IDENTIFICATION OF OILSEED RAPE CULTIVARS USING AFLP MARKERS
IDENTIFIKACE ODRŮD ŘEPKY OLEJNÉ POUŽITÍM AFLP MARKERŮ

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ABSTRAKT

Nový typ molekulárního markeru AFLP, založeného na fluorescenci, byl používán pro svou schopnost identifikovat odrůdy a homogenitu DH linií řepky olejné. Každá ze šesti testovaných AFLP kombinací detekovala polymorfismy, nejlepší kombinace (M-CAA/E-ACT) měla 26% polymorfních píků z celkového počtu 90 a bylo možné rozlišit analyzované odrůdy a 4 z 5 DH linií. Genetická podobnost byla vypočítána použitím klastrové analýzy (metody UPGMA). Ukázalo se, že genetická podobnost mezi DH liniemi je nízká, i když tyto linie byly získány mikrosporogenezí. PCO analýza odrůd ukázala dva uzavřené klastery. Pouze u odrůdy Arabela byla zaznamenána větší odlišnost.

KLÍČOVÁ SLOVA: AFLP, řepka olejná, Brassica napus

ABSTRACT

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

KEY WORDS: AFLP, oilseed rape, Brassica napus

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DETAILED ABSTRACT

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 ([17]). 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 new cultivar for farmers and merchants.

A new type of molecular marker, fluorescence-based AFLP, was evaluated for its ability to identify oilseed rape cultivars and homogeneity in DH lines. Each of the six tested AFLP combinations detected polymorphisms, the best combination (M-CAA/E-ACT) had 26% of polymorphic peaks from a total of 90 peaks and could distinguish analysed cultivars and 4 out of 5 DH lines. The result of AFLP analysis showed that this method is an efficient method because of its capacity to reveal many polymorphic bands per assay, although they did not offer the highest level of polymorphism.

Genetic similarity was calculated using cluster analysis (UPGMA method). We demonstrate that genetic similarity between DH lines is low although these lines were obtained using microsporogenesis. PCO analysis of cultivars showed two close clusters. More differences were found only in the position of cultivar Arabella.

The results presented here show that fluorescence - based AFLP is, for the purposes of fingerprinting of oilseed rape cultivars, a very suitable method. But only limited numbers of cultivars and primer combinations were analysed. It is necessary to analyse 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.

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 ([17]).

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 80's, mainly isoenzymes and storage proteins have been tested as markers for cultivar characterisation of various crops including oil seed rape ([9], [19]). 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 ([2]).

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 ([10], [4], [5]). 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 ([26]). RAPD analysis has been widely used for detection of genetic polymorphisms in *Brassica* species, especially at the beginning of the last decade ([18], [13], [15]). 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 ([16], [22], [24]). 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 ([25]). Recent results of AFLP analysis as a tool for oil seed rape cultivar identification are the most promising compared to other available methods ([17], [3]). This is due to the high multiplex ratio, which is the number of information points analysed per experiment ([20]). 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.

Table 1: List of oilseed rape cultivars

Cultivar	Parents	Country of origin
1 – Solida	Rod 1129/75 x Rod 3981 x BNW-17 NDR x KM2	Czech Republic
2 – Arabella	Lines 142/79 x A3/82	Germany
3 – Sonata	(Bronowski x Zero) x K2040	Czech Republic
4 – Falcon	Ledos x (Rapol x Hector) x Jet Neuf	Germany
5 – Lirajet	Lindora x Jet Neuf	Germany
Five DH lines from cv. Slapska Stela	KM x Jet Neuf	Czech Republic

MATERIAL 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 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).

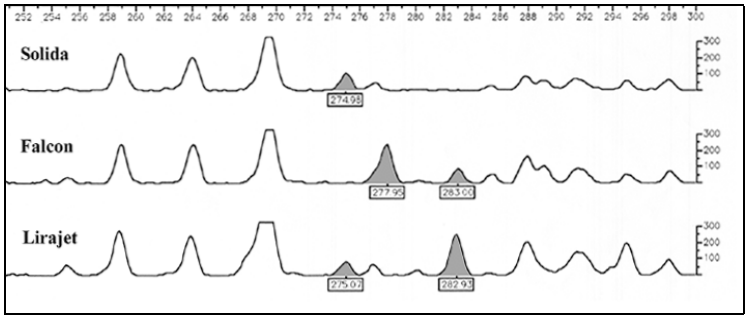
AFLP assays

AFLP profiles ([25]) 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 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 Multi-Color fluorescent system were also tested. Primer combinations are listed in Table 2. AFLP electrophenograms 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).

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

Type of AFLP reaction	Primer combination		No. of scoreable peaks	No. of polymorphic peaks	Percentage of polymorphic peaks
Single primer	MseI-CAA	EcoRI-AAG	55	3	5.5
	MseI-CAA	EcoRI-ACC	30	2	6.7
→	MseI-CAA	EcoRI-ACT	90	23	26
Multiple primer	MseI-CCC	EcoRI-AAC	25	3	12
	-	EcoRI-AGT	30	5	17
	-	EcoRI-AAG	45	7	16
			Total – 275	Total – 43	

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



RESULTS AND DISCUSSION

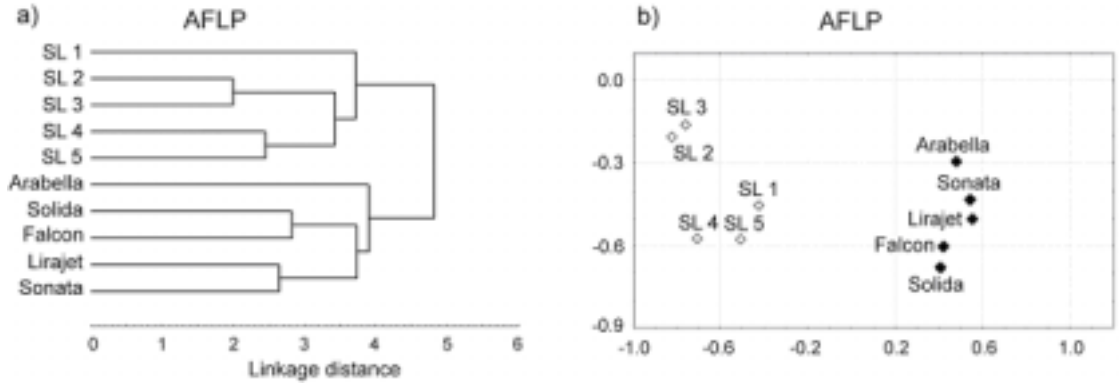
AFLP analysis

Six primer combinations (see Table 2) were tested. Each tested primer combination showed polymorphism. Combination M-CAA + R-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 similarity

Genetic similarity was calculated using cluster analysis (UPGMA method). UPGMA similarity matrixes 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, because these lines were obtained using microsporogenesis. Three cultivars ('Falcon', 'Lirajet' and 'Slapska Stela') had cultivar 'Jet Neuf' as one of their ancestors. 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'.

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



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 ([20]). Lombard et al. ([17]) 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. ([11]) 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. ([3]) evaluated genetic relationship among nine cultivar 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 ([7]). Russell et al. ([21]) reported results of 23.2 versus 3.2 for barley and similar results have been observed in other crops, for instance in rice ([6]) or apple ([8]). 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 ([14]).

Automated fluorescence dye-labelled AFLP techniques offer significant improvements over radioactive labelling methods by increasing the scoring accuracy and the typing efficiency ([23]). 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 oilseed rape is bred according to the pedigree system, certain levels of variation within oil seed rape cultivars is present ([1]). Chartes et al. ([12]) 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. ([18]). 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, for the purposes of fingerprinting of oilseed rape cultivars, a very suitable method. But only limited numbers of cultivars and primer combinations were analysed. It is necessary to analyse 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.

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