [6-8].

Within the 21 studied primers applied on flax germplasm, we did not notice any single-stranded DNA species which disappeared from the ISSR pattern after reamplification. Also, we did not obtain any smear in the pattern of any ISSR primer reaction. Yet we could sharply distinguish two types of reamplification effect on ISSR patterns (Fig. 1, 2).

The insertion of a reamplification step either resulted in an even (or slightly stronger/weaker) intensity of the bands on the gel pattern when the 2nd round was compared to the 1st round. Having yielded no new bands, these cases were classified as "no reamplification effect" (Fig. 1). Alternatively, we obtained a totally new band pattern in the 2nd round, which brought new ISSR polymorphism (Fig. 2). Those primers with a "strong reamplification effect" were 3PCT6, PCT6, PCT2an3W, TETRA1 and TETRA5 (see Tab. 1 for the primer sequences) and represented 23.8% of all the primers tested here. The ISSR patterns of the primers obtained from the 1st round were completely blank (Fig. 2).

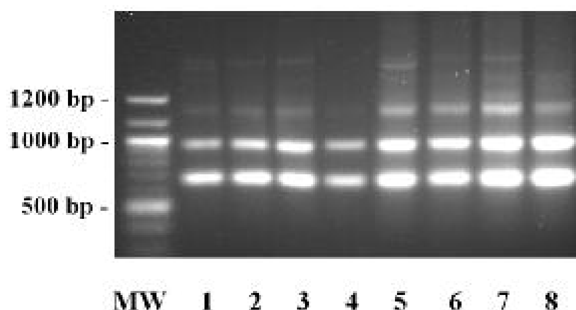

Fig.1. No reamplification effect. A sample ISSR pattern (agarose gel with ethidium bromide staining) using the primer TETRA4 (5'- KKB NVS S[GACA]$_6$) demonstrating the effect of reamplification. This is the example of the reamplification variant in which the reamplification round of ISSR-PCR brings no increase in the number of bands. 1 ... VENIKA (1st round), 2 ... WIKO (1st round), 3 ... BILTSTAR (1st round), 4 ... ARIANE (1st round), 5 .. VENIKA (2nd round), 6 ... WIKO (2nd round), 7 ... BILTSTAR (2nd round), 8 ... ARIANE (2nd round).

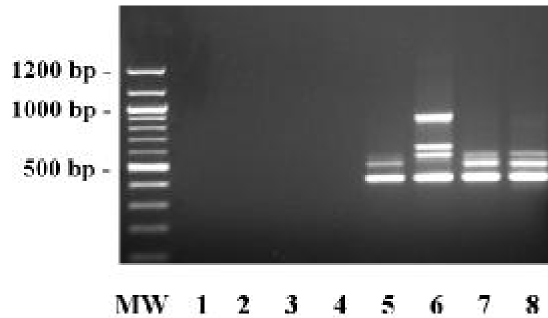

Fig.2. Strong reamplification effect. A sample of the ISSR pattern (agarose gel with ethidium bromide staining) using the primer 3PCT6 (5'- VSS[GATA]$_6$) demonstrating the effect of reamplification. This is the example of the reamplification variant providing a totally new fingerprinting pattern in the 2nd round of ISSR-PCR (the 1st round ISSR pattern was completely band-free). 1 ... VENIKA (1st round), 2 ... WIKO (1st round), 3 ... BILTSTAR (1st round), 4 ... ARIANE (1st round), 5 .. VENIKA (2nd round), 6 ... WIKO (2nd round), 7 ... BILTSTAR (2nd round), 8 ... ARIANE (2nd round).

These "reamplification-prone" primers would usually be removed from the screened primer set as "non-perspective" during the initial primer selection when only 1st round ISSR-PCR screening is performed. Consequently, a substantial portion of the genetic markers obtainable from ISSR polymorphism would be definitely lost. Within the set of 21 primers, the insertion of the reamplification step into ISSR-PCR protocol increased the total number of bands 1.98 times, and the number of polymorphic bands 5.00 times. These statistics justify the regular insertion of a reamplification round into the ISSR-PCR protocol.
We can conclude that the data discussed above document the benefit from the insertion of a reamplification step into the standard one-round ISSR-PCR protocol, thus making the primer screening scheme more effective in the selection of highly informative ISSR primers suitable for the identification of plant germplasm accessions required in basic ecological, population, and phylogenetic studies as well as in crop authentication [9].

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