Fluorescence-based AFLPs occur as the most suitable marker system for oilseed rape cultivar identification

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Abstract. Three different types of molecular markers, RAPD, SSR and fluorescence-based AFLP, were evaluated and compared for their ability to identify oilseed rape cultivars. The direct comparison of RAPD, SSR and AFLP approaches in cultivar identification showed that the AFLP methodology detected polymorphisms more efficiently than either RAPD or SSR methods. For the characterisation of six oilseed rape cultivars, 60 RAPD primers were tested and only eight of them (14%) detected sufficient levels of polymorphism. Five microsatellites out of fifteen tested were polymorphic, but in all loci, except one, only two different alleles were detected. This result indicated the limited degree of polymorphism found in Brassica napus. Each of the six tested AFLP combinations detected polymorphisms, the best combination (M-CAA/E-ACT) had 26% polymorphic peaks from a total of 90 peaks and could distinguish the analysed cultivars and 4 out of 5 core lines of cultivars. The results presented show that fluorescence-based AFLP is, for the purposes of oilseed rape cultivar fingerprinting, a more suitable approach than either RAPD or SSR.

Key words: AFLP, Brassica napus, oilseed rape, SSR, RAPD.

Introduction

Agricultural crops have been improved over a long period of time. The genetic basis of new cultivars has been decreasing, nowadays it is therefore very difficult to characterise cultivars of many crops on the basis of phenotypic characters (for instance in wheat, maize, sugar beet, fodder crops etc.) (Nielsen 1985). Likewise, the registration and protection of oilseed rape cultivars relies on a relatively small number of morphological characters and as the number
of cultivars increases, the possibility to distinguish them on the morphological basis alone becomes less feasible (Lombard et al. 2000).

So although morphological traits, quality traits and yielding characteristics are currently explored for cultivar protection (ISTA and UPOV directions), new markers are being developed to maintain the efficacy of registration and Distinctness Uniformity Stability (DUS) testing, which guarantees the quality of the new cultivar for farmers and merchants. In the 1980’s, mainly isoenzymes and storage proteins were tested as markers for cultivar characterisation of various crops, including oilseed rape (Gupta, Röbbeelen 1986, Mündges et al. 1990). A disadvantage of these biochemical markers seems to be their relatively low level of polymorphism, probably as a result of the genetic similarity of modern cultivars. They are suitable for the differentiation of *B. napus* from other Brassicas (*B. oleracea, B. rapa* etc.), but for the identification of individual oilseed rape cultivars it is necessary to use additional marker systems for precise cultivar description (Čurn, Sáková 1997).

The DUS testing would benefit from the use of DNA markers, several types of which have been used to assess genetic diversity in the genus *Brassica*, and they may also be used as potential tools for cultivar identification. The restriction fragment length polymorphism (RFLP) analysis has been shown to be a valuable technique for detecting patterns of DNA polymorphism among and within *Brassica* species (Figdore et al. 1988, Hallden et al. 1994, dos Santos et al. 1994). However, this procedure is laborious, expensive, only a few loci are detected per assay and automation is difficult.

The three newer DNA marker systems are based on PCR technology and for this reason are more suitable for routine cultivar identification, due to the small amount of DNA required, and their being generally fast and simple. Random amplified polymorphic DNA (RAPD) analysis allows a large number of markers to be assayed inexpensively using PCR and oligonucleotide primers of arbitrary sequence (Williams et al. 1990). RAPD analysis has been widely used for the detection of genetic polymorphisms in *Brassica* species, especially at the beginning of the last decade (Kresovich et al. 1992, Jain et al. 1994, Mailer et al. 1994). Microsatellites or simple sequence repeats (SSR) are co-dominant, highly polymorphic PCR-based markers and may be expected to be very powerful in cultivar discrimination. Although the development of locus-specific oligonucleotide primers is time-consuming and expensive, recently a range of specific primer pairs for Brassicas has been made available. These primers may prove valuable for cultivar identification (Kresovich et al. 1995, Szewc-McFadden et al. 1996, Uzunova, Ecke 1999). Alternatively, amplified fragment length polymorphism (AFLP) analysis can be employed. AFLP analysis is a technique by which selected fragments from the digestion of total plant DNA are amplified by PCR (Vos et al. 1995). Recent results of the AFLP analysis as a tool for oilseed rape cultivar identification are the most promising compared to the other available methods (Das et al. 1999, Lombard
This is due to the high multiplex ratio, which is the number of information points analysed per experiment (POWELL et al. 1996).

The aim of this study was to compare the results of these three molecular techniques, which can be potentially utilised for oilseed rape cultivar identification. The most suitable method should, in addition to showing high levels of detectable polymorphisms, be rapid, reproducible, labour- and cost-efficient. Important parameters are also time consumption and the necessary laboratory equipment and instrumentation. Our results showed that although fluorescence-based AFLPs are relatively expensive and they require special equipment, they are more suitable for cultivar identification than RAPDs or SSRs.

### Material and methods

#### Plant material

Five registered cultivars and five doubled haploid (DH) lines of oilseed rape were analysed (for description see Table 1). Seeds of the cultivars were obtained directly from breeding stations. DH lines (SL1-SL5) were regenerated via a microspore embryogenesis procedure from the elite half-sib family of the cultivar Slapska Stela. Lines were kindly provided by V. Kučera from the Research Institute of Crop Production in Prague.

#### Table 1. List of investigated oilseed rape cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Parents</th>
<th>Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – Solida</td>
<td>Rod 1129/75 × Rod 3981 × BNW-17 NDR × KM2</td>
<td>Czech Republic</td>
</tr>
<tr>
<td>2 – Arabella</td>
<td>Lines 142/79 × A3/82</td>
<td>Germany</td>
</tr>
<tr>
<td>3 – Sonata</td>
<td>(Bronowski × Zero) × K2040</td>
<td>Czech Republic</td>
</tr>
<tr>
<td>4 – Falcon</td>
<td>Ledos × (Rapol × Hector) × Jet Neuf</td>
<td>Germany</td>
</tr>
<tr>
<td>5 – Lirajet</td>
<td>Lindora × Jet Neuf</td>
<td>Germany</td>
</tr>
<tr>
<td>6 – Slapska Stela</td>
<td>KM × Jet Neuf</td>
<td>Czech Republic</td>
</tr>
</tbody>
</table>

#### DNA extraction and purification

Bulk genomic DNA of oilseed rape cultivars was extracted from young leaves of 2-week-old seedlings by the DNeasy Plant Mini Kit (QIAGEN). We used a minor modification to the manufacturer’s protocol (plant DNA was mixed together to form one bulk). DNA from each of the DH lines was extracted only from the leaves of one seedling.
RAPD analysis

The protocol for RAPD analysis was adapted from that of WILLIAMS et al. (1990). The volume of the final PCR reaction (25 μL) composed of 1× buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 1% Triton X-100), 100 μM dNTP, 10 pM of primer (Operon Technologies, series A, B and F), 1 U DyNAzyme II Taq polymerase (Finzyme) and 25 ng of template DNA. Amplifications were carried out in an MJ Research Thermocycler PTC 100 with 45 cycles of 1 min at 92°C, 2 min at 35°C and 3 min at 72°C. PCR products were separated on 1.5% agarose gel in TBE buffer, and DNA bands were visualized after ethidium bromide staining under UV light.

AFLP assays

AFLP profiles (VOS et al. 1995) were obtained following the Perkin-Elmer Protocol (Part number 402083, Rev. A, 1995). DNA was double-digested with EcoRI and MseI and the resulting fragments were ligated to adaptors specific for the EcoRI and MseI restriction sites. A preselective amplification was carried out with EcoRI+A and MseI+C primers, and the PCR products were then diluted 15-fold with water and used as the template for selective amplifications using both EcoRI+3 and MseI+3 primers. The EcoRI+3 primers were fluorescent labelled with yellow, green and blue dyes (PE – Applied Biosystems). Amplified fragments were separated on an ABI PRISM 310 Genetic Analyser and analysed by GeneScan and Genotyper (PE – Applied Biosystems). In addition to single primer reactions, multi primer reactions based on the Multi-Color fluorescent system were also tested. Primer combinations are listed in Table 2.

<table>
<thead>
<tr>
<th>Type of AFLP reaction</th>
<th>Primer combination</th>
<th>No. of scoreable peaks</th>
<th>No. of polymorphic peaks</th>
<th>Percentage of polymorphic peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single primer</td>
<td>MseI-CAA</td>
<td>EcoRI-AAG</td>
<td>55</td>
<td>3</td>
</tr>
<tr>
<td>Multiple primer</td>
<td>MseI-CAA</td>
<td>EcoRI-ACC</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>MseI-CAA</td>
<td>EcoRI-ACT</td>
<td>90</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>MseI-CCC</td>
<td>EcoRI-AAC</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>EcoRI-AGT</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>EcoRI-AAG</td>
<td>45</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Total – 275</td>
<td>Total – 43</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Number of polymorphic fragments obtained with six AFLP primer pairs in oilseed cultivars
SSR analysis

SSR (microsatellite) loci were amplified using 15 primer pairs: Bn6A2, Bn9A (KRESOVICH et al. 1995), Bn12A, Bn19A, Bn25C2, Bn26A, Bn35D, Bn38A, Bn59A1, Bn68/1, Bn72A, Bn80/3, Bn83/1 (ZEWCE-MCFADDEN et al. 1996), nga129-1, nga139-1 (WESTMAN et al. 1998) under the following conditions: 50 ng DNA template, 1× buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 1% Triton X-100), 100 μM dNTP, 10 pM of primer, 1 U DyNAzyme II Taq polymerase (Finzyme). A total of 30 PCR cycles were performed, with 30 s of denaturation at 94°C, 30 s of annealing at 57°C, and 1 min of polymerisation at 72°C. PCR products were visualised by ethidium bromide staining after electrophoresis on 4% agarose and 10% polyacrylamide gels in TBE buffers.

Data analysis

RAPD and SSR gels were digitised and analysed by the Bioprofil 1D+ software (Vilber Lourmat, France) with manual correction. AFLP electrophenograms ranging in size from 50 to 400 bp and analysed by the Genotyper software, were scored manually for the presence (1) or absence (0) of polymorphic bands across genotypes. Dendrograms were constructed using the UPGMA (Unweighted Pair Group Mean Average) method. PCO plots show components 1 (vertical axis) vs 2 (horizontal axis). Both analyses were calculated using the STATISTICA 6 software package (Statsoft).

Results

RAPD analysis

A total of 60 primers were surveyed across five genotypes. Six primers (10%) did not amplify any product; 70% primers exhibited no or only low levels of polymorphism (polymorphic bands had weak staining intensity); 8 primers (OPA-01, OPA-03, OPA-09, OPA-13, OPB-01, OPB-06, OPB-11, OPB-17) (14%) detected a minimum of 3 polymorphic bands. These polymorphic primers amplified 87 bands and 27 (31%) of this number were polymorphic. This gave an average 3.36 polymorphic products per one polymorphic primer and 0.45 polymorphic products per the total number of primers.

AFLP analysis

A total of 6 primer combinations (Table 2) were tested. Each tested primer combination showed polymorphism. Combination M-CAA + R-ACT was the most polymorphic. Detecting 23 polymorphic products, this should distinguish all the analysed cultivars (Figure 1). A comparison of RAPD and AFLP methods is given in Table 3.
SSR analysis

A total of 15 primer pairs, which were published in original papers, were tested: 13 primer pairs were derived from the genomic library of *B. napus* (KRESOVICH et al. 1995), while 2 pairs (nga) originated from *Arabidopsis* (BELL, ECKER 1994). The analysis confirmed that these two primer pairs amplified microsatellites also in *B. napus* (WESTMAN et al. 1998). Each primer pair gave at least one PCR fragment. The number of fragments differed depending on the separation method. Five polymorphic loci were detected, but only one of these (Bn12A) had more than two alleles. It was not possible to distinguish between all the analysed cultivars, even using combinations of all the five polymorphic primer pairs. Because the level of detectable polymorphism was very low, cluster analysis was not carried out.

Table 3. Characteristics of the RAPD, AFLP and SSR approaches used in analysing oilseed cultivars

<table>
<thead>
<tr>
<th>Marker</th>
<th>Number of primers</th>
<th>Number of polymorphic primers</th>
<th>Percentage of polymorphic primers</th>
<th>Number of polymorphic bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPDs</td>
<td>60 (primers)</td>
<td>8</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td>AFLPs</td>
<td>6 (pr. combination)</td>
<td>6</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>SSRs</td>
<td>5 (primers pairs)</td>
<td>5</td>
<td>30</td>
<td>*</td>
</tr>
</tbody>
</table>

* different depending on the separation system applied

Figure 1. Record of approx. 15% products of primer combination *MseI*-CAA + *EcoRI*-ACT analysed using GENOTYPER software. Shaded areas represent polymorphic bands between cultivars Solida, Falcon and Lirajet.
Genetic similarity

Genetic similarity was calculated using cluster analysis (UPGMA method). UPGMA similarity matrixes were calculated from the total number of polymorphic bands (RAPD 27, AFLP 43, see Figures 2a,b) and from randomly selected 25 polymorphic bands (data not shown). Results for RAPD and AFLP were very similar. In both cases the position of each cultivar in dendrograms was the same. AFLP methods distinguish all the individual DH lines of cultivar Slapska Stela.

The level of genetic similarity between these individuals (DH lines) is surprisingly low and is comparable with those of different cultivars. This fact was not expected, because these core lines were obtained using microsporogenesis. Three cultivars (Falcon, Lirajet, Slapska Stela) had as one of their ancestors cultivar Jet Neuf. This fact was not evident, cultivar Slapska Stela even formed an independent cluster. The PCO analysis in both marker systems showed two close clusters. More considerable differences are only in the position of cultivar Arabella.
Discussion

In this study we evaluated three, recently developed marker systems (RAPD, SSR and AFLP), as tools for oilseed rape cultivar identification. Although each technique differs in the detected levels of polymorphism, our comparison of the RAPD and AFLP approaches confirmed that the results obtained by different marker systems (RAPD, AFLP, SSR, RFLP) are often similar from the point of view of genetic similarity (THORMANN et al. 1994, POWELL et al. 1996, RUSSELL et al. 1997).

Out of 60 RAPD primers tested for the description of six oilseed rape cultivars only eight (14%) detected sufficient levels of polymorphism. The number of polymorphic bands was 3.36 per one polymorphic primer and 0.45 per the total number of primers. MAILER et al. (1994) obtained similar values. They found 6 suitable primers from the total number of 100 and it was essential to use all the 6 primers in order to distinguish 22 *B. napus* cultivars. The number of polymorphic bands per these six primers was 3.8. HALDEN et al. (1994) achieved a higher level of polymorphism. They tested 92 RAPD primers and 76% of these was polymorphic. However, in this case only three lines of *B. napus* were utilized and while two were very similar to each other, one line was more distantly related to the others.

In contrast to RAPDs, each of the six tested combinations of AFLP primers detected polymorphisms. The best combination M-CAA/E-ACT (26% polymorphic peaks of the total number of 90 peaks) can distinguish all the analysed cultivars and also 4 of 5 DH lines from cultivar Slapska Stela. This result showed that AFLPs are the most efficient because of their capacity to reveal many polymorphic bands per assay; however, they do not offer the highest level of polymorphism (POWELL et al. 1996). LOMBARD et al. (2000) found that only two combinations of AFLP primers from the total number of 17 tested could distinguish 83 oilseed rape cultivars. HILL et al. (1996) evaluated the use of AFLP markers for determining phylogenetic relationships in 44 lines of *Lactuca sativa* and 13 accessions of the wild species. They identified a total of 320 polymorphic AFLP loci using only three pairs of primers and only 5 fragments were monomorphic across all the genotypes tested. AFLP markers are used in the last few years for the molecular characterisation and genetic diversity evaluation of *B. napus* cultivars (SEYIS et al. 2003). Another application of this technique is the detection of hybrids between *B. napus* and its relatives (HANSEN et al. 2001, 2003) and transgenic oilseed rape and wild relatives (WARWICK et al. 2003).

Our comparison of RAPD and AFLP showed that the AFLP methodology detected polymorphism more efficiently than the RAPD approach. In this work AFLPs yielded 7.3 polymorphic bands per primer(s), whereas RAPDs only 0.45. DAS et al. (1999) evaluated the genetic relationship between nine cultivars of *B. rapa*. They reported that the level of polymorphism for both RAPD and AFLP approaches was considerably higher than our results, but the number
of polymorphic bands per AFLP assay was 5.6-fold higher compared to that for RAPD (42.6 vs 7.6). The detected number of polymorphic bands per assay in melon samples was 15.08 for AFLPs and only 0.73 for RAPDs (GARCIA-MAS et al. 2000). The range of these values is comparable to our own. RUSSELL et al. (1997) reported 23.2 versus 3.2 for barley, and similar results have been observed in other crops, for instance in rice (FUENTES et al. 1999) or apple (GOULAO et al. 2001). The number of polymorphic loci per assay is important for cultivar identification. The newly registered cultivars need not display polymorphism in analysis with the established cultivars and it may be then necessary to test other tens of primers. Because the evaluation of RAPD gels is relatively subjective, only products with a moderate-to-dark staining intensity and well scored bands are suitable for cultivar identification. These requirements influenced an increasing number of RAPD primers. In addition, the low reproducibility of RAPDs is well known (JONES et al. 1997).

Automated fluorescence dye-labelled AFLP techniques offer significant improvements over radioactive labelling methods by increasing scoring accuracy and typing efficiency (SCHWARZ et al. 2000). On the other hand, these techniques require special equipment and are more expensive in comparison with RAPDs and SSRs, although the automated DNA sequencer is now practically the standard equipment of most molecular biology laboratories. Another approach is the use of a simplified AFLP method with band separation on agarose gels (TYRKA 2002). The automated fluorescence dye-labelled AFLP method has made it possible to detect higher numbers of bands with a higher sensitivity and resolution. RAPD methods are simple, cheap, rapid and also with no special requirements in terms of equipment, as they only require the PCR technology.

SSRs have usually revealed the highest level of polymorphism of all the DNA markers tested (MAUGHAN et al. 1995, SALIMATH et al. 1995, POWELL et al. 1996). A range of specific SSR primers has been developed for Brassica and related species (KRESOVICH et al. 1995, SZEWC-MCFADDEN et al. 1996, UZUNOVA, ECKE 1999) and it is also possible to use known primers from related species (Arabidopsis) (WESTMAN et al. 1998). However, our results confirmed a limited availability of this marker system for oilseed rape cultivar identification. Five microsatellites were found to be polymorphic, but except for Bn12A only two different alleles were detected. UZUNOVA and ECKE (1999) used 11 sequence-specific SSR primer pairs for the genetic analysis of 31 conventional oilseed rape varieties, but only one tested primer pair came up to expectations. KRESOVICH et al. (1995) and SZEWC-MCFADDEN et al. (1996) also reported that a limited degree of polymorphism was detected by SSR in B. napus.

Indeed in SSR the detectable level of polymorphism is more dependent on the separation and visualisation system. Sensitive separation techniques are capable of distinguishing alleles differing in only a small number of bases, but they are not suitable for practical routine usage (sequencing gel, radiolabeling, silver staining). However, the separation of fluorescent labelled products
on an automatic DNA analyser is beside this order. Two separation techniques were used for SSRs in this study: 4% agarose and 10% polyacrylamide gel. In both cases these were stained with ethidium bromide. Under PAGE, the gel showed a higher number of fragments; however, the levels of polymorphism were still low.

An interesting modification of the standard procedure of microsatellite polymorphism detection is the usage of the 5'-anchored SSR primer. Anchored SSR primers are complementary to genomic microsatellites and contain short oligonucleotide ‘anchor’ sequences that ensure the primers being annealed to either the 5’ or 3’ end of genomic repeats. An advantage is the detection of a greater number of loci assayed per reaction. CHARTERS et al. (1996) distinguished all the 20 analysed oil seed rape cultivars using one pair of 5’-anchored SSR primers, and each primer pair was capable of distinguishing only 16 cultivars. Specific primers combined with 5’-anchored primer detect higher levels of polymorphism than only single specific pairs (VARGHESE et al. 2000).

The sensitivity of the AFLP method was shown by the detection of a high degree of intra-cultivar polymorphism in the cultivar Slapska Stela (a Czech variety, registered in 1996). These doubled haploid lines originated from a half-sib family and thus this material should be genetically very uniform. Although oilseed rape is bred according to the pedigree system, a certain level of variation within oilseed rape cultivars is present (BANGA 1993). CHARTES et al. (1996) analysed variation in 20 cultivars using 5’-anchored SSRs, and 14 of these 20 cultivars revealed variability, whereas 3 cultivars were extremely polymorphic. A total of 20 individuals of cultivar Libravo exhibited variability at 10 of the 21 bands scored. Intra-cultivar polymorphism in oil seed rape was also reported by MAILER et al. (1994) in their RAPD analysis. The observed polymorphisms within half-sib families of cultivar Slapska Stela, however, are still surprising, and probable causes should be considered, based on the breeding system used for their development. However, the implementation of advanced methods of cultivar identification requires only maximally uniform material and consequently an adequate approach to plant breeding.

**Conclusions**

The results presented here show that fluorescence-based AFLP is, for the purposes of fingerprinting of oilseed rape cultivars, more suitable than RAPD and SSR. However, only limited numbers of cultivars and primer combinations were analysed. It is necessary to analyse a larger number of AFLP primer combinations on a wide range of cultivars to confirm the results presented in this paper. The Multi-Color fluorescence approach, which allows the separation of three primer combinations in one analysis, could detect polymorphism more
effectively. A comparison of the AFLP and 5’-anchored SSR approaches, which may be similarly effective, but cheaper than AFLP, might also yield interesting results.

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