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Application of fluorescence-based semi-automated AFLP analysis in barley and wheat

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Abstract Genetic mapping and the selection of closely linked molecular markers for important agronomic traits require efficient, large-scale genotyping methods. A semi-automated multifluorophore technique was applied for genotyping AFLP marker loci in barley and wheat. In comparison to conventional ^{33}P-based AFLP analysis the technique showed a higher resolution of amplicons, thus increasing the number of distinguishable fragments. Automated sizing of the same fragment in different lanes or different gels showed high conformity, allowing subsequent unambigous allele-typing. Simultaneous electrophoresis of different AFLP samples in one lane (multimixing), as well as simultaneous amplification of AFLP fragments with different primer combinations in one reaction (multiplexing), displayed consistent results with respect to fragment number, polymorphic peaks and correct size-calling. The accuracy of semi-automated codominant analysis for hemizygous AFLP markers in an F_2 population was too low, proposing the use of dominant allele-typing defaults. Nevertheless, the efficiency of genetic mapping, especially of complex plant genomes, will be accelerated by combining the presented genotyping procedures.

Key words Automated genotyping · Fluorescence-based DNA analysis · AFLP · Barley · Wheat

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Introduction

The application of PCR-based marker techniques like SSRs (simple sequence repeats, Weber and May 1989), RAPDs (random amplified polymorphic DNAs, Williams et al. 1990) and AFLPs (amplified fragment length Polymorphisms, Vos et al. 1995) provides a powerful tool for commercial breeding applications, e.g. marker-assisted backcrossing and gene-pyramiding. The recently developed AFLP fingerprint technique is based on the digestion of genomic DNA with at least one restriction enzyme, the ligation of appropriate adaptors to the revealed fragments, and amplification with adaptor-homologous primers. The selectivity originates from additional nucleotides at the 3' primer termini, generating only a specific subpopulation of fragments. Therefore, the AFLP technique exhibits several advantages for the analysis of complex genomes including: (1) the generation of a large number of anonymous, randomly distributed molecular markers in a single reaction, (2) a high level of polymorphism, and (3) a high reproducibility and reliability due to stringent PCR conditions. In plants, the AFLP technique was recently used to construct high-resolution maps (Becker et al. 1995; Van Eck et al. 1995; Schondelmaier et al. 1996; Keim et al. 1997), as well as to find closely linked genetic markers for a specific phenotype (Meksem et al. 1995; Thomas et al. 1995; Cnops et al. 1996; Büschges et al. 1997; Schwarz et al. 1999).

Conventional visualisation by autoradiography or silver-staining shows several limitations. Throughput is low, because only one sample per gel lane can be analysed. Also accurate allele-typing is often not feasible due to poor the resolution of large fragments and/or migration-variability from lane to lane, as well as from one run to another. Moreover, optical scoring of segregation data and manual input into a table sheet compatible for linkage-analysis programs is time-consuming and represent an additional source of mistakes. To overcome such restrictions fluorescence-based, semi-automated methods were recently developed for fragment analysis. The availability of fluorescence dyes with distinguishable

wavelength emissions allows one to electrophorese different samples simultaneously in a single lane. Co-electrophoresis of an internal size standard in every lane, labelled with another fluorescent dye, provides a precise size-calling to correct band shifting within and between gel runs. A four-colour fluorescence-based technique was first used for the genotyping of SSR loci by Ziegle et al. (1992). In comparison to conventional radiolabelling techniques, it exhibited a higher efficiency and at least the same accuracy for allele-sizing of SSRs (Schwengel et al. 1994). Two investigations have employed this technique for mapping microsatellite markers in humans (Reed et al. 1994; Pritchard et al. 1995). An acceleration of genotyping was also demonstrated for SSRs in *Brassica* ssp. by combining several fluorescently labelled primers in a single PCR reaction (Mitchell et al. 1997).

In the present study a procedure for the genotyping of AFLP loci is described and its resolution and accuracy compared with a conventional [γ-^{33}P]ATP radiolabelling technique. The reliability of semi-automated genotyping of an AFLP locus was confirmed by comparison to known allele configurations from closely bordered RFLP markers. Its application for large-scale mapping in barley and wheat is discussed.

Materials and methods

Plant material and DNA extraction

DNA from four barley genotypes, 'P01', 'P10' (Kølster et al. 1986), 'Golf' and 'Alexis', and two wheat cultivars, 'Chinese Spring' and 'Chiyacao' (Huang et al. 1997), was used for AFLP analysis. Segregation analysis was performed with an F_2 population derived from a cross between 'P01 × P10'. Genomic DNA was isolated from young leaf material according to the CTAB method of Saghai-Maroof et al. (1984).

Multifluorophore AFLP analysis

AFLP analysis was carried out as described in the AFLP plant mapping kit from PE/Applied Biosystems. A 0.5 µg quantity of genomic DNA was digested with 1 U of *Mse*I and 5 U of *Eco*RI, and simultaneously 5 pmol of *Eco*RI adaptors and 50 pmol of *Mse*I adaptors were ligated with 1 U of T4 DNA ligase (all enzymes New England Biolabs, Beverly, Mass., USA) in a buffer containing 10 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, 50 mM $NaCl_2$ and 50 ng/µl of bovine serum albumin in a total volume of 11 µl for 2 h at 37°C. The restriction-ligation samples were diluted with 189 µl of H_2O to give an appropriate concentration for subsequent PCR reactions. To enrich the fragments of interest, pre-selective amplification of target sequences was performed with *Eco*RI and *Mse*I adaptor-homologous primers each possessing one additional nucleotide at the 3' primer end. PCR reactions were set up with 4 µl of diluted restriction-ligation DNA, 0.125 µM of *Eco*RI+1 primer, 0.125 µM of *Mse*I+1 primer, 0.4 U of *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany), 0.2 mM of each dNTP (Amersham-Pharmacia Biotech, Uppsala, Sweden) and 1 × Qiagen PCR buffer in a total volume of 20 µl. For amplification the following cycle profile was used: 20 cycles of 1-s denaturation at 94°C, 30-s annealing at 60°C and 2-min extension at 72°C. To verify successful amplification, 10 µl of the PCR reaction were separated on a 1.5% agarose gel: a smear of amplified target fragments was visible in a range from 100–1500 bp. The remaining 10 µl were diluted 20-fold by adding 190 µl of H_2O and stored at 4°C.

Selective amplification was achieved with *Eco*RI and *Mse*I primers having three additional nucleotides. For multifluorophore fragment analysis, *Eco*RI primers were either labeled with 5-carboxy-fluorescein (5-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-carboxy-fluorescein (JOE), or N,N,N',N'-tetramethyl-6-carboxyrhodamin (TAMRA), while the *Mse*I primer was unlabelled. PCR reactions were carried out using 3 µl of diluted pre-amplified DNA, 0.05 µM of *Eco*RI+3 primer, 0.25 µM of *Mse*I+3 primer, 0.4 U of *Taq* DNA polymerase, 0.2 mM of each dNTP and 1 × Qiagen PCR buffer in a 20-µl volume. High selectivity was obtained with following cycle profile: one cycle of 30 s at 94°C, 30 s at 65°C, 2 min at 72°C, followed by eight cycles of a 1.0°C decreasing annealing temperature per cycle and finally 23 cycles of 1 s at 94°C, 30 s at 56°C, 2 min at 72°C. All PCRs were performed on a Perkin Elmer 9600 thermocycler (PE/Applied Biosystems, Foster City, Calif., USA). For multifluorophore analysis 0.4 µl of 5–FAM-labelled PCR products, 0.5 µl of JOE-labelled PCR products and 0.7 µl of TAMRA-labelled PCR products were pooled. The combined sample was mixed with 0.2 µl of a 6-carboxy-X-rhodamin (ROX)-labelled internal length standard GeneScan-500 ROX (PE/Applied Biosystems) and 0.8 µl of formamide dye (98% formamide, 0.005% dextran blue), denatured for 3 min at 90°C and quickly chilled on ice. Multiplex PCR was conducted as described above except for the concentration of primers; 0.05 µM of each of the three selective *Eco*RI primers labeled with 5-FAM, JOE and TAMRA, respectively, and 0.5 µM of one unlabeled *Mse*I primer were used. Then 1.5 µl of the PCR products, internal length standard and loading dye were combined as described for multimixing.

Electrophoresis of 36 samples was carried out using 5% denaturing polyacrylamide gels (Long Ranger™, FMC Bioproducts, Rockland, Me., USA) in 1 × TBE electrophoresis buffer (89 mM Tris-base, 89 mM boric acid, 2.0 mM EDTA, pH 8.3) on an ABI Prism™ 377 DNA sequencer (PE/Applied Biosystems) at 2500 V for 4 h. For raw data collection, the ABI Prism™ Collection Software version 1.1 was employed.

Semi-automated AFLP fragment analysis was performed with GeneScan™ analysis software version 2.0.2 and Genotyper™ DNA fragment analysis software version 2.0 (PE/Applied Biosystems), as described in the user's manuals. The genotyping procedure was modified as follows: manual control of lane tracking was necessary, a local Southern method (Southern 1979) was used for size-calling of AFLP fragments, the analysis range started after the primer peak and polymorphic peaks were defined and labelled by creating categories with a tolerance of ± 0.5 bp. For allele-typing, the peak heights were divided by a scale factor defined as the sum of signals of all fragments. The scaled peak heights of the parental lines were set as a reference. For dominant allele-typing, the category members were defined as follows: for homozygous allele 'A' when scaled the height was 0–50% of the reference, and for homozygous allele 'B' when scaled the height was 50–120% of the reference. For co-dominant allele-typing: for homozygous allele 'A' when scaled the height was 0–22% of the reference, for heterozygous allele 'H' when scaled the height was 22–65% of the reference, and for homozygous allele 'B' when the scaled height was 65–120% of the reference.

Radiolabelled AFLP analysis

For conventional AFLP analysis the *Eco*RI primer was labelled with [γ-^{33}P]ATP (Amersham-Pharmacia Biotech) using 0.1 U of T4 polynucleotide kinase (Amersham-Pharmacia Biotech) for 30 min at 37°C. Selective amplification was performed using the same pre-amplified DNA and PCR conditions as described above. The samples were prepared for loading by mixing 2 µl of each sample with 2 µl of formamide dye (98% formamide, 0.005% bromophenol blue, 0.005% xylene cyanol), denaturing for 3 min at 90°C and quickly chilling on ice. The gel was run at a constant 50 W on a standard electrophoresis apparatus (Gibco BRL Life

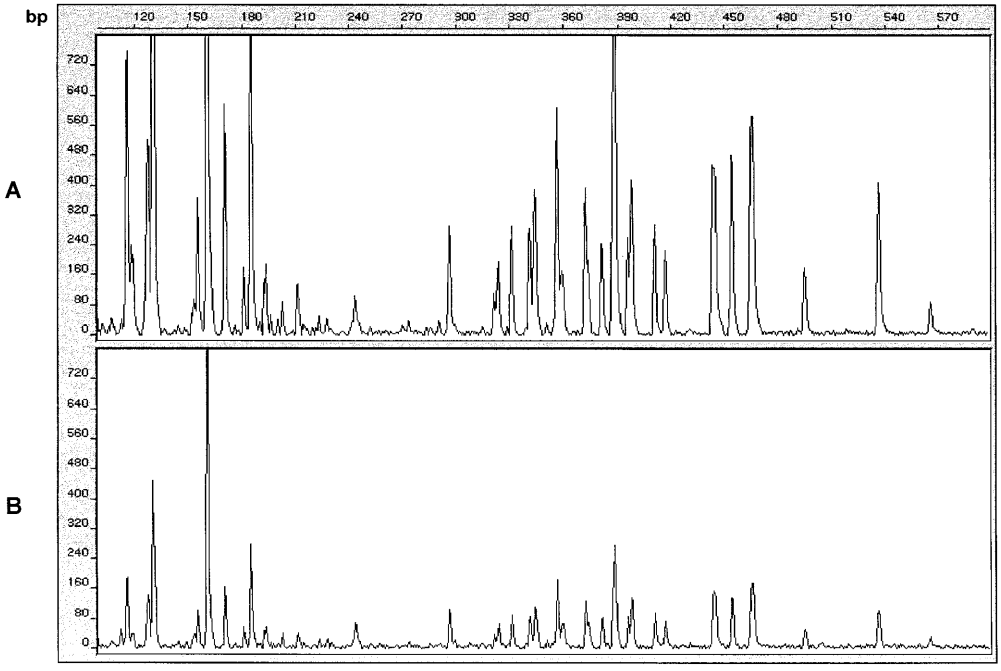

Fig. 1A, B Multiplex versus simplex PCR. Genomic DNA from barley cultivar 'Golf' served as a template for AFLP reactions using the primer combination *Eco*RI-ACT/*Mse*I-CAA. Electropherograms display PCR products obtained by (**A**) multiplex PCR and (**B**) simplex PCR. Vertical scales display the relative signal intensity of fluorescently labelled fragments

Technologies, Rockville, Md., USA). Gels were subsequently fixed in 10% acetic acid, dried at 80° C for 1 h and exposed to X-ray film (Biomax MR, Eastman Kodak, Rochester, N.Y., USA) at room temperature overnight.

Results and discussion

Application of fluorescence-based AFLP analysis in wheat and barley

The multifluorophore detection system on an ABI Prism™ 377 DNA sequencer allows the concurrent analysis of three DNA samples labelled with different fluorescence dyes. To test whether the detection system can correctly distinguish between multimixed AFLP reactions, three samples labelled with either 5-FAM, JOE or TAMRA were analysed separately and in all possible combinations. Subsequent electropherograms obtained with GeneScan™ software showed no difference in peak detection, despite the observation of co-migrating fragments which carried different dyes. Therefore, the throughput of AFLP samples can be enhanced three-fold compared to conventional electrophoresis systems. Furthermore, accurate size-calling of identical samples was examined within and between gels. As reported for microsatellites by Mitchell et al. (1997), likewise for AFLP fragments, inter-gel differences in size-calling were greater than intra-gel differences. Examining automated fragment-size estimates for three primer combinations in 78 F_2 individuals, deviations between lanes and between gels never exceeded 0.2 and 0.5 nucleotides, respectively, which is a meaningful pre-requisite for accurate allele-calling with Genotyper™ software.

All possible 256 primer combinations resulting from 16 selective *Eco*RI+ANN and 16 *Mse*I+CNN primers were used to generate AFLP fragments from DNA of barley and wheat. For each primer combination the number of amplification products from barley DNA ranged between 17 and 172 with an average of 83, whereas for wheat the number of fragments varied from 18 to 226 with a mean of 103. The primer combinations *Eco*RI-ATT/*Mse*I-CNN and *Eco*RI-AAA/*Mse*I-CNN produced a smear with DNA from both species and were excluded from further analysis. Non-specific amplification using these primer combinations is probably due to the mismatch-tolerating order of the three selective nucleotides at the *Eco*RI 3' primer end. The combination of the multifluorophore detection system with the power of AFLP analysis provides an efficient tool for large-scale genetic mapping because up to 8600 fragments in barley and 11 200 fragments in wheat (number of fluorescent dyes × mean value of fragments/primer combination × gel capacity) can be analysed with a single gel run. A 96-lane upgrade for all ABI Prism™ 377 instrument models is now available, therefore maximizing the efficiency for genetic-mapping purposes.

The reliability of multiplex PCR was tested using 30 arbitrarily chosen AFLP primer combinations with DNA from barley cultivars 'Golf' and 'Alexis'. Products of multiplex PCR and reactions with single primer-pair combinations were compared with respect to the number of detected bands and polymorphisms, as well as their reproducibility. With multiplex PCR, it is not possible to balance different dye intensities by loading appropriate volumes of single reactions as done for multimixing analysis, resulting in more-intense fluorescence emission (Fig. 1). Regardless of this, all polymorphisms found by the analysis of single AFLP reactions were confirmed for multiplex PCR. Therefore, multiplexing accelerates the mapping of AFLP markers while minimizing time and consumables.

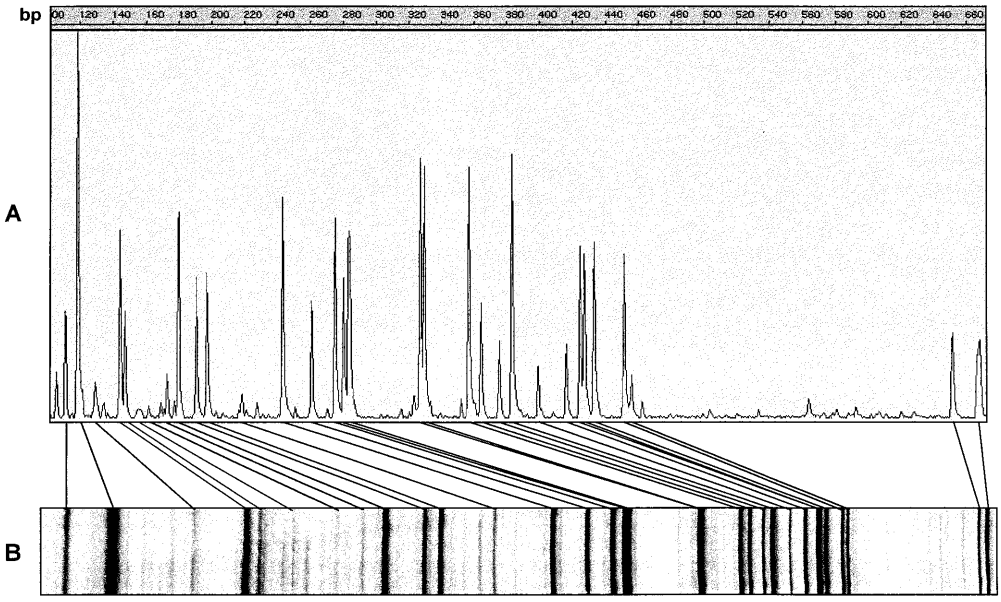

Fig. 2A, B Resolution of AFLP fragments in a size range of 100–670 bp. Fragments were amplifed with primer combination *Eco*RI-ACA/*Mse*I-CGT using genomic barley DNA of line 'P01' as a template. **A** Electropherogram showing 39 distinguishable 5-FAM labelled AFLP fragments. The vertical scale displays the relative signal intensity of fluorescently labelled fragments. **B** Autoradiograph showing 32 distinguishable ^{33}P-labelled AFLP fragments

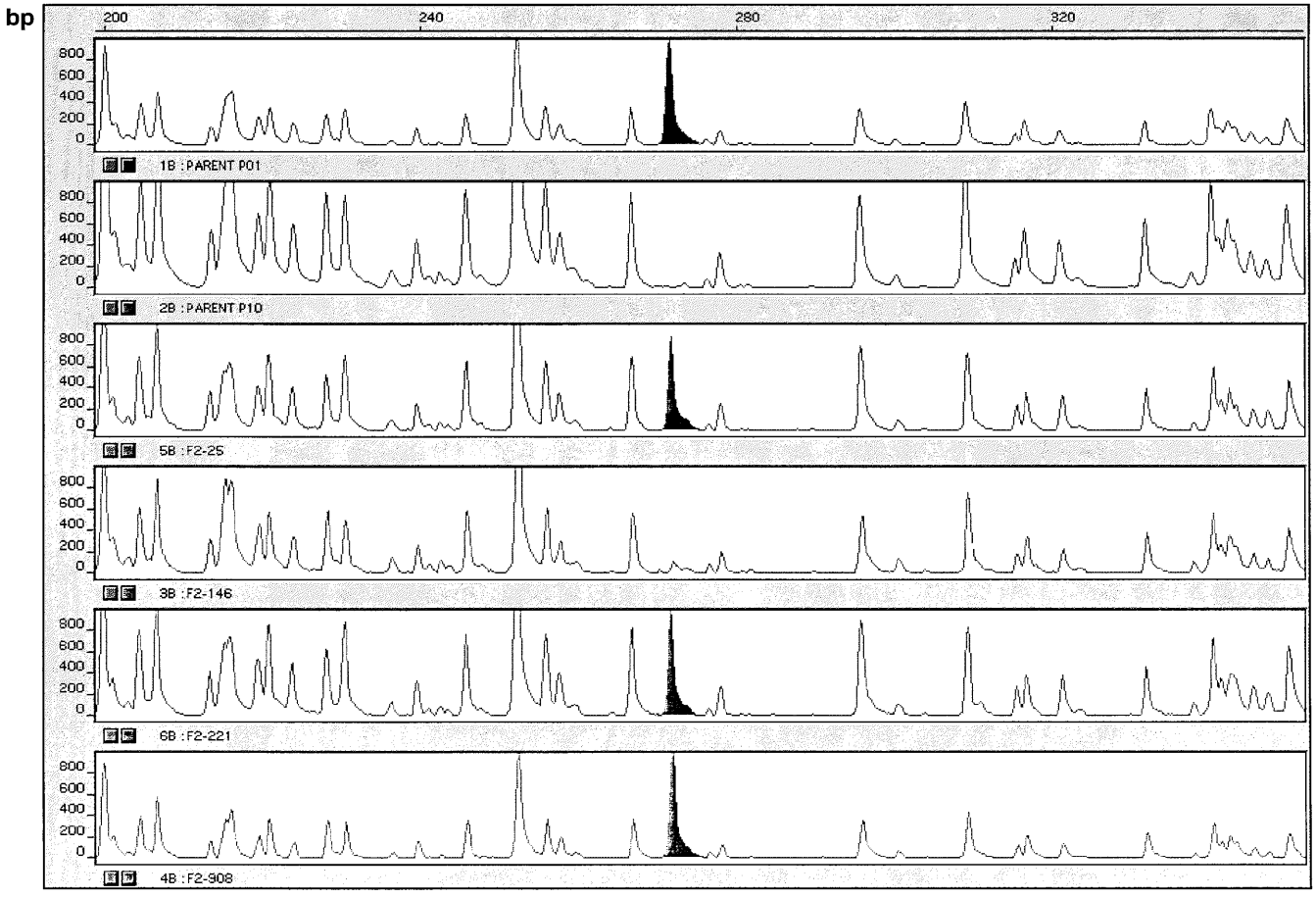

Fig. 3 Accurate size-calling of AFLP fragments with GeneScan software. Electrophorograms of parental lines 'P01' and 'P10' and four randomly chosen F_2 individuals in a size range of 200–350 bp. The segregating AFLP marker ACT/CAA-271 is marked as a *solid, black peak*. Vertical scales display the relative signal intensity of fluorescently labelled fragments

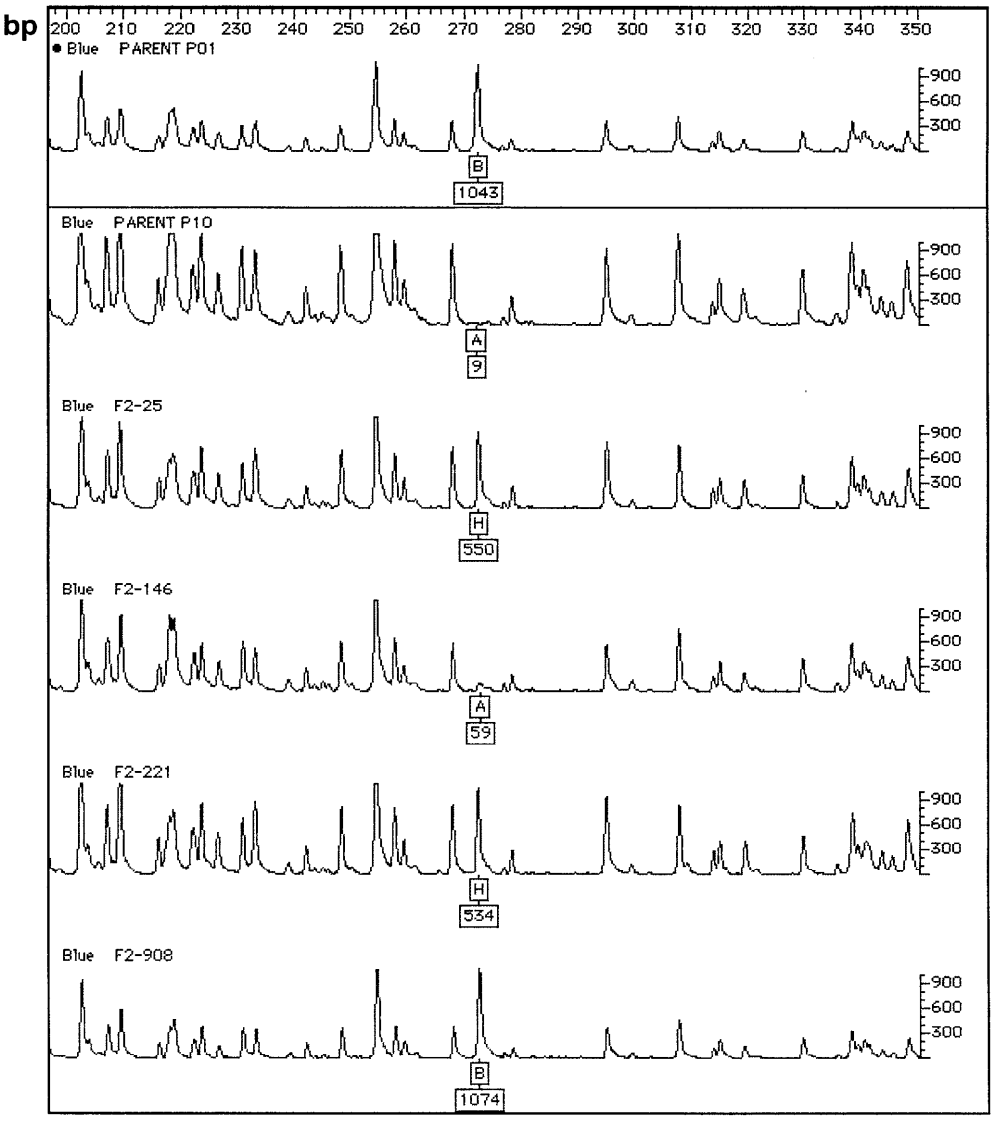

Fig. 4 Semi-automated allele-typing with Genotyper software. The segregating AFLP marker ACT/CAA-271 is specified by *labels* indicating peak heights and allele classes. The vertical scales display the relative signal intensity of fluorescently labelled fragments

Comparison of AFLP detection systems

An important aim of our study was to examine whether the fluorescence-based AFLP technique is comparable to a conventional ^{33}P-based method. Both techniques were applied to detect AFLP products generated from barley DNA with 17 different primer combinations. We focussed on differences concerning the detectable number of bands, the power of resolution and detection sensitivity. Radiolabelled samples were analysed in a size range of 100–700 bp, whereas the semi-automated size-calling of fluorescently-labelled reactions was limited to a range of 100–600 bp, depending on the use of internal length standard GeneScan-500 ROX. Differences were apparent in fragment profiles between fluorescent versus radiolabelled AFLPs. On average, 82.2 fragments per primer combination were detected by scoring fluorescent products with the ABI Prism™ 377 DNA sequencer and GeneScan™ analysis software version 2.0.2. This clearly exeeds the mean value of 64.7 detected-fragments when using the radiolabelled method, though scoring involved a greater size range and many high-molecular-weight fragments are lost using fluorescent AFLPs. As the sensitivity of both detection systems is about 10^{-18} – 10^{-17} mol/DNA fragment, we propose the following explanations for the observed differences. Radiolabeled fragments of a size greater than 400 bp, that differ in length by only a few nucleotides, were not distinguishable due to the logarithmic spacing of fragments. Additionally, due to the dispersion of strong signals, neighbouring fragments cannot be precisely scored (Fig. 2). In contrast, on an ABI Prism™ 377 DNA sequencer, fluorescently labelled fragments pass the detector near the bottom of the gel resulting in a linear spacing of DNA fragments, thus improving the resolution over the whole size range. Nevertheless, conventional electrophoresis and visualisation systems are still necessary for conversion of interesting AFLPs into STS markers, because access to fragments is not possible using automated DNA sequencers. This is a limitation of DNA sequencer-based

applications, since AFLP markers are a valuable source for the development of user-friendly, sequence-specific PCR markers.

Semi-automated segregation analysis

The majority of *Eco*RI/*Mse*I AFLP markers in barley are hemizygous, being null allelic in one or the other parent (Becker et al. 1995). Co-dominant scoring of such hemizygous bands is achievable by the measurement of signal intensity. While the signal quantification of ^{33}P isotopes by exposure to film is nonlinear with respect to the amount of radioactivity in the sample and detection time, fluorescence signal detection shows linearity over a much-greater range (Schwengel et al. 1994) thus allowing correct quantification of signals. Additional normalisation of signal amplitudes of all samples enables co-dominant genotyping of AFLP marker loci. Accurate size-calling in a range of 100–600 bp was checked using GeneScan™ electropherograms of the parental lines and the F_2 mapping population (Fig. 3). GeneScan-result files were imported into Genotyper™ software and polymorphic peaks between the parental lines were labelled manually. In Fig. 4 normalised peak heights and the resulting allele classes are shown as labels for marker ACT/CAA-271 of the parental lines and four randomly chosen F_2 individuals. For progeny lines F_2-25 and F_2-221 a scaled peak height about half of parent 'P01' was calculated. Therefore, they were grouped in the heterozygous category. Previous mapping data of tightly bordered co-dominant RFLP markers (data not shown) allowed us to predict the most-probable allele configuration of the AFLP marker ACT/CAA-271 for 78 F_2s, ignoring rare double-crossover events. Semi-automated, co-dominant genotyping showed 6.4% deviation from predicted allele-typing with flanking markers. This high percentage of mis-scoring is alarming. Therefore, default threshold levels of Genotyper™ software for automated genotyping of hemizygous AFLP marker loci are not usable for unambigous mapping. An individual default-setting for each polymorphic band would be necessary. Therefore, the co-dominant analysis feature is not yet a valuable tool because it would require as much time as is needed for manual scoring. By using category default-settings for dominant allele-typing, deviation was reduced to 1.3%, as presented from the results of the manual-genotyping procedure of the dominant, AFLP-derived STS marker aACT/CAA-271 (Fig. 5) (Schwarz et al. 1999).

The amount of false positives produced by co-dominant allele-typing can be tolerated for the construction of whole-genome maps considering a 50% increase in genotype information when heterozygous marker loci can be recognised in F_2s. Also, the error-detection features of some genetic-mapping programs exclude improbable marker orders caused by false positives and minimize genotyping errors resulting from semi-automated analysis. On the other hand, true orientation of markers in the vicinity of a target gene is the most crucial step in a map-based cloning approach, for which we recommend manual scoring.

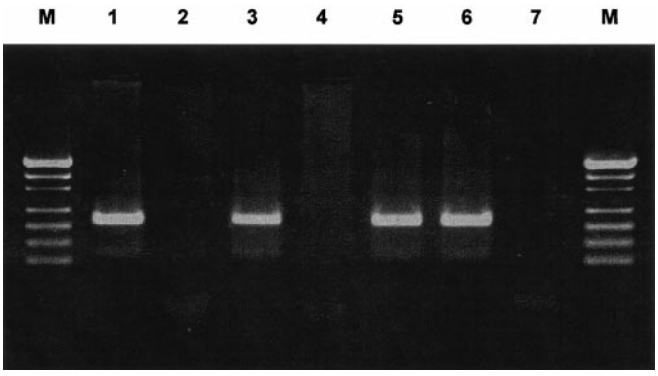

Fig. 5 Segregation of AFLP-derived dominant STS marker aACT/CAA-271. *M* pUC19 DNA/*Msp*I, *1* 'P01', *2* 'P10', *3* F_2-25, *4* F_2-146, *5* F_2-221, *6* F_2-908, *7* H_2O

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