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APPLICABILITY OF AMPLIFIED FRAGMENT LENGTH POLYMORPHISM MARKERS FOR STRAWBERRY VARIETY IDENTIFICATION

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APPLICABILITY OF AMPLIFIED FRAGMENT LENGTH POLYMORPHISM MARKERS FOR STRAWBERRY VARIETY IDENTIFICATION

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Abstract

Amplified fragment length polymorphism (AFLP) markers were evaluated for their applicability to the strawberry variety identification. Using 8 primer combinations, 150 repeatable AFLP markers were generated in ten strawberry varieties. The number of polymorphic fragments per fingerprint (primer combination) ranged from 13 to 25, with an average of 18. Cluster analysis clearly separated the varieties according to different genetic pools and their geographic origins. Genetic relationships of varieties, estimated by the AFLP markers generated with three primer combinations (50 polymorphic bands) were similar to those estimated with 150 markers (eight primer combinations), indicating the high discriminant power of these markers. The different steps of the AFLP procedure (restriction of DNA, ligation of adapters, pre-amplification and selective amplification) were tested for their reproducibility. The results showed a high stability of the observed patterns as long as the restriction of DNA could be controlled. These results are discussed in terms of the choice of an appropriate protocol for variety identity evaluation.

Introduction

Commercial strawberry plants are generated by vegetative propagation. The invasive nature of stolons can lead to cultivar mixing when different cultivars are in close proximity. A rapid and precise cultivar identification method becomes necessary in the event of these circumstances. Unambiguous identification of varieties is also necessary in order to protect breeders' rights. Identification based only on the observation of morphological characteristics is time consuming because most morphological characteristics used for distinction testing are found in the flower and fruit.

The use of isozymes for cultivar identification was investigated in strawberry (Nehra *et al.*, 1991). This method showed only a limited degree of polymorphism and is very sensitive to developmental variation.

Many new possibilities for the identification of cultivars have been opened up with the development of DNA markers. Randomly amplified polymorphic DNA (RAPD) analysis was carried out on strawberry cultivars of various origins in order to differentiate these varieties at the molecular level (Graham *et al.*, 1996; Landry *et al.*, 1997). However this technique has the major disadvantage that it's very sensitive to experimental conditions (MacPherson *et al.*, 1993; Schierwater and Ender, 1993; Skroch and Niehuis, 1995; Chen *et al.*, 1997), which limits its application.

The stability and reproducibility of fingerprinting profiles is very important when the objective reached is to use them as a tool for plant variety identification.

Recently a new PCR-based method called AFLP has been developed (Vos *et al.*, 1995). This DNA fingerprinting technique has already been used with success for studies of genotyping, genome mapping and phylogenetics in other species such as soybean (Maughan *et al.*, 1996),

lettuce (Hill *et al.*, 1996), sunflower (Hongtrakul *et al.*, 1997), tea (Paul *et al.*, 1997), hop (Hartl and Seefelder, 1998) and rice (Zhu *et al.*, 1998).

The objective of the study presented here was to evaluate the applicability of AFLP markers in strawberry variety identification. To achieve this, we have determined the usefulness of this technique to reveal genetic polymorphism on many strawberry varieties which represent different genetic pools and we have examined in detail the reproducibility of generated fingerprints.

Materials and methods

Plant material and DNA extraction

A total of ten strawberry varieties (*Fragaria ananassa* L., cv. Elsanta, Mara des Bois, Cigaline, Garriguette, Miranda, Cigoulette, Pajaro, Dorit, Chandler and Gento) which represent different geographical origins and a wide range of genetic variation in strawberry were used in this research.
Total DNA from leaf tissue was extracted using three different methods: according to Cheung *et al.*, (1993) by the CTAB method (Rogers and Bendich, 1988) and using a commercial kit from QIAGEN (Dneasy Plant Mini Kit). DNA concentration was estimated in comparison with known concentrations of lambda DNA in a 1% agarose gel.

AFLP protocol and gel electrophoresis

AFLP analysis was performed according to the procedure of Vos *et al.* (1995) and following the protocol described by Life Technologies (AFLP™ Analysis System I), with some minor modifications. Genomic DNA (250 ng) was digested by EcorI And MseI enzymes for 3 h instead of 2 h. Pre-amplified templates were diluted 30-fold with distilled water instead of being diluted 50-fold with TE buffer. All the primers used contained tree selective bases at the 3' end. The EcorI primers were end-labelled with [δ–^{33}P] ATP using T4 polynucleotide kinase. Selective amplification was performed with 0.75 U of Taq DNA polymerase by sample instead of 0.1 U.
The PCR products were mixed with an equal volume of loading buffer (98% formamide, 10mM NaOH, 12.5% saccharose, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated for 4 min at 93°C. Four microliters of each sample were loaded into pre-warmed 5% polyacylamide gel with 8M urea and 0.5X TBE running (50 mM Tris, 50mM boric acid, 1mM EDTA, pH 8.0). Gels were run a constant wattage (50 W) for 2 h, dried at 80°C for 30 min and exposed to X-ray film for about 4-7 days.

by scoring autoradiographs visually for the presence/absence
biguous bands were scored. If there was any doubt about the
he ten varieties, the band was not considered.

The genetic similarity between varieties was estimated using Jaccard's coefficient of similarity. Cluster analysis were performed using the unweighted pair-group method with an arithmetical average (UPGMA) algorithm.

Reproducibility analysis

The reproducibility of each AFLP procedure step (restriction of the DNA, ligation of adapters, pre-amplification and selective amplification) was examined by comparing in three different strawberry varieties, the fingerprint obtained with a same DNA preparation but with 3 digestions, 2 ligations, 2 pre-amplifications and 2 selective amplifications independents. A total of 24 profiles were compared by variety for each primer combination (3x2x2x2=24). Tree different primer combinations were tested. This study was repeated two times.

Results

Identification of DNA polymorphism in strawberry cultivars

64 primer combinations were screened in the ten strawberry varieties, in order to choose those giving readable electrophoretique profiles and a high number of markers.
The eight best primer combinations were chosen to analyze the stability of bands and to measure the relationships among the varieties. The repeatability of each AFLP marker was assessed by comparing, for each variety, three new profiles obtained with different DNA extraction. Only the bands present in the three profiles were retained.
These eight AFLP primer combinations produced 150 repeatable AFLP markers. Each primer combination produced between 70 to 100 fragments with sizes ranging from 30 to 700 bp. The number of polymorphic fragments per fingerprint (primer combination) ranged from 13 to 25, with an average of 18.
Genetic similarities between lines ranged from 0.4 to 0.75. Cluster analysis separated varieties into two major groups which correspond to different genetic pools (American and French). This analysis permitted the distinction of two groups of closely related varieties: the varieties Cigaline and Garriguette on one hand, and the varieties Pajaro and Cigoulette on the other hand. These results are consistent with the genealogy based data. A dendogram constructed using only the AFLP markers generated with tree primer combination (50 polymorph bands) gave similar results.

Reproducibility of AFLP technique

For the three varieties analyzed, all the polymorph bands selected during the preliminary study of repeatability were reproducible on all the profiles.
However the 24 samples tested of each variety are not identical for all the bands constituting the profile. In effect, we have observed that, in addition to some minor bands of weak intensity, the profiles differ also for some intense bands of weak molecular weight (lower thar 100 pb). Our analysis shows that these differences are related to the DNA restriction.

Discussion

In order to apply fingerprinting techniques for cultivar identification it's important to known the levels of polymorphism revealed. Our results show that AFLP methodology permits the revealation of a relatively high polymorphism in Strawberry varieties. Indeed, the average number of AFLP markers generated by primer combination is 18, which corresponds to an efficiency 3 times higher compared to RAPD methodology (Graham *et al.,* 1996). These results confirm the high discriminant power of the AFLP technique reported in other plant species (Hartl and Seefelder, 1998; Hill *et al.,* 1996; Hongtrakul *et al.,* 1997; Maughan *et al.,* 1996, Paul *et al.,* 1997; Zhu *et al.,* 1998).

A prerequisite necessary for the use of a DNA profiling technique for cultivar identification is that the markers generated are reproducible. Our results shown that AFLP markers are highly reproducible as long as some precautions are taken. One should avoid selecting weak bands which are not always reproducible and not retain as markers the bands whose stability has not already been confirmed by repeating all the procedure (from the DNA restriction to the amplification). Moreover we have ascertained that the reproducibility of profiles is tightly linked to the DNA quality. In effect we have noted that DNA restriction, which depends on DNA quality, is the more sensitive step of the AFLP procedure. Strawberry is a specie with a lot of polysaccharides and polyphenols in the leaves. Three different methods of DNA extraction have been tested and compared. The CTAB's (Rogers et Bendich, 1988) and Cheung's *et al.* (1993) methods didn't give satisfactory results. The DNA obtained by the purification method on silica resin (KIT QIAGEN) is in general of good quality. However erroneous profiles (non-correspondence between the three repetitions) have been observed for several varieties. This shows that in some instances, problems linked to the extract quality remains. In the perspective of the using the AFLP tool for plant identity control, it's necessary that a protocol, aiming to avoid any error source, is drawed up. For the Strawberry, it's therefore necessary that some restrictions (and so different amplifications) are performed for each sample in order to identify erroneous profiles linked to incomplete restrictions.

Compared with the only other multilocus fingerprinting systems used in strawberry (RAPD methodology; Graham *et al.,* 1996), the AFLP technique shows many advantages. It permits the generation of a higher number of markers per primer combination used and is more reproducible. Our results show that AFLP technique can be a very useful tool for the identification of strawberry varieties. In effect, with a relatively small number of primer pairs, it should be possible to assess with sufficient precision and reasonable cost the cultivar identification.

In the future, we are going to analyze all varieties registered or protected in France with AFLP markers in order to set out a type profile for each variety. This work will permit the standardization of the tool for an scale of the different marker number between varieties in relationship with the genealogy, the belonging to different genetic pools and the phenotyping difference. Which will permit in this way the estimation and the definition of the fluctuation allowed during the variety controls.

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