Biological Forum – An International Journal **9**(2): 87-91(2017)

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

A Multiplex PCR assay for Discriminating Charlock from Rapeseed: Implications for Seed Testing

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(Received 17 June 2017, Accepted 27 July, 2017)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: So far, a number of specific markers have been introduced for genome identification and phylogenic studies in *Brassicaceae* family. We introduced a new application for these markers *i.e.* identifying charlock as a noxious weed in seed samples of rapeseed. A multiplex PCR assay was designed based on 625, 190 and 325 bp fragments of *Brassica Transparent Testa Glabra 1 (TTG1)* gene, 5S ribosomal DNA, and cruciferin gene as internal control. The resulting presence/absence amplification pattern could differentiate *Sinapis arvensis* genome (SarSar) from that of *Brassica napus* (AACC). As an alternative to chemical tests, this assay can complement visual identification wherein charlock seeds are discerned based on seed morphology by seed analysts. However, the discriminating characteristics are completely hidden once working samples are taken from film-coated seeds. Moreover, interaction of chemicals used in film coating might result in misclassification. Therefore, the introduced molecular assay can reliably differentiate charlock seeds from rapeseed and be used in determination of other seeds by counting as a part of seed testing.

Keywords: charlock, rapeseed, noxious weed, seed testing

INTRODUCTION

In spite of being a valuable source of resistance to diseases and pod shattering (Snowdon *et al*., 2000; Liu *et al*., 2014) and recently novel type of cytoplasmic male sterility (Liu *et al*., 2015), charlock or wild mustard (*Sinapis arvensis* L.) is a notorious weed of Brassica crops. Moreover, when it comes to determining other seeds in rapeseed (*Brassica napus* L.) seed samples, it is also considered as a noxious weed along with other seeds like wild radish (*Raphanus raphanistrum*) and turnip weed (*Rapistrum rugosum*). Maximum number of restricted seeds in a noxious weed exam varies between countries as defined in national seed standards. For instance, Iran's seed standard tolerate up to seven weed seed (including charlock) in a 100g sample of rapeseed. There is also much more stringent standard by restricting up to two seeds in a half pound sample of certified rapeseed bag as mandated by Oregon Seed Certification Service, USA. Although rapeseed and charlock could be roughly distinguished from each other by leaf, stem, and petal characteristics in the field, there is more resemblance between seeds of two species. Morphologically, black and reddish seeds of charlock are spherical that could be easily rolled, compared to seeds of rapeseed that are similarly spherical with color range from reddish or reddish-brown to gray or grayish-black. There is also other similarity in their reticulum that gives a smooth and fine appearance to both seeds. However, these subtle differences of surface characteristics are completely covered when working samples are of film-coated seeds. As a complement to morphological identification, suspected charlock seeds could be chemically discriminated by chloral hydrate (Allen, 1917), hydrogen chloride (Dhesi *et al*., 1970) and potassium hydroxide (van der Berg & Vierbergen, 1979) tests from other *Brassica* seeds. In the latter test, as reported in International Seed testing Association's (ISTA) handbook of variety testing (ISTA, 1993), charlock seeds have yellow-green to bluish-green fluorescence after 30-45 minutes under ultraviolet exposure compared to *B. napus* and *B. rapa* seeds, once placed on a filter paper moistened with fresh 5% KOH at 20°C in a closed petri dish.

A number of studies have been conducted at genome level in order to dissect phylogenic and taxonomic relationship between species of Brassicaceae family.

Yan *et al*. (2014) introduced genome-specific markers derived from Brassica Transparent Testa Glabra 1 (TTG1) gene for distinguishing AA, BB, and CC genomes of six U-triangle *Brassica* species (U, 1935). Therefore, it is speculated that diploid genome of charlock (SarSar, 2n=18) is different with that of allotetraploid rapeseed (AACC, 2n=38) while being more relevant to B-genome based on cytological studies (Schelfhout *et al*., 2004). Other studies also suggested that B-genome may have fewer resemblance to A- and C- genomes (Hosaka *et al*. 1990; Chèvre *et al*. 1991; Song *et al*. 1988; Quiros *et al*. 1991; Truco *et al*. 1996).This assumption was further supported by introducing specific sequence characterized amplified region (SCAR) markers specific to B-genome. While these microsatellite-based markers were also common in charlock, they did not amplify in other species with A- or C-genomes (Pankin & Khavkin, 2011). Besides to nuclear simple sequence repeats (SSRs), a number of plastid SSR markers have been also introduced for detecting polymorphism between *Brassica* species (Flannery *et al*. 2006). Moreover, other tandemly repetitive blocks of DNA like ribosomal DNA (rDNA) were deployed for generation of specific markers to Brassicas and charlock genomes (La Mura *et al*. 2010). With recent transcriptome sequencing of *Sinapis arvensis*, a number of unigenes are also available that could be utilized for development of genome specific markers to charlock (Liu *et al*., 2014).

We developed a multiplex PCR assays based on the presence and absence of respective genome-specific markers in rapeseed and charlock developed in previous studies. This assay, as a complement to visual inspection, can reliably differentiate charlock seeds with SarSar genome from AACC of rapeseed and be used in determination of other seeds by counting as a part of seed testing.

MATERIALS AND METHODS

A. Plant Materials

Hybrid seeds of rapeseed were taken from varieties Neptune (Euralis Semences, France) and Hyola 50 (Pacific Seeds, Australia). In addition field-collected seeds of charlock were used in present study.

B. DNA Extraction from single seed

Isolation of DNA from single seeds of rapeseed or charlock was carried out on small scale basis using sodium dodecyl sulfate (SDS) method. Firstly, single seed was crushed in a 2-mL safe lock tube using a sampler tip. After effective disruption of seed, 300µL of EB (0.5 M Tris pH 7.5, 0.35 M NaCl, 0.5% SDS) was added to the tube, vortex mixed and heated at 68°C for 45 minutes in water bath. Then, the tubes were centrifuged in 5430 R (Eppendorf, Germany) for 10 minutes at 13,000 rpm. After that, the supernatant was transferred to a fresh tube and one-tenth volume of potassium acetate (0.5M) was added to it. After vortex mixing and placing the tube for 15 minutes on ice, prior to centrifuging for 10 minutes at 13,000 rpm. The resulting supernatant again was transferred to a fresh tube. Two-volume of pre-chilled ethanol and one-tenth volume of 3 M sodium acetate were added to the tube. After gently inverting for 10 times, the tube was kept in freezer (-20°C) for at least one hour. The tube then was centrifuged for 7 minutes at 7,000 rpm. After discarding the supernatant the resulting pellet was washed with pre-chilled ethanol 70% and centrifuged for 3 minutes at 7,000 rpm. Then discarding the supernatant was repeated and the pellet was air dried. Finally, the pellet was dissolved in 50µL of TE buffer (1M Tris, 0.5 M EDTA, pH 8.0). Quality and quantity of DNAs were checked by Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, the USA).

C. Selection of markers

A number of genome-specific markers developed in other studies were utilized in discriminating rapeseed and charlock seeds (Table 1). Namely, three primer pairs i.e. DA and DC targeting TTG1 gene (Yan *et al.* 2014), two SSR markers i.e. BniB.Ni2-H06.2 (hereafter H06) and BniB.Na12-A02.3 (hereafter A02) (Pankin & Khavkin, 2011), two plastid SSR markers i.e. MF3 and MF7 (Flannery *et al*. 2006), and 5S rDNA-based unique markers for A,C and Sar genomes (La Mura *et al*. 2010) were used for discrimination analysis. Moreover, forward and reverse primers targeting 5S rDNA were used as internal control in PCR reactions.

D. Design of primers

A pair of primer (named as Cruc) for amplifying a 325bp fragment of BnC1 crucifer in storage protein gene (GenBank Acc. No. X59294) were designed by Primer3 (http://bioinfo.ut.ee/primer3/) and synthesized by Bioneer, Inc. South Korea. (Table 1).

E. Multiplex PCR assay

Multiplex PCR assays were performed in a volume of 20 µL containing 50 ng template DNA, 1X PCR Master Mix (0.025 unit Taq DNA polymerase, 2mM $MgCl_2$, 0.2mM dNTPs) (CinnaGen, Iran), and 0.3 pmol (Cruc), 0.7 pmol (DC) and 0.3 pmol (S-5S) forward and reverse primers.

Table 1: List of selected primers, fragment size, and expected target genome or gene.

Primer Name	Sequence (5'-3')	fragment size	Expected target Genome/Gene	Annea l temp	References
DA	F: GGGTTTTCGCCTCGGTCTCC R: ACTCCCCTGGTGCCGCTGC	239	AA	58	Yan *et al.* 2014
DC	F: ACTCCGACTCCATGTCCCTCA R: ACACTCCCCTGGTGCCTTTCA	625	CC	58	
H06	F: AAGCGTCGAGGATGAACCCTAG R: CATTGTCCTTTTGTTGCATTGCTGTTAC	190-200	BB, SarSar	60	Pankin & Khavkin, 2011
A02	F: ACCGCCATTGAAACCTTCTTTTTACCCAC R: AGTGAATCGATGATCTCGCCGTTGTCC	170-180	BB, SarSar	68	
Cruc	F: CCATCCTTCGCTTCCTTCGT R: GTCGAACACTCTGTCACCGT	325	*BnC1*	58	This study, GenBank accession X59294
S-5S	F:CTTGGTCGGTGATAGCTCAT R:GCAACGGAAGTGCACCGT	190	SarSar	60	La Mura *et al*. 2010
5S rDNA	F:CTGGGAAGTCCTCGTGTTG R:TTAGTGCTGGTATGATCGCA	400	5S rDNA	56	
MF3	F: AATGGTATGACTAGCTTATAAGG R:CTTAACAATGAGATGAGGCAATC	273-311	*trnE-trnT*	60	Flannery *et al*.2006
MF7	F:CGGCAGGAGTCATTGGTTCAAA R:GATTTTGTAACTAGCTGACG	142-171	*TrnM-atpE*	60	

The amplification consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C (denaturation) for 35 seconds, 60°C (annealing) for 30 seconds and 72°C (extension) for 30 seconds, and a final extension at 72°C for 5 min. An electrophoresis unit (SCIE-PLAS, the UK) was used for resolving PCR products on a 2% agarose gel containing GelRed™ (Biotium, the USA). Image of gels was taken by G:BOX (SYNGENE, the UK) gel documentation system.

F. Sequence analysis

Sequencing of H06 amplicon were performed by Bioneer Inc., South Korea, using 3730xl DNA Analyzer (Applied Biosystems, USA). Multiple sequence alignment of H06 microsatellite locus in *B. napus* and *S. arvensis* with *B. nigra* (Genbank Acc. No. EF196659), *B. juncea* (Genbank Acc. No. 196658), and *B. carinata* (Genbank Acc. No. EF196657) was conducted in T-Coffee program (http://www.ebi.ac.uk/Tools/msa/tcoffee/).

RESULTS

A. Identifying charlock by SSR markers

In contrast to results of discerning BB and SarSar genomes from other genomes by amplification of SSR markers H06 and A02 (Pankin & Khavkin, 2011) we showed that these loci are also amplified in rapeseed with AACC genome. Among them, H06 appeared to be polymorphic between rapeseed and charlock compared to monomorphic pattern of A02. Alignment of sequencing result indicated that the H06 amplicon has a (CT)8 motif in rapeseed compared to (CT)6 in charlock that resulted in a 11bp size difference (Table 2, Fig. 1). Noteworthy to say that, no polymorphic pattern was detected between 10 varieties of rapeseed (data not shown).

As regards plastid SSRs, amplification of MF3 and MF7 loci that target respective trnE-trnT and TrnM-atpE spacer gene regions resulted in detecting variation between *B. napus* and *S. arvensis* species.

Table 2: Number of CT motif and amplicon size of H06 SSR marker in four *Brassica* species and *Sinapis arvensis*.

Species	$(CT)_n$	Amplicon size (bp)	Reference
S. arvensis	12	168	This study
B. napus	16	179	This study
B. nigra	52	215	EF196659, Pankin & Khavkin, 2011
B. juncea	42	203	EF196658, Pankin & Khavkin, 2011
B. carinata	20	179	EF196657, Pankin & Khavkin, 2011

The repetitive mononucleotide motif (Tn) of these loci resulted in detection of 10 and 8 bp difference respectively as resolved in polyacrylamide gel. These suggest that plastid SSRs (MF3 and MF7) and nuclear SSR (H06) could be used in discriminating seeds of charlock from rapeseed.

B. Multiplex PCR assay based on genome-specific markers

Genome-specific amplification of *Brassica* TTG1 gene with DA and DC primers was verified in present study by amplification of 239 and 625 bp fragments respectively. Thus, DA and DC primers were amplified in AACC genome of rapeseed but in SarSar genome of charlock. On the other hand, amplification of S-5S primer was only successful in seed-extracted DNA of *S. arvensis* whilst failed in rapeseed. We developed a multiplex PCR assay consisting DC and S-5S primers together with Cruc forward and reverse primers as internal control, based on presence/absence amplification pattern. The results of assay showed that rapeseed could be discriminated with 625 and 325 bp fragments targeting C-genome and crucifer in gene respectively. On the other hand, charlock seeds could be identified with 325 and 190 bp bands in agarose gel from rapeseed by targeting respective cruciferin gene (internal control) and SarSar genome (Fig. 1).

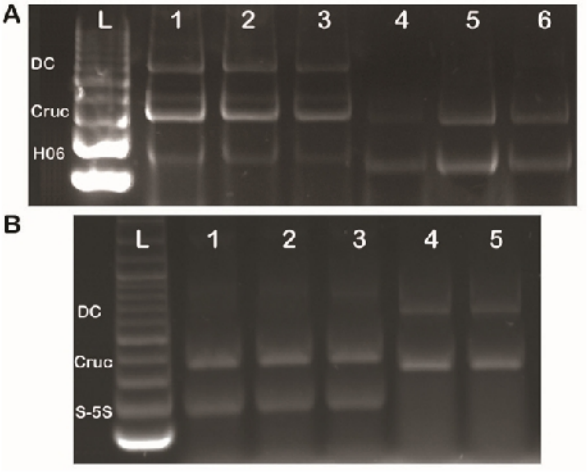

Fig. 1. Agarose gel demonstrating results of two multiplex PCR assay for discriminating charlock from rapeseed. (A) Multiplex PCR of a 625 bp of C-genome specific marker (DC) with an SSR marker (H06) in rapeseed (Lanes 1-3) and charlock (Lanes 4-6). (B) Multiplex PCR of a 625 bp of C-genome specific marker (DC) with a 190 bp of SarSar genome specific marker (S-5S) in charlock (Lanes 1-3) and rapeseed (Lanes 4-5). A 325 bp of cruciferin gene (Cruc) was used as internal control in both assays. L: 100 bp ladder

DISCUSSION

In the present study, a new application was adopted for genome-specific markers in other seeds exam besides to other applications of genome identification and phylogenic studies (Schelfhout *et al*. 2004; Pankin & Khavkin, 2011), interspecific hybridization (Schelfhout *et al*. 2004; La Mura *et al*. 2010; Yan *et al*. 2014) and tracing pollen flow and risk assessment of genetically modified rapeseeds (Yan *et al*. 2014).

Indication of positive amplification for a set of 8 specific SCAR markers in B- and SarSar genomes against negative in A- and C-genomes was rejected in our study. This was shown by amplification of two selected SSR markers i.e. H06 and A02 in rapeseed with AACC genome. Among them, the polymorphic pattern of H06 between rapeseed and charlock in agarose gel introduced it as a discriminating marker. As a presence/absence figure was much more favorable, we utilized DC and S-5S markers for discerning CC and SarSar genomes respectively along with an internal control that targets BnC1 gene in Brassicaceae family. However, DC marker could be replaced with DA with an amplicon size of 239bp to target AA genome of rapeseed in the introduced multiplex PCR assay.

This assay could be used as an alternative to chemical test in order to complement morphological identification of charlock in seed samples of rapeseed. Compared to chemical method, it takes more time and imposes a considerable cost for seed testing laboratory in terms of materials used in DNA extraction, PCR, and electrophoresis. However, this molecular test gives a considerably more reliable diagnosis since the genomes of seeds are targeted by specific markers. It is also free from any observational bias that may arise from interaction of materials (including colorants) used in seed coating with like potassium hydroxide in chemical test.

Therefore, the introduced molecular assay can reliably differentiate charlock seeds from rapeseed and be used in determination of other seeds by counting as a part of seed testing.

ACKNOWLEDGEMENTS

The authors gratefully thank Seed & Plant Certification & Registration Institute (SPCRI) for funding the project.

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