Identification of oilseed rape cultivars using fluorescence-based AFLP markers

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Abstract

Fluorescence-based AFLP molecular marker was evaluated for its ability to identify oilseed rape cultivars. Each of the six tested AFLP combinations detected polymorphisms, the best combination (MCAA/E-ACT) had 26% of polymorphic peaks from a total of 90 peaks and could distinguish analyzed cultivars, and 4 out of 5 DH lines. The results indicated that fluorescence-based AFLP is a very suitable approach for the purposes of oil seed rape cultivar fingerprinting.

Key words: AFLP, oilseed rape, Brassica napus

Introduction

Registration and protection of oil seed rape cultivars relies on a relatively small number of morphological characters and as the number of cultivars increases, the ability to distinguish them on a morphological basis alone becomes more difficult (Lombard et al., 2000). So although morphological traits, quality traits and yielding characteristic are currently explored for cultivar protection (ISTA and UPOV directions), new markers are being developed to maintain the efficacy of registration and DUS (Distinctness, Uniformity, Stability) testing which guarantees the quality of a new cultivar for farmers and merchants. In the eighties, mainly isoenzymes and storage proteins have been tested as markers for cultivar characterisation of various crops including oil seed rape (Gupta and Robbelen, 1986; and Mundges et al., 1990). A disadvantage of these biochemical markers seems to be their relatively low levels of polymorphism, probably as the result of the genetic similarity of modern cultivars. They are suitable for the differentiation of Brassica napus from other Brassicas (B. oleracea, B. rapa etc.), but for the identification of individual oil seed rape cultivars it is necessary to use additional marker systems for precise cultivar description (Curn and Sakova, 1997). DUS testing would benefit from the use of DNA markers, of which several types have been used to assess genetic diversity in the genus Brassica, and they can also be used as potential techniques for cultivar identification. Restriction fragment length polymorphism (RFLP) analysis has been shown to be a valuable tool for detecting patterns of DNA polymorphism among and within Brassica species (Halleden et al., 1994; Santos et al., 1994 and Figdore et al., 1988). However, this procedure is laborious, expensive, only a few loci are detected per assay, and automation is difficult. The newer three 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 requested, generally fast and simple. Random amplified polymorphic DNA (RAPD) analysis allows large numbers 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 detection of genetic polymorphisms in Brassica species, especially at the beginning of the last decade (Mailer et al., 1994; Jain et al., 1994 and Kresovich et al., 1992). Micro satellites 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 and Uzunova and Ecke, 1999). Alternatively, amplified fragment length polymorphism (AFLP) analysis can be employed. AFLP analysis is a technique by which selected fragments from a digestion of total plant DNA are amplified by PCR (Vos et al., 1995). Recent results of AFLP analysis as a tool for oil seed rape cultivar identification are the most promising compared to other available methods (Lambord et al., 2000 and Das et al., 1999). 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 introduce AFLP approach to description of oil seed rape cultivars and to compare polymorphism of registered cultivars and DH lines.

Materials and methods

Plant material: Five registered cultivars and five doubled haploid lines of oilseed rape were analysed (Table 1). Seed of the cultivars was obtained directly

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from the breeding stations. DH lines (SL1-SL5) were regenerated via a microspore embryogenesis procedure from the Czech cultivar Slapska Stela. The lines were kindly provided by V. Kucera and M. Vyvadilova from the Research Institute of Crop Production in Prague.

**DNA extraction and purification:** Genomic DNA of oilseed rape cultivars was extracted from young leaves of 2-week-old seedlings by the DNeasy Plant Mini Kit (QIAGEN).

**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. EcoRI+3 primers were fluorescent labeled with yellow, green and blue dyes (PE–Applied Biosystems). Amplified fragments were separated on an ABI PRISM 310 Genetic Analyser and analysed by Gene Scan and Genotyper (PE–Applied Biosystems). In addition to single primer reactions, multi primer reactions based on Multi-Color fluorescent system were also tested. Primer combinations are listed in Table 2. AFLP electrophorograms ranging in size from 50 to 400 pb were analysed by the Genotyper software. Then they were scored manually for the presence (indicated by the value 1) or absence (indicated by the value 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) against 2 (horizontal axis). Both analyses were calculated using the STATISTICA 6 software package (Statsoft).

**Results and discussion**

**AFLP analysis:** Six primer combinations (see Table 2) were tested. Each tested primer combination showed polymorphism. Combination M-CAA + E-ACT was the most polymorphic (denoted by an arrow in Table 2). Detecting 23 polymorphic products, this should distinguish all analysed cultivars (Figure 1).

**Genetic diversity:** Genetic diversity was estimated using cluster analysis (UPGMA method). UPGMA similarity matrices were calculated from the total number of polymorphic bands (AFLP 43, see Figure 2a). The AFLP method distinguished all individual DH lines originated from 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,

### Table 1: List of oilseed rape cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Parents</th>
<th>Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solida Rod</td>
<td>1129/75 x Rod 3981 x BNW-17 NDR x KM2</td>
<td>Czech Republic</td>
</tr>
<tr>
<td>Arabella</td>
<td>Lines 142/79 x A3/82</td>
<td>Germany</td>
</tr>
<tr>
<td>Sonata</td>
<td>(Bronowski x Zero) x K2040</td>
<td>Czech Republic</td>
</tr>
<tr>
<td>Falcon</td>
<td>Ledos x (Rapol x Hector) x Jet Neuf</td>
<td>Germany</td>
</tr>
<tr>
<td>Lirajet</td>
<td>Lindora x Jet Neuf</td>
<td>Germany</td>
</tr>
<tr>
<td>Five DH lines from cv. Slapska Stela</td>
<td>KM x Jet Neuf</td>
<td>Czech Republic</td>
</tr>
</tbody>
</table>

### Table 2: Number of polymorphic fragment obtained six AFLP primer pairs

<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 EcoRI-AAG</td>
<td>55</td>
<td>3</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>MseI-CAA EcoRI-ACC</td>
<td>30</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>MseI-CAA EcoRI-ACT</td>
<td>90</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>Multiple primer</td>
<td>MseI-CCC EcoRI-AAC</td>
<td>25</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>– EcoRI-AGT</td>
<td>30</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>– EcoRI-AAG</td>
<td>45</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Total – 275</td>
<td>Total – 43</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

because these lines were obtained using microsporogenesis. Three cultivars (‘Falcon’, ‘Lirajet’ and ‘Slapska Stela’) had ‘Jet Neuf’ as one of their ancestor cultivar. This fact was not manifested, as cultivar ‘Slapska Stela’ alone forms a separated cluster. Also PCO analysis showed two close clusters. More considerable differences were found only in the position of cultivar ‘Arabella’.

Each of the six tested combinations of AFLP primers detected polymorphisms. The best combination M-CAA/E-ACT (26% of polymorphic peaks from a total number of 90 peaks) can distinguish all analysed cultivars and also 4 from 5 DH lines out of cultivar ‘Slapska Stela’. This result showed that AFLPs form an efficient method because of their capacity to reveal many polymorphic bands per assay, although they did not offer the highest level of polymorphism (Powell et al., 1996).

Lombard et al. (2000) found that only two combinations of AFLP primers from a total number of 17 tested ones could distinguish 83 oil seed 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 genotypes tested. Das et al. (1999) evaluated genetic relationship among nine cultivars of Brassica rapa. They reported that the level of polymorphism 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 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 (Garacia et al., 2000). Russell et al. (1997) reported results of 23.2 versus 3.2 for barley and similar results have been observed in other crops, for instance in rice (Fuentes et al., 1999) and apple (Goulao et al., 2001). The number of polymorphic loci per assay is important for cultivar identification. The new registered cultivars need not display polymorphism in analysis with established cultivars and it may then be necessary to test other tens of primers. Because the evaluation of RAPD gels is relatively subjective, for cultivar identification they are only suitable with moderate-to-dark staining intensity with well scored bands.

These requirements influenced the 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 the scoring accuracy and the typing efficiency (Schwarz et al., 2000). On the other hand, this technique requires special equipment and is more expensive when compared with RAPDs and SSRs, although now the automated DNA sequencer is almost the standard equipment of most molecular biology laboratories. RAPD methods are simple, cheap, rapid and they mean no special requirements in terms of equipment, requiring only PCR technology. The sensitivity of the AFLP method was shown by the detection of a high degree of intra-cultivar polymorphism in the cultivar ‘Slapska Stela’ (Czech variety, registered in 1996). These double haploid lines were originated from maintenance breeding lines and thus this material should be genetically very uniform. Although oiseed rape is bred according to the pedigree system, certain levels of variation within oil seed rape cultivars are present (Banga, 1993). Charters et al. (1996) analysed variation in 20 cultivars using 5'- anchored SSRs, and 14 of these 20 cultivars revealed variability, and 3 cultivars were extremely polymorphic. 20 individuals of cultivar Libravo exhibited variability at 10 of the 21
bands scored. Intra-cultivar polymorphism in oil seed rape was reported also in the RAPD analysis done by Mailer et al. (1994). Observed polymorphisms under lines of cultivar ‘Slapska Stela’, however, is still surprising, reasons should perhaps be considered based on the breeding system used for development. But implementation of advanced methods of cultivar identification requires only highly uniform material and consequently an adequate approach to plant breeding. The results presented here show that fluorescence-based AFLP is a very suitable method, for the purposes of fingerprinting of oilseed rape cultivars. But only limited numbers of cultivars and primer combinations were analyzed. It is necessary to analyze larger numbers 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 separation of three primer combinations in one analysis, could detect polymorphism more effectively.

References


Figure 2: Dendrogram (a) and PCO plot (b) from AFLP data.


