Sequence homology of polymorphic AFLP markers in garlic (*Allium sativum* L.)

Meryem Ipek, Ahmet Ipek, and Philipp W. Simon

Abstract: Linkage mapping and genetic diversity studies with DNA markers in plant species assume that comigrating bands are identical, or at least that they have homologous sequences. To test this assumption in a plant with a large genome, sequence identities of 7 polymorphic amplified fragment length polymorphism (AFLP) markers of garlic, previously used to estimate similarity in genetic diversity studies, were characterized. Among 37 diverse garlic clones, 87 bands from these 7 polymorphisms were excised, amplicons were cloned, and 2 to 6 colonies were sequenced from each band, to yield a total of 191 DNA amplicons. Of these 87 bands, 83 bands (95.4%) contained AFLP amplicons that were identical or highly homologous to the typical marker of that band; only 4 bands contained amplicons with little homology to the same-sized amplicons of other garlic clones. Of these 83 bands, 64 (73.6%) contained only highly homologous amplicons (>90% sequence identity), whereas 19 (21.8%) contained both homologous and nonhomologous amplicons, with sequence identities less than 60%. Of the 37 nonhomologous amplicons identified, 25 (67.5%) differed in length from other amplicons in the band. Sequence conservation of AFLP amplicons followed patterns similar to phylogenetic relationships among garlic clones, making them useful for developing simple PCR-based markers in genetic mapping and diversity assessment.

Key words: AFLP bands, large genome size, garlic, sequence homology assessment.

Résumé : Les études de cartographie ou d’analyse de diversité génétique à l’aide de marqueurs moléculaires chez les plantes prennent pour acquis que des bandes migrant de façon identique sont identiques ou au moins homologues. Afin de vérifier cette hypothèse chez une plante à grand génome, l’ail, l’identité des séquences de sept marqueurs AFLP employés précédemment dans des études visant à estimer la similarité a été examinée. Chez 37 clones d’ail différents, 87 amplicons issus des sept polymorphismes ont été excisés, clonés et entre deux et six colonies ont été séquencées pour chaque amplicon pour un total de 191 séquences. Des 87 amplicons, 83 (95.4 %) correspondaient à des séquences identiques ou très homologues au marqueur typique de cet amplicon, tandis que pour quatre amplicons des séquences sans grande homologie ont été trouvées. Des 83 amplicons, 64 (73.6 %) comprenaient uniquement des séquences très homologues (>90 % d’identité), tandis que pour 19 amplicons (21.8 %), tant des séquences homologues que non-homologues (affichant des identités <60 %) ont été observées. Des 37 séquences non-homologues, 25 (67,5 %) étaient de taille différente des autres amplicons présents dans une bande. La conservation de séquence au sein d’amplicons AFLP était conforme aux relations phylogénétiques entre les clones d’ail ce qui les rend utiles pour le développement de marqueurs PCR en vue de travaux de cartographie ou d’analyse de la diversité.

Mots clés : bandes AFLP, génome de grande taille, ail, évaluation de l’identité des séquences.

[Intaduit par la Rédaction]

Introduction

DNA molecular markers, such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR), have been extensively used in a wide range of applications, including genetic diversity assessment, genetic mapping, plant breeding, and cultivar fingerprinting. The choice of molecular marker for comparative biological and genetic studies depends on their abundance, speed and ease of development, robustness, and cost (Karp et al. 1996).

AFLP is a PCR-based DNA fingerprinting technique (Vos et al. 1995) that requires no prior sequence information (Tohme et al. 1996). Data generation is fairly rapid, and cost per polymorphic AFLP marker is comparable to that of...
Table 1. Garlic clones in which 7 amplified fragment length polymorphism (AFLP) markers were sequence-characterized.

<table>
<thead>
<tr>
<th>Clones</th>
<th>ACGACAT304 (304 bp)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ACGACAT97 (97 bp)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ACGACAT169 (169 bp)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ACGACAT70 (70 bp)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ACGGCTC460 (460 bp)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ACGGCTC103 (103 bp)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ACGGCTC132 (132 bp)&lt;sup&gt;b&lt;/sup&gt;</th>
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| DDRGRU2      | I                                | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds                             | h                              | ss                             | ds 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RAPD markers, because of its high multiplex ratio in a single reaction. Consequently, AFLP has been widely used to evaluate diversity and genetic linkage in many plant species (Garcia-Mas et al. 2000; Sharma et al. 1996; Spooner et al. 1996; Tohme et al. 1996; Zhu et al. 1998), including garlic (Ipek et al. 2003; Lampasona et al. 2003; Volk et al. 2004; Ipek et al. 2005).

AFLP DNA fragments can be amplified during the PCR reaction only if the nucleotides flanking the restriction sites match the nucleotides of the selective amplification primers. This technique can detect polymorphisms due to differences in the restriction sites, small insertions or deletions between restriction sites, and noncomplementation between selective nucleotides in the primer sequences that flank the restriction sites (Folkertsma et al. 1996). Because stringent reaction conditions are used for primer annealing during the selective amplification of the DNA fragments, AFLPs are considered to be reliable, robust, and reproducible markers (Vos et al. 1995; Huys et al. 1996).

It has been assumed that comigrating AFLP and RAPD amplified DNA fragments (amplicons) are homologous. Based on this assumption, these comigrating amplicons are scored as 1 allele. However, this assumption has not been tested in many studies, and it has been demonstrated, on an agarose gel, that comigrating RAPD markers might not share the same sequences (Rieseberg 1996; Adams and Rieseberg 1998). Furthermore, Santos and Simon (2002) analyzed the homology between AFLP markers developed in 2 different F2 mapping populations and found that, although the majority of AFLP amplicons that shared the same position on a PAGE gel had more than 91% sequence identity, some had a sequence identity as low as 24%, indicating that nonhomologous amplicons can share the same position on a PAGE gel. In another study, comigrating AFLP amplicons in Echinacea were found to share a sequence identity of only 23.6% to 45% within a species (Mechanda et al. 2004).

In our previous study (Ipek et al. 2003), we analyzed the genetic diversity of garlic, using AFLPs, and compared the results with those obtained with RAPDs and isozymes. In the current study, we tested the assumption that AFLP amplicons sharing the same position on a polyacrylamide gel also share sequence identity in the very large garlic genome. To do this, we cloned and sequenced 7 polymorphic AFLP markers from the 37 diverse garlic clones used in our previous study, and evaluated their sequence homology.

### Materials and methods

#### Plant material and AFLP procedure

Thirty-seven garlic clones, representing the breadth of diversity for *Allium sativum* L., were grown, DNA was extracted, and AFLP was carried out, in accordance with the methods described in Ipek et al. (2003) (Table 1). Most are cultivated clones, but U079 and U094 are wild clones from Central Asia. AFLP gels were transferred onto 3 mm Whatman filter paper, dried with a vacuum at 80 °C for 60 min, and exposed for 2 d on Biomax MR film (Kodak, Rochester, N.Y.). Polymorphic AFLP amplicons were excised from the 3 mm Whatman paper with the polyacrylamide gel, using a scalpel (Fig. 1). Seven polymorphic AFLP markers, ranging from 70 to 460 bp (4 from primers EcoRI + AC/GA/MseI +
CAT and 3 from EcoR I + ACGG/Mse I + CTC), were selected for sequence characterization (Table 2). These bands were chosen because they represented a range of size and primer combinations, and they collectively were present across the entire range of AFLP groups identified in Ipek et al. (2003).

Cloning and sequencing of AFLP amplicons

Excised AFLP amplicons were transferred into 1.5 mL microcentrifuge tubes, soaked into 200 μL TE buffer (10 mmol Tris/L and 1 mmol EDTA/L (pH 8.0)) overnight at 4 °C, and crushed. After centrifugation in a microcentrifuge, at maximum speed for 5 min, the liquid phase was transferred to new tubes, and AFLP amplicons were reamplified, using the same selective amplification primers. Each 25 μL PCR reaction contained 3 μL of eluted AFLP amplicon, 0.75 units of Taq polymerase (Promega, Madison, Wis.), with supplied MgCl2 reaction buffer (at 1.0 mM concentration), 0.8 μmol/L of each primer, and dNTPs at 200 μmol/L. The reactions were heated to 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 56 °C for 1 min, and 68 °C for 2 min. They were then held at 4 °C. The products of PCR were separated by subjecting them to electrophoresis through 1.5% (m/v) agarose gels in 1× TAE buffer (40 mmol Tris-acetate/L and 1 mmol EDTA/L). Amplicons were purified from agarose gels, using the QIAquick gel extraction kit (QIAGEN, Valencia, Calif.), and cloned into the pGEM-T vector system (Promega); vectors in the ligation mix were transformed into JM109 competent cells (Promega), in accordance with the manufacturer’s protocols.

Two to 4 bacterial colonies, carrying inserts from each reamplified AFLP band, were grown for plasmid extraction. Plasmids were extracted from 5 mL of overnight-grown bacterial cultures, using the Wizard Plus Miniprep DNA Purification system (Promega), in accordance with the method described by the supplier, and 3 μL of plasmid solution was used for sequencing. Taq DNA polymerase cycle-sequencing reactions were performed, in accordance with the conditions recommended by Applied Biosystems (Foster City, Calif.), with fluorescent dye terminator, but half-volume reactions were used. DNA was sequenced with a PE-Biosystem 377 XL automated DNA sequencing instrument at the Biotechnology Center at the University of Wisconsin—Madison. Sequences of selected AFLP amplicons that shared the same position on the polyacrylamide gel were aligned, and identities among the sequences were calculated, using BioEdit sequence alignment editor (Hall 1999), and displayed, using GENEDOC (Nicholas and Nicholas 1997). If the sequences for consensus AFLP marker were not recovered in these colonies, 2 more bacterial colonies were picked and inserts were sequenced. Each amplicon sequence was compared with those in the GenBank databases, using both BLASTN and BLASTX programs at the National Center for Biotechnology Information.

Results

From 7 polymorphic AFLP markers, a total of 87 bands were excised from 37 garlic clones (Tables 1 and 2; Fig. 1), and amplicons in these bands were reamplified (Fig. 2). A total of 191 amplicons from these 87 bands were sequence-characterized, to yield 124 independent sequences (Table 1). Many of the amplicons cloned from a band had sequences identical to other amplicons in that band; only 1 of them is presented in our results. Overall, 67 of the 191 amplicons sequenced were identical to others in a band. All sequences contained the expected nucleotide sequences of AFLP selective amplification primer sequences, including selective bases, indicating no mismatch occurrence of selective bases during the AFLP amplification of amplicons. No significant matches were found between sequences of AFLP amplicons and those in the GenBank database. We did not analyze any monomorphic AFLP markers from the polyacrylamide gel because it is not common to use them as informative markers in comparative biological studies. Among the 87 bands, 64 (73.6%) contained only AFLP amplicons that had highly homologous sequences relative to their respective...
AFLP markers (>90% sequence identity), 19 bands (21.8%) contained both homologous and nonhomologous amplicons, and 4 bands (4.6%) had only amplicons with little sequence homology (<60% sequence identity) to the consensus sequence of their respective AFLP markers (Table 1). Of the 124 independent amplicons from the 87 bands, 37 (29.8%) had nonhomologous sequences, with sequence identities <60% (Table 2). Although the sizes of 12 nonhomologous AFLP amplicons (9.7%) were the same as their respective AFLP markers, the remaining 25 (20.2%) varied, by up to 8 bp, and the sizes of 13 (10.5%) of these differed from those of their respective AFLP marker by ±1 bp (Table 2).

Consensus sequences of respective AFLP markers were found in 83 (95.4%) of the 87 bands characterized, but marker ACGGCTC103 in garlic clones DDR7087 and M/PIT and marker ACGGCTC132 in garlic clones U079 and PI383831 had no amplicon with the homologous sequence of their respective AFLP markers, although a band of the same size was observed in the polyacrylamide gel for these clones (Table 1). Therefore, these 4 AFLP bands represented a 4.6% scoring error; they did not have the amplicon with the homologous sequence of their AFLP marker, although they were scored as present.

Although only 1 consensus sequence was observed for AFLP markers ACGGCTC460 (Fig. 3), ACGACAT304, ACGACAT169, ACGGCTC103, and ACGACAT97, 2 different consensus sequences were detected for markers ACGACAT70 and ACGGCTC132 (Figs. 4 and 5). In marker ACGGCTC132, garlic clones in group V of Ipek et al. (2003) had the same homologous sequences, whereas a different homologous sequence occurred in this AFLP marker from the garlic clones grouped in AFLP groups I, IX, and X (Fig. 4). In contrast, garlic clone DDR7099, which clustered in group IX, had both homologous sequences of this AFLP marker. Similarly, there were 2 different homologous nucleotide sequences detected for AFLP marker ACGACAT70, on the basis of the consensus sequences, but their sizes differed from each other by 1 bp (Fig. 5). One group of 71-bp sequences had homology among garlic clones clustered in AFLP group III; the other group of homologous 70-bp sequences was composed of AFLP amplicons from the garlic clones that were clustered in AFLP groups II, V, VI, VII, VIII, and IX of Ipek et al. (2003) (Fig. 5). It is interesting that some garlic clones in groups I and X contained both homologous sequences (Table 1; Fig. 5).

**Discussion**

In genetic diversity or mapping studies, it is assumed that comigrating DNA bands consist of homologous or identical amplicons. Our results suggest that sequence homology of AFLP amplicons among the garlic clones for a given AFLP marker was similar to the phylogenetic relationships among the garlic clones. Although the sequences of DNA amplicons for AFLP markers ACGACAT70 and ACGGCTC132 were homologous among more closely related garlic clones within each AFLP group, the sequences of AFLP amplicons were nonhomologous among the less-related AFLP groups described in Ipek et al. (2003) (Figs. 4 and 5). Mechanda et al. (2004) reported 33.3%–100% sequence identity of an
AFLP marker in 1 variety of *Echinacea*, and only 23.6%–45% identity within a species. The authors also reported sequence identity of an AFLP marker as low as 1.2% within this genus. These results suggest that AFLP markers should be sequence-characterized when phylogenetically distant plant materials are being evaluated.

Our results demonstrated the presence of up to 5 different amplicons in some bands, including, in 19 (21.8%) of 83 bands, some that are nonhomologous. The presence of multiple amplicons with different sequence identity in a band could lead to the misscoring of an AFLP marker. Because of this, scoring based on the presence or absence of a band should be considered phenotypic rather than genetic, as was suggested by Mechanda et al. (2004). The probability of multiple amplicons in a band might be higher in plant species with a large genome, such as the garlic genome (Bennett and Leitch 2004), than in those with smaller genomes. In fact, the incidence of multiple amplicons with different sequence identity in garlic AFLP bands is comparable to that observed in the soybean, which has a genome size about 93% smaller than that of garlic (Meksem et al. 2001). Nevertheless, for phenetic analysis, it has been demonstrated that random error will not change the relative similarities among taxa, but will result in the overestimation of absolute relatedness (Rieseberg 1996; Adams and Rieseberg 1998). We observed that 4 of 87 bands scored (4.6%) lack homologous AFLP sequences, and we surmise that this random error will not change conclusions about relative similarity in most studies.

In mapping studies, distorted segregation ratios, accounting for 15% to 30% of segregating AFLP markers, are often reported. This can be explained in part by the misscoring of segregating AFLP markers in a mapping population in which bands being scored include multiple comigrating amplicons. In fact, in previous research, we found that 15.3% to 24.3% of the segregating AFLP markers scored over 2 garlic populations had disturbed genetic ratios (Ipek et al. 2005). Furthermore, 89% to 98.7% of the disturbed segregations fit a duplicate gene ratio. Consequently, nonhomologous segregating AFLP amplicons of the same size will behave like duplicate markers if they originate from unlinked loci and, in this case, they would be excluded from mapping analysis. Indeed, a relatively high proportion of unlinked AFLP markers has been reported in the *Allium roylei × Allium cepa* interspecific hybrid (27%) (Van Heusden et al. 2000), and in oat (17%) (Groh et al. 2001), olive (7.6%–20.6%) (La Rosa et al. 2003), and garlic (30.5%–41.9%) (Ipek et al. 2005). We found that 21.8% of the bands we analyzed contained both homologous and nonhomologous amplicons. Independent linkage analysis of nonhomologous amplicons will help confirm or refute duplicate genes as an explanation for disturbed segregations in garlic.

When multiple amplicons occur in the same AFLP band, they are not always the same size. The presence of multiple nonhomologous AFLP amplicons in a band of varying sizes has been reported in earlier studies and was also observed in garlic. Meksem et al. (2001) detected AFLP amplicons with size differences ranging from 1 to 2 bp within a band, and the authors indicated that only 1 AFLP amplicon corresponded to the original AFLP marker in their mapping population; Mechanda et al. (2004) reported even larger size differences in polymorphic AFLP makers. The presence of amplicons on a polyacrylamide gel with sizes different from those expected could result from errors during the excision of AFLP amplicons from the gel or from the physical properties of the denaturing polyacrylamide gel that affect the mobility of DNA fragment in the electric field (Rousseau et al. 2000; Brahmasandra et al. 2001). In this study, 13 amplicons (10.5%) had sequences that differed from the consensus AFLP amplicon by ±1 bp, which could be due to excision error (Table 2). We evaluated AFLP markers with sizes ranging from 70 to 460 bp and found 2 different consensus sequences for AFLP marker ACGACAT70 (70 bp); their sizes differ from each other by 1 bp (Fig. 5). Therefore, the need for caution in scoring AFLP markers is reinforced. Comigration of DNA fragments with different base pair lengths might be detected with new band migration techniques, but nonhomologous bands of the same size will remain unresolved without sequence characterization.

Our results demonstrated that some AFLP bands given the same score in a study of garlic diversity analysis contained multiple amplicons that differ in sequence and size. Fig. 2. Picture of reamplified ACGACAT304 and ACGACAT169 amplified fragment length polymorphism (AFLP) markers in selected garlic clones. S.M. indicates DNA molecular-weight markers.
Fig. 3. Alignment of sequences in DNA fragments amplified in AFLP marker ACGGCTC460 for 8 garlic clones. All clones have homologous fragments, and JN/EG also has an additional 458-bp AFLP amplicon with nonhomologous sequence (indicated with *). Black, dark grey, and light grey shading indicates 100%, 80%, and 60% sequence identities, respectively.
Fig. 4. Alignment of the sequences of DNA fragments amplified from AFLP marker ACGGCTC132 for 18 garlic clones. A subgroup of 6 clones (top 6 sequences) share 1 homologous sequence, of 132 bp, with >98% identity. A second subgroup of 10 clones (next 10 sequences) shares another homologous sequence, of 132 bp, with >94% identity within the subgroup. The remaining sequences (bottom 16 sequences) were nonhomologous, except for the first 19 and last 20 bases of the selective amplification primers, and they varied in size from 127 to 140 bp. Note that some garlic clones had multiple amplicons of this fragment size (e.g., DDR6811 and PI493116). Roman numerals in the name of the garlic clones indicate the AFLP groups described in Ipek et al. (2003). Black, dark grey, and light grey shading indicates 100%, 80%, and 60% sequence identities, respectively.
Although some of these multiple amplicons are nonhomologous within a band, we detected the presence of AFLP amplicons with homologous sequences in 83 of 87 (95.4%) bands. However, in 4 of 87 bands (4.6%), amplicons with homologous sequences were not recovered. These 4 bands would be misscored in diversity analysis. Nevertheless, we conclude that, overall, AFLP is a very reliable marker system for genetic diversity assessment, mapping, and selective breeding programs of plant species, even those with large genomes, such as garlic. Scoring error does occur, but at a relatively low rate for evaluation of intraspecific variation. AFLP sequences analyzed in this study are being developed for use as simple PCR-based markers, such as site-specific target sequences and sequence-characterized amplified region markers; together with other simple PCR-based markers, such as microsatellites, these molecular tools will be useful for germplasm diversity analysis and map merging in garlic.

References


