Characterization of oilseed genotypes using microsatellite based DNA barcode

Katalin Pilinszky[1], János Falusi[2], Antal Szőke[1], Erzsébet Kiss[1], László Heszky[1]
[1]Institute of Genetics and Biotechnology, Szent István University, Gödöllő, Hungary
[2]Cereal Research Non-Profit Ltd., Táplánszentkereszt, Hungary

Summary

Nowadays, a new oilseed rape variety must undergo distinctness, uniformity and stability (DUS) testing that constitutes the protection of plant breeders' intellectual property. A cost effective and reliable test method would be desirable to identify the parent-progeny relationship or the pedigree of new varieties and hybrids. In this work, twenty-four rapeseed varieties and hybrids bred by Cereal Research Non-Profit Ltd. (Táplánszentkereszt, Hungary) were examined to assess genetic diversity and to specify fingerprintings for each sample. Seventeen SSR primers were tested, but only twelve SSR primers amplified appropriate patterns. Altogether, 64 alleles were scored and 5.3 alleles per locus on average were detected. It was found that these markers showed individual fingerprints for each genotype, which proved the parent-progeny relationship of the pedigree of new hybrids and varieties. Based on SSR profiles, barcodes were constructed to make the comparisons of different genotypes easier.

Introduction

Brassica napus L. is an important oilseed and fodder crop that originated in a limited geographic region. Currently, oilseed rape is the most important source of vegetable oil in Europe and the second most important source after sunflower in Hungary. Last year the total area of oilseed rape was 290.000 ha with 581.000 tons yield in Hungary and the area is gradually increasing year by year since there is an increasing interest in vegetable oil as biofuel.

New crop varieties must undergo distinctness, uniformity and stability (DUS) testing that constitutes the protection of plant breeders' intellectual property. In DUS testing, each candidate variety must differ from others and be uniform and stable in the characteristics used to show distinctness. Furthermore, the comparison of the increasing number of new varieties is based on morphological characters. However, the morphological traits which are related to certain phenological phases and are highly limited in numbers, are not always sufficient to differentiate between distinct genotypes. Moreover, this current testing system is expensive and time-consuming. To complement the above-mentioned method, a cost effective and reliable test method would be desirable.

At first, different biochemical features have been used for fingerprinting *B. napus* varieties, using GLC analysis that includes the oil fatty acid profile of the seeds (White and Law 1991), the HPLC analysis of leaf glucosinolates (Adams et al. 1989) and starch-gel electrophoresis of cotyledon isoenzymes (Mundges et al. 1990). Biochemical and morphological markers can detect only a limited degree of polymorphism, moreover, they are sensitive to environmental impacts. Nevertheless, molecular markers offer a potential for distinguishing different varieties. RAPD (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) markers have been extensively used for fingerprinting *Brassica* varieties (Kresovich et al. 1992, Mailer et al. 1994, Lee et al. 1996a, 1996b).

Firstly Charters et al. (1996) applied 5'-anchored simple sequence repeats to compare various *B. napus* and *B. rapa* cultivars. Two of the primers could distinguish 16 out of 20 cultivars. It was concluded that anchored SSR analysis was an informative and reproducible method for fingerprinting. Kresovich et al. (1995) screened for GA-, CA-, GATA SSRs motifs in rapeseed. They designed primer pairs and evaluated seven selected SSRs in *B. napus*, *B. rapa*, *B. olearcea*. Szewc-McFadden et al. (1996) evaluated *B napus* and its putative progenitors *B. oleracea* and *B. rapa* using dinucleotid SSRs. Out of 21 SSR markers, 17 amplified product in the three species. Furthermore, 13 showed differences among species. Bond et al. (2004) tested 83 SSRs only half of which could assess the relationship between the cultivars. They categorized SSR amplification by screening the number of amplified alleles. Tommasini et al. (2003) composed fifteen markers that can be applied in multiplex SSR. They observed and analyzed the significance the correlation between SSRs, pedigree, and five morphological characters. Based upon their results, three groups could be distinguished: winter, spring and forage types. Moreover, they did not find any correlation between SSR markers and morphological data. Lowe et al. (2004) developed 398 SSR markers to characterize *Brassica* species, only 53 SSRs of which were used for identification. Piquemal et al. (2005) created a *Brassica napus* genetic map. Fifty-two out of 240 SSR primers were developed by them. 572 F2 individual plants

containing six parental lines were examined. As a result, they came up with a consensus map of 19 linkage groups, which clearly showed the *B. napus* originates from two different ancestors, confirming that A genome belongs to *B. rapa*, while C genome derives from *B. oleracea* in the amphidiploid *B. napus*. This map is available at http://ukcrop.net/perl/ace/search/BrassicaDB.

Materials and Methods

Twenty-four rapeseed varieties and hybrids provided by Cereal Research Non-Profit Ltd. were examined in this study. The material consisted of eight registered varieties and eight candidate hybrids with their maternal and paternal lines. DNA was extracted from young leaf samples using the QIAGEN® DNeasy Plant Mini Kit according to the manufacturers' protocol. PCR was performed with BioRad iCycler. The reaction mixture contained 15-30 ng DNA template, 1 µM of each primer, 75 µM of dNTP, 2 mM $MgCl_2$, 1x PCR buffer and 1 unit Taq polymerase (WestTeam). The PCR profile consisted of the following cycles: precycle 4 min at 94 °C, 36 cycle of denaturation 30s at 94 °C, 30 s annealing at 65-56 °C and 1 min extension at 72 °C, postcycle: 5 min at 72 °C. The primer sequences applied in our experiment are available at http://brassica.bbsrc.ac.uk/BrassicaDB/. The allele sizes of microsatellites were estimated automatically and measured by the ALFexpress II DNA Fragment Analyzer (Amersham Bioscience, Little Chalfont, England, UK) using Cy-5 labelled markers on 8% acrylamide gels. Allele frequencies and DNA barcodes were constructed using the Microsoft Excel 2008 software.

Results and discussion

Microsatellite polymorphisms were determined in 24 rapeseed genotypes, including varieties, candidate hybrids and their parental lines. Altogether, 17 SSR primers were tested in our analyses. Markers showing multiple PCR product or poor amplification are: Na12E02, MR176, Na12H09, Na12A08, Brass061. In some cases, the problem was that the sensitive scoring system showed stuttering, however it was only visualizable with ALFexpress II DNA Fragment Analyzer. Bond et al. (2004) also demonstrated that more than half of the 83 tested and published primers did not amplify appropriately. Tomassini et al. (2003) stated that the separation or distinction between varieties mostly depended on the number and the quality of the markers. Only the primers which amplified clearly were used to analyze the rapeseed varieties and hybrids. Finally, the twelve applied SSR primers were: Na12A02, Na10F06, Na10E02, MR33, Na10D03, Na10D09, Na10G06, Na12C06, Na10C06, Na12A01, Na12C08, Na12B05. The range of different allele sizes at the twelve loci is listed in Table 1. All primers amplified consistently and showed unique genetic fingerprints through the rapeseed samples. Altogether 64 alleles were scored. On average, six alleles per locus were observed. Microsatellite Na12A02, Na10F06, Na10E02, MR33, Na10C06, Na12A01, Na12C08, Na12B05 markers were effective in proving the parent-progeny relationship.

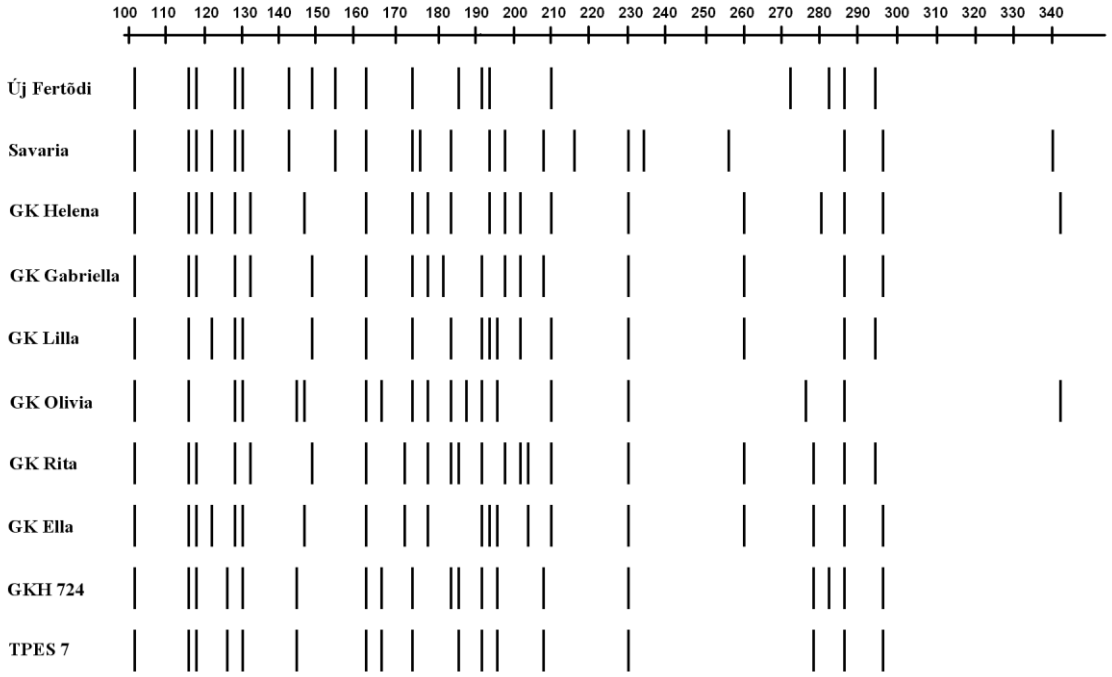

Figure 1. SSR based DNA barcodes of ten genotypes

Infact, many microsatellite markers amplify more than 2 alleles per loci in oilseed rape, thus the analysis and the comparison of genotypes in most cases are difficult. For these reasons, SSR alleles sizes were converted to barcode, which enables us to illustrate the investigated oilseed rape genotypes more easily (see Figure 1).

Firstly Galbács et al. (2009) introduced this SSR based barcoding in grape, by converting SSR data into barcodes. This method was very suitable for comparison of numerous genotypes. The DNA barcode system combined with DUS testing could be more accurate in distinguishing and analyzing new candidate varieties. We proved also that the barcode could be a useful tool for easier identification.

Table 1. Characteristics of Brassica SSR amplification product in the test array

SSR	Size rage of product (bp)	Number of amplified fragments
Na12A02	162-196	7
Na10F06	102-242	5
Na10E02	122-148	5
MR 33	182-192	5
Na10D03	162	1
Na10D09	286	1
Na10G06	172-230	4
Na12A01	116-184	8
Na12C08	282-342	5
Na12B05	130-202	10
Na12C06	210-234	4
Na10C06	202-286	9
		∑=64

References

Adams, H., Vaughan, J.G., Fenwick, G.R. 1989. The use of glucosinolates for cultivar identification in Swede, *Brassica napus* L. var *napobrassica* (L.) Peterm. J Sci Food Agric **46**:319-324

Bond, J.M., Mogg, R.J., Squire, G.R., Johnstone, C. 2004 Microsatellite amplification in *Brassica napus* cultivars: Cultivar variability and relationship to a long-term feral population. Euphytica **139**:173-178

Charters, Y.M., Robertson, A., Wilkinson, M.J., Ramsay, G. 1996. PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5'-anchored simple sequence repeat (SSR) primers. Theor Appl Genet **92**:442–447

Galbács, Zs., Molnár, S., Halász, G., Kozma, P., Hoffmann, S., Kovács, L., Veres, A., Galli, Zs., Szőke, A., Heszky, L., Kiss, E. 2009. Indentification of grapevine cultivars using microsatellite-based DNA barcodes. Vitis **48**:17-24

Kresovich, S., Williams, J.G.K., McFerson, J.R., Routman, E.J., Schaal, B.A. 1992. Characterization of genetic identities and relationships of *Brassica oleracea* L. via a random amplified polymorphic DNA assay. Theor Appl Genet **85**:190-196

Kresovich. S., Szewc-McFadden, A.K., Bliek, S.M., McFerson, J.R. 1995. Abundance and characterization of simple sequence repeats (SSR) loci isolated from a size-fractionated genomic library of *Brassica napus* L. (rapeseed). Theor Appl Genet **91**:206–211

Lee, D., Reeves, J.C., Cooke, R.J. 1996a. DNA profiling and plant variety registration. 1. Restriction fragment length polymorphism in varieties of oilseed rape. Plant Var Seeds **9**:181–190

Lee, D., Reeves, J.C., Cooke, R.J. 1996b. DNA profiling and plant variety registration. 2. The use of random amplified DNA polymorphisms to discriminate between varieties of oilseed rape. Electrophoresis **17**:261–265

Lowe, A.J., Moule, C., Trick, M., Edwards, K.J. 2004. Efficient largescale development of microsatellites for marker and mapping applications in *Brassica* crop species. Theor Appl Genet **108**:1103–1112

Mailer, R.J., Scarth, R., Fristensky, B. 1994. Discrimination among cultivars of rapeseed *(Brassica napus* L.) using DNA polymorphisms amplified from arbitrary primers. Theor Appl Genet **87**:697-704

Mundges, H,. Kohler, W., Friedt, W. 1990. Identification of rape seed cultivars *(Brassica napus)* by starch-gel electrophoresis of enzymes. Euphytica **45**:179-187

Piquemal, J., Cinquin, E., Couton, F., Rondeau, C., Seignoret, E., Doucet, I., Perret, D., Villeger, M.J., Vincourt, P., Blanchard, P. 2005. Construction of an oilseed rape (*Brassica napus* L.) genetic map with SSR markers. Theor Appl Genet **111**:1514-1523

Szewc-McFadden, A., Kresovich, S., Bliek, S., Mitchell, S., McFerson, J. 1996. Identification of polymorphic, conserved simple sequence repeats (SSR's) in cultivated *Brassica* species. Theor Appl Genet **93**:534–538.

Tommasini, L., Batley, J., Arnold, G.M., Cooke, R.J., Donini, D., Law, J.R., Lowe, C., Moule, C., Trick, M., Edwards, K.J. 2003. The development of multiplex simple sequence repeat (SSR) markers to complement distinctness, uniformity and stability tetsting of rapeseed (*Brassica napus* L.) varieties. Theor Appl Genet **106**:1091-1101

White, J., Law, J.R. 1991. Differentiation between varieties of oilseed rape *(Brassica napus* L.) on the basis of the fatty acid composition of the oil. Plant Var Seeds **4**:125-132