Full Paper

Application of the AFLP method to differentiate *Genista tinctoria* microsymbionts

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The high-resolution amplified fragment length polymorphism technique (AFLP), with single *Pst*I restriction endonuclease and two selective primers (*Pst*II-G and *Pst*II-GC), was used for genotyping and study of the genomic relationships between *Genista tinctoria* microsymbionts sampled in England, Poland, and Ukraine. Out of 906 amplification products obtained with both selective primers, 537 markers were polymorphic and could be used to differentiate studied nodule isolates. Cluster analysis, based on AFLP patterns from PCR reaction with *Pst*II-G and *Pst*II-GC primers, separated *Genista tinctoria* rhizobia into three subgroups according to their geographic origin. The results presented in this paper emphasize the role of AFLP analysis in taxonomic and ecological studies of rhizobia.

Key Words—AFLP; *Genista tinctoria*; genomic diversity; rhizobia

Introduction

Rhizobia, capable of living in nitrogen-fixing symbiosis with leguminous plants are phenotypically and genomically very diverse (Małek and Sajnaga, 1999; Young et al., 2001). They have been intensively studied for many years and their classification has changed several times. So far, for nodule bacteria classification and identification such methods as phage typing, intrinsic antibiotic resistance profiling, enzyme activity analysis, plasmid and protein pattern analyses have been commonly used (Małek and Sajnaga, 1999). In recent years, several new techniques have been developed for typing and identification of prokaryotic organisms at the DNA level. These methods differ in their discriminatory power, reproducibility,

easiness, and procedure costs (Caetano-Anolles et al., 1991; Hulton et al., 1991; Stern et al., 1984; Świącicka and de Vos, 2003; Tenover et al., 1995; Williams et al., 1990). One of the newest and most promising techniques is AFLP analysis (Blears et al., 1998; Mueller and Wolfenbarger, 1999; Savelkoul et al., 1999). DNA patterns created by this method are polymorphic due to: (i) mutations at restriction sites that are dispersed over the genome, (ii) mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and (iii) length differences of restriction fragments.

The AFLP method provides a fast approach for measuring the extent of species diversity and is very useful for grouping bacteria according to their relationship (Duim et al., 2004; Gibson et al., 1998; Savelkoul et al., 1999; Terefework et al., 2001; Valsangiacomo et al., 1995). There are four major steps in the AFLP procedure: (i) restriction endonuclease digestion of genomic DNA, (ii) ligation of double stranded oligonucleotide adaptors to each sticky end of the digestion

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fragments, (iii) amplification of adaptor “tagged” restriction fragments using primers complementary to the adaptor and restriction site sequence with additional selective nucleotides at their 3' ends, (iv) analysis of amplified fragments by gel electrophoresis. The sequences of the primers applied for PCR reaction define the number of the restriction fragments that are to be amplified. Primers fully complementary to the adaptors (nonselective primers) amplify indiscriminately all “tagged” fragments, whereas primers with one to three arbitrary nucleotides (selective primers), amplify only part of them.

Since the original publication of the AFLP procedure by Vos et al. in 1995, some modifications of this method, based on one restriction enzyme and a single adaptor, have been described (Suazo and Hall, 1999; Tyrka et al., 2002).

In this study, simplified AFLP analysis with one endonuclease *Pst*I and two selective primers was used for typing of *G. tinctoria* microsymbionts and for assessing their genomic diversity.

Materials and Methods

Bacterial strains. The rhizobial strains used in this paper are listed in Table 1. They were grown and stored on YEM medium (Vincent, 1970).

DNA isolation. For DNA isolation bacteria were grown in 20 ml of liquid YEM medium (Vincent, 1970) for 3–5 days at 28°C. DNA was extracted and purified according to the method of Pitcher et al. (1989). Iso-

lated DNA was dissolved in 50 µl of MilliQ water (Millipore S.A., Molsheim, France) and treated with DNase-free RNase at a final concentration 50 µg ml⁻¹ for 1 h at 37°C. The DNA was then purified of proteins, using the chloroform:2-pentanol mixture (24:1), precipitated with ethanol as in the Pitcher et al. procedure (1989), and dissolved in 100 µl of distilled MilliQ water. The concentration and purity of DNA samples were determined spectrophotometrically by measuring absorbance at 260 and 280 nm.

Restriction-ligation reaction. Two micrograms of genomic DNA was digested with 2.5 U of *Pst*I (Fermentas, Lithuania) in the 1× Buffer O⁺ (provided with the enzyme) at 37°C for 18 h in a final volume of 20 µl. A 16-µl aliquot containing 1.6 µg of digested DNA was used in the ligation reaction containing 10 µM of double stranded *Pst*I adaptor (prepared according to Waugh et al. 1997), 1 U of T4 DNA ligase, and single strength ligation buffer (Fermentas) in a final volume of 20 µl and incubated at 37°C for 4 h. The adaptor “tagged” restriction fragments were then precipitated with 3 vol of mixture containing chilled absolute ethanol and 3 M sodium acetate pH 5.2, at 24:1 ratio, respectively, at -20°C for 2 h. After washing in 70% ethanol, the DNA was dissolved in 50 µl of MilliQ water and used as template for PCR reaction. Oligonucleotides used in the ligation reaction were adaptors *Pst*I_AF, 5'-CTCGTAGACTGCGTACATGCA-3' and *Pst*I_AR, 5'-CATCTGACGCATGT-3' (Proligo, USA). An adaptor was designed in such a way that it contains, besides a sequence complementary to sticky ends of restriction

Table 1. Bacterial strains used in this study.

Strains	Host	Origin	Source
Polish <i>G. tinctoria</i> isolates JAN: 1–16	<i>G. tinctoria</i>	Poland	Kalita and Małek (2004)
Ukrainian <i>G. tinctoria</i> isolates J.UKR: 1.1, 1.2, 2.1, 2.2, 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 7.2, 8.1, 8.2, 9.1, 9.2	<i>G. tinctoria</i>	Ukraine	Kalita and Małek (2004)
English <i>G. tinctoria</i> isolates J.ANG: 91.1, 91.3, 91.6, 92.1, 92.2, 92.3, 92.4, 92.6	<i>G. tinctoria</i>	England	Kalita and Małek (2004); J.P.W. Young
<i>Bradyrhizobium japonicum</i> USDA110	<i>Glycine max</i>	United States	USDA ^a
<i>Bradyrhizobium japonicum</i> USDA6 ^T	<i>Glycine max</i>	United States	USDA
<i>Bradyrhizobium</i> sp. (<i>Lupinus</i>) USDA3045	<i>Lupinus</i> sp.	United States	USDA
<i>Bradyrhizobium liaoningense</i> USDA3622 ^T	<i>Glycine max</i>	United States	USDA
<i>Bradyrhizobium elkanii</i> USDA76 ^T	<i>Glycine max</i>	United States	USDA

^aUSDA, United States Department of Agriculture, Beltsville, MD, USA.

fragments, also an additional base pair in order to eliminate the restriction site after ligation of the adaptor to a restriction fragment (Bleas et al., 1998; Mueller and Wolfenbarger, 1999). This trick allows the performance of a restriction-ligation reaction in the same mixture.

PCR reactions. Nonselective amplification was performed to check the restriction digestion reaction. It was carried out in a 20 µl volume containing: 5 µl of 10× diluted adaptor “tagged” template DNA from the restriction-ligation reaction, 12.5 µl of 2× PCR Ready-Mix (Sigma, USA), 1.5 µl of 1.5 mM MgCl₂, 1.5 µl of 10 µM of forward adaptor *Pst*I_AF sequence as primer, and 4.5 µl of H₂O. Amplification was performed in GeneAmp PCR System 2400, Perkin Elmer under the following program conditions: 2 min 30 s at 94°C initial denaturation, 45 cycles of 45 s at 94°C, 45 s at 54°C, 45 s at 72°C, with final extension 7 min at 72°C. Amplified fragments were separated by electrophoresis in a 1.5% (w/v) agarose gel (ICN, USA) in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and stained with ethidium bromide (0.5 µg ml⁻¹). Selective amplification of restriction fragments was performed in the standard reaction mixture as above, with a minor modification concerning primers. The oligonucleotides used in the PCR reaction were primers *Pst*I-GC, 5'-GACTGCGTACATGCAGGC-3' and *Pst*I-G, 5'-GACTGCGTACATGCAGG-3'. They were synthesized by Oligo, IBB, Poland. The thermal profile in selective amplification was more stringent: initial denaturation for 2 min 30 s at 94°C, 7 cycles with 30 s at 94°C, 30 s at 59°C–53°C (decreasing 1°C per cycle) and 30 s at 72°C followed by 33 cycles of 45 s at 94°C, 45 s at 53°C, 45 s at 72°C, and final extension of 7 min at 72°C. The amplified products were resolved and identified on gel as described above.

Data analysis. The AFLP analysis was performed with the BIO-GENE program version 11.01 (Vilber-Lourmat, France). Pairwise comparison for band-based Dice analysis was done between all strains with the coefficient of Nei and Li (1979). For cluster analysis of AFLP patterns the unweighted-pair-group method (UPGMA) was used.

Results

The AFLP technique was evaluated for its usefulness in the study of genome diversity and in the genotyping of *G. tinctoria* microsymbionts coming

from England, Poland, and Ukraine. Experiments were performed according to the protocol of Tyrka et al. (2002). In the AFLP method the endonuclease used should be adapted to the G+C content and size of the genome to be tested. With regard to a high G+C content in the DNA of nodule bacteria, specially bradyrhizobia (61.8–64.5 mol%, Yao et al., 2002) as well as the large size of their genomes (*B. japonicum* USDA110 genome size was estimated to be 9.1 Mb, Kaneko et al., 2002), *Pst*I endonuclease, recognizing the consensus sequence 5'-CTGGAG-3', was chosen for AFLP typing of *G. tinctoria* microsymbionts.

The efficiency of DNA digestion was checked by nonselective amplification of the restriction fragments. The obtained smears of amplification products proved a good cutting of *G. tinctoria* genome by *Pst*I endonuclease (data not presented). In selective amplification of *Pst*I restriction fragments of *G. tinctoria* microsymbionts and reference bradyrhizobia DNA, two primers were used, i.e. *Pst*I-G and *Pst*I-GC with G and GC as selective bases at their 3' ends, respectively. From the total of 505 amplicons created by *Pst*I-G primer, 300 were polymorphic for studied bacteria and differentiated them (Fig. 1). A more selective primer *Pst*I-GC produced 401 bands of which 237 bands were polymorphic (Fig. 2). The amplification products varied in length from approximately 0.28 kb to 2.15 kb (Figs. 1, 2). Analysis of the DNA gel patterns showed that 5 and 4 DNA bands, from PCR reaction with *Pst*I-G and *Pst*I-GC primers, respectively, were common for nearly all *G. tinctoria* rhizobia independently of their geographic origin (Figs. 1, 2). On the basis of the banding patterns it was possible to discriminate all *G. tinctoria* isolates. Both primers produced unique amplification patterns for each strain (Figs. 1, 2), except J.UKR3.2 and J.UKR4.2 strains, which exhibited identical AFLP profiles with *Pst*I-G primer (Fig. 2).

To compute the rate of genome similarity of *G. tinctoria* rhizobia, a rectangular matrix was used. A high genome heterogeneity, expressed by a similarity coefficient from 0.17 to 1.00, was found among *G. tinctoria* rhizobia. The levels of genome diversity for strains from the individual countries were similar, although Ukrainian isolates were the most homogeneous. Their genome similarity coefficients ranged from 0.17 to 0.97, 0.27 to 0.95, and 0.48 to 1.00, for Polish, English, and Ukrainian isolates, respectively (data not presented).

Genome relationships between *G. tinctoria* nodula-

Fig. 1. Diagram illustrating AFLP patterns obtained with *PstI*-GC primer.

Lanes 1, 48, molecular size standard, 14 fragments (in bp): (from the top) 3,000, 2,000, 1,500, 1,200, 1,031, 900, 800, 700, 600, 500, 400, 300, 200, 100 (Gene Ruler 100 bp DNA Ladder Plus, Fermentas); lanes 2–17, Polish *G. tinctoria* isolates JAN1–JAN16; lanes 18–34, Ukrainian *G. tinctoria* isolates J.UKR1.1, 1.2, 2.1, 2.2, 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 7.2, 8.1, 8.2, 9.1, 9.2, respectively; lanes 35–42, English *G. tinctoria* isolates J.ANG 91.1, 91.3, 91.6, 92.1, 92.2, 92.3, 92.4, 92.6, respectively; lane 43, *B. japonicum* USDA110; lane 44, *B. japonicum* USDA6; lane 45, *B. elkanii* USDA76; lane 46, *Bradyrhizobium* sp. (*Lupinus*) USDA3045; lane 47, *B. liaoningense* USDA3622. Arrows indicate DNA bands common for *G. tinctoria* rhizobia.

Fig. 2. Diagram illustrating AFLP patterns obtained with *PstI*-G primer.

Lanes 1, 48, molecular size standard, 14 fragments (in bp): (from the top) 3,000, 2,000, 1,500, 1,200, 1,031, 900, 800, 700, 600, 500, 400, 300, 200, 100 (Gene Ruler 100 bp DNA Ladder Plus, Fermentas); lanes 2–17, Polish *G. tinctoria* isolates JAN1–JAN16; lanes 18–34, Ukrainian *G. tinctoria* isolates J.UKR1.1, 1.2, 2.1, 2.2, 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 7.2, 8.1, 8.2, 9.1, 9.2, respectively; lanes 35–42, English *G. tinctoria* isolates J.ANG 91.1, 91.3, 91.6, 92.1, 92.2, 92.3, 92.4, 92.6, respectively; lane 43, *B. japonicum* USDA110; lane 44, *B. japonicum* USDA6; lane 45, *B. elkanii* USDA76; lane 46, *Bradyrhizobium* sp. (*Lupinus*) USDA3045; lane 47, *B. liaoningense* USDA3622. Arrows indicate DNA bands common for *G. tinctoria* rhizobia.

Fig. 3. UPGMA dendrogram obtained from AFLP analysis of *G. tinctoria* isolates and reference bradyrhizobia done with *Pst*I-G primer.

The scale at the top of the dendrogram shows the genome similarity rates (%).

tors, including also reference bradyrhizobia, are demonstrated on the dendrograms constructed by UPGMA cluster analysis (Figs. 3, 4). Based on AFLP patterns, obtained by amplification with *Pst*I-G primer, all *G. tinctoria* rhizobia were separated into two main groups. One of them comprised English and Polish isolates, except JAN15, which was included in the second genomic group comprising the Ukrainian strains as well as *Bradyrhizobium* sp. (*Lupinus*) USDA3045, *B. japonicum* USDA110, and *B. japonicum* USDA6. Two remaining reference bradyrhizobia entered the cluster of Polish and English isolates as the most external branch (Fig. 3). *G. tinctoria* microsymbionts were grouped into two main clusters also by the amplification products obtained with *Pst*I-GC primer (Fig. 4). One of them consisted of English (except one strain) and Ukrainian isolates as well as Polish strain JAN16 and *Bradyrhizobium* sp. (*Lupinus*) USDA3045,

whereas another cluster comprised the English strain J.ANG91.6 and Polish rhizobia except the earlier mentioned strain JAN16 and JAN15, which was included in the cluster of reference bradyrhizobia at a similarity coefficient of 17%.

Discussion

Many researchers suggest that host plants and environmental factors are the most important agents that determine the genomic structure of rhizobial populations (Long, 1989; Preston et al., 1998; Wdowiak et al., 2000; Wernegreen and Riley, 1999). The rhizobia described so far are very differentiated, although their heterogeneity is limited by symbiotic interaction with legumes. To survey the biodiversity of *G. tinctoria* microsymbionts we chose the simplified AFLP technique with one restriction enzyme *Pst*I, one double stranded

Fig. 4. UPGMA dendrogram obtained from the AFLP analysis of *G. tinctoria* isolates and reference bradyrhizobia done with *Pst*I-GC primer.

The scale at the top of the dendrogram shows the genome similarity rates (%).

adaptor and two *Pst*I primers with one and two selective nucleotides at their 3' ends. As was expected, the number of polymorphic bands in PCR reactions, with the less selective primer *Pst*I-G was higher than with the more selective *Pst*I-GC, although both primers created a sufficient number of polymorphic markers to distinguish all *G. tinctoria* microsymbionts. The number of markers produced in a single AFLP-PCR reaction can be increased not only by making the primer less selective but also by using the more frequently cutting endonucleases (Bleas et al., 1998). In the standard AFLP technique with two restriction enzymes, the DNA bands had a length from 50 bp to 500 bp (Bleas et al., 1998; Vos et al., 1995). The size of fragments generated in our experiments ranged from 280 bp to 2,150 bp (Figs. 1, 2). The differences in the length of amplicons resulted largely from the facts that: (i) we used one average cutting restriction endonuclease instead of two cutters, recognizing four and six nu-

cleotide sequences and (ii) the separation of amplicons in our experiments was done on agarose in contrast to the standard procedure, where polyacrylamide gel of a higher resolution power was used.

Analysis of the AFLP patterns revealed a considerable genomic diversity of the studied rhizobia. Nearly all *G. tinctoria* isolates yielded unique and complex DNA fingerprints with each primer used (Figs. 1, 2). Noted high genomic diversity of the rhizobial population could be explained by a high frequency of mutations and horizontal gene transfer between bacteria combined with selection through continually changing environmental conditions (Cohan, 2002).

To analyze the reproducibility of the AFLP fingerprints, bacteria were subjected three times to PCR reactions under the same conditions. The DNA were quite consistent, supporting the robustness of AFLP technique (data not presented). AFLP analysis is superior not only in terms of reproducibility and reliability

but also in terms of the number of sequences amplified per reaction. The number of discernible bands per strain in one reaction was between 5 and 15 (Figs. 1, 2). A virtually unlimited number of markers can be generated using various restriction enzymes and different selective nucleotides in the primers.

All *G. tinctoria* microsymbionts studied in this work for genomic diversity were earlier assessed by numerical analysis of the phenotyping properties. By this approach they were placed in the bradyrhizobium cluster and separated into three groups according to their geographic origin (Kalita and Małek, 2004). The dendrograms constructed by cluster analysis of AFLP data (Figs. 3, 4) corresponded to those generated by phenotyping. On the basis of the DNA banding patterns, *G. tinctoria* rhizobia were also allocated into three well-defined subgroups. Each subcluster comprised strains from the particular country (with only some exceptions) as in phenogrouping (Kalita and Małek, 2004), clearly indicating a high potential of AFLP analysis for taxonomic identification and classification of *G. tinctoria* nodulators. The AFLP technique applied by us was used successfully earlier to study the diversity of bradyrhizobia specific for *Faidherbia albida* and *Aeschynomene* species (Willems et al., 2000) as well as *Rhizobium galegae* strains (Terefework et al., 2001). It has also been broadly applied in taxonomy, diagnostics and epidemiology (Savelkoul et al., 1999). One of the first bacterial genera studied by AFLP analysis was *Aeromonas* (Esteve, 1997). The results concerning *Aeromonas* sp. showed a good agreement in strain grouping by AFLP banding patterns and DNA hybridization which is now considered the "gold standard" in determination of bacterial species. AFLP fingerprinting played a big role in analysis of the clonal expansion and persistence of pathogenic bacteria such as *Acinetobacter baumannii*, *Staphylococcus epidermidis*, *Legionella pneumophila*, *Campylobacter* sp., *Helicobacter pylori* and *Streptococcus pyogenes* in the hospital environment (Gibson et al., 1998; Picardeau et al., 1997; Valsangiacomo et al., 1995; Wassenaar and Newell, 2000). The superior discriminative power and great reproducibility of AFLP profiles (analyzed bionumerically), might be utilized to generate a new specific genomotype database accessible by Internet for the whole scientific community.

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