Theor Appl Genet (2003) 106:1293–1299
DOI 10.1007/s00122-003-1197-0

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# Identification of AFLP and STS markers closely linked to the *def* locus in pea

Received: 20 September 2002 / Accepted: 11 November 2002 / Published online: 12 March 2003


**Abstract** The recessive mutation of the *def* gene of pea (*Pisum sativum* L.) leads to the loss of the hilum, the abscission zone between the seed and the pod. Thereby, it reduces the free dispersal of the seeds through pod shattering. As a prerequisite for a gene isolation via a map-based cloning approach, bulked segregant analysis followed by single plant analyses of over 200 homozygous individuals of a population of 476 F2 plants derived from a cross between 'DGV' (*def* wild-type) and 'PF' (*def* mutant), were used to detect markers closely linked to the *def* locus. The AFLP technique in combination with silver staining was used to maximize numbers of reproducible marker loci. Fifteen AFLP loci showed a genetic distance less than 5 and two of them less than 1 centiMorgans (cM) to the gene of interest. AFLPs were converted into sequence tagged sites (STSs) and into a newly refined AFLP-based single locus marker named the 'sequence specified AFLP' (ssAFLP).

**Keywords** Pea · *def* locus · Pod shatter · AFLP · Sequence-tagged-site (STS) marker

Communicated by C. Möllers

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## Introduction

In the angiospermae the fruits are classified according to the way they open in the nondehiscent and the dehiscent fruits. The Ranunculaceae, Cruciferae, Brassicaceae and Leguminosae belong to the latter class, and the process by which their seeds are dispersed is called fruit dehiscence or pod shatter. At ripeness, the seeds, released from the connection to the ovary, the funiculus, through an abscission zone, the hilum, shed from the plants after the carpel valves, open at a dehiscence suture. The dehiscence of the carpel suture as well as the hilum is correlated to the decreasing humidity of the ripening fruit.

Pod shattering causes harvest losses from 10% up to 50% under severe weather conditions in a number of crop plants, including oilseed rape (MacLeod 1981; Child et al. 1998) and legumes like soybean (Philbrook and Oplinger 1986), pea (Jackson and Miller 1999), lentil (Moden et al.1986) and birdsfoot trefoil (Garcia-Diaz 2000). As harvest time approaches, drying seed pods become fragile and may split, and disperse the seeds with only a small energy input from e.g. wind or harvesting. Due to uneven ripening the harvesting timepoint is crucial but difficult to fix. Moreover, crops often have to be harvested late at night or early morning due to increased relative humidity and appearing dew deposits (Muehlbauer et al. 1997). Apart from chemicals, organic polymers forming a covering film are applied to keep the moisture in the seed pods (ENGAGE Agro Corporation 2002).

For many decades plant organ abscission has been an objective of intensive research. Mainly the morphology of the abscission zone, the involved phytohormones like abscissic acid (ABA) and ethylene, and more recently the molecular mechanisms underlying the process, have been investigated. Comprehensive overviews can be found in Roberts et al. (2000) and Patterson (2001).

Concerning fruit-dehiscence recent molecular studies revealed the effect of the *SHATTERPROOF* MADS-box genes (*SHP1*, *SHP2*) on dehiscence-zone differentiation of carpel valves in *Arabidopsis thaliana* L. (Liljegren et al. 2000). In *shp1 shp2* double mutants fruits failed to

dehisce due to the absent carpel valve dehiscence zone, and the possibility to reduce pod shattering in crops by genetic engineering is proposed.

We have chosen the second essential component of the fruit dehiscence process for molecular characterisation, namely the abscission zone between seed and funiculus, the hilum. In pea (*Pisum sativum* L.) a spontaneous monogenic recessive mutation has been selected, that leads to a slightly changed morphology of the funiculus, including the loss of the hilum (Rozental 1966; Swiecicki and Wolko 1990). As a result, the seeds stay connected to the pod even if the carpel valves burst open (see Fig. 1). The character was named *development funiculus* with the gene symbol *def* (Khangildin and Khangildin 1969). *Def* as a simple monogenic mutation has unique importance, since to our knowledge it is the only known gene, up to now, which solely affects the hilum abscission zone with no pleiotropic effects. In that way it is comparable to the *SHATTERPROOF* genes, which seem to affect only the carpel valve dehiscence zones of *A. thaliana*. Other known genes involved in organ abscission like *DELAYED DEHISCENCE* 1 (Sanders et al. 2000) or *JOINTLESS* in tomato (Mao et al. 2000) act in a more multifaceted manner. In the current consensus map for *P. sativum* L. the *def* gene is located on the bottom end of linkage group VII which corresponds to chromosome no. 4 (Weeden et al. 1998).

Our long-term aim is to isolate the *def* gene using a map-based cloning approach. This requires a fine map of the close region around the gene of interest. In a second step the genetic map is transferred into a physical map, based on a contig of BAC/YAC-clones spanning the corresponding chromosomal part. In the so called chromosome-walking step, possible gene candidates are identified by a combination of subcloning and sequencing the contig. Finally gene function has to be verified by a complementation analysis.

Here we present markers closely linked to the *def* gene as an outset for map-based cloning.

To establish a local map around the *def* gene the AFLP marker technique was used (Vos et al. 1995), which allows fast and reproducible amplification and visualization of many genetic loci. The AFLP technique was used in combination with bulked segregant analysis (BSA, Michelmore et al. 1991) to limit detectable polymorphisms to the region around the gene of interest.

## Material and methods

### Plant material

Plants were grown in the greenhouse under a 16-h-long day. The line 'Pajbjergfonden 42403' (PF) is a homozygous carrier of the *def* mutation. PF was crossed with the homozygous wild-type line 'Dippes Gelbe Viktoria' (DGV, John Innes Center, Pisum Accession no. JI2413). PF as well as DGV were used as the male and as the female parent. Autogamous pollination was allowed in the F1 and the F2. A F2 population of 476 plants was grown and further analysed. Up to 14 F3 plants of each F2 plant showing the wild-type phenotype were analysed to distinguish their heterozygous or homozygous dominant genotypes. Chi-square tests with a 0.05 type I error were perfomed to prove the goodness-of-fit of the Mendelian segregation of the *def* locus. Leaf material of plants was harvested and stored at –80 °C.

### DNA preparation

Total DNA was extracted from leaves following the protocol of Doyle and Doyle (1990). The DNA concentration was measured in a Versafluor fluorometer (Bio-Rad) using the Fluorescence DNA quantitation kit (Bio-Rad). The quality and intactness of the DNA was examined using agarose-gel electrophoresis.

### AFLP analysis

The AFLP technique was carried out according to Vos et al. (1995) with the following modifications: 400 ng of total DNA were digested with four units of *Eco*RI and *Mse*I (GIBCO-BRL) in a total volume of 25 $\mu$l for 2 h at 37 °C. The selective amplification reaction was carried out in a final volume of 20 $\mu$l containing 5 $\mu$l of a 1:50 dilution of the preamplification reaction, 20 ng of the *Eco*RI primer, 30 ng of the *Mse*I primer, 0.2 mM of all four dNTPs, one unit of *Taq* polymerase and 2 $\mu$l of a 10 × *Taq* buffer. All PCR reactions were performed using the RED-*Taq* polymerase system (SIGMA-Aldrich) and the T-3 Thermocycler (Biometra). Five microliters of a formamide loading dye (98% formamide, 10 mM EDTA pH 8.0, bromo phenol blue and xylene cyanol) were added to the reaction. The DNA was denatured for 5 min at 99 °C and chilled in an ice water bath. Five decimal five microliters of the reaction were separated in a 4% denaturing polyacrylamide gel (29% acryl:1% bisacryl, 7.5 M urea in 1 × TBE buffer). Electrophoresis was carried out with a Sequi Gen Cell sequencing gel apparatus (Bio-Rad). To fix the gel matrix for the staining procedure, one glass plate was treated for 5 min with a binding solution of 50 ml absolute ethanol, 1.5 ml of acetic acid (8%) and 150 $\mu$l of metacryloxypropyltrimethoxysilane (SIGMA-Aldrich), washed with absolute ethanol and dried prior to casting and pouring the gel. The DNA was visualized by silver staining following the protocol of Bassam et al. (1991). Eight *Eco*RI and eight *Mse*I selective primers were used in 64 different combinations. Each primer was specified by three selective bases of which the first selective base of the *Eco*RI and the *Mse*I primer were a Adenine and a Cytosine, respectively. AFLP loci were named first with the two final specific bases of the *Eco*RI primer, and second with the two final specific bases of the *Mse*I primer followed by the size of the AFLP band. For AFLP loci specific for the wild-type parent (DGV), upper-case letters and specific for the mutant parent (PF) lower-case letters were used.

### Sequence tagged sites (STS) and sequence specified AFLPs

AFLP bands to be further analysed were cut out of dried silver-stained gels and incubated in 100 $\mu$l of TE buffer pH 8.0 for 1 h at room temperature. Five microliters of the solution were used as a template in a re-amplification PCR with the corresponding selective primers, following the AFLP selective amplification described above. The reaction was separated in a 1.5% agarose gel and PCR products of the expected size were cut out of the gel, extracted from the gel matrix with a QIAEX II gel extraction kit (Qiagen), directly cloned in the T/A-cloning vector pGEM-T-Easy (Promega) and transformed into *Escherichia coli*. Clones with the expected insert size were sequenced and specific primers with a length from 17 to 25 basepairs were derived from the sequence starting at the distal end of either the *Eco*RI or the *Mse*I restriction site and leading into the specific sequence. Amplification reactions were optimized according to the respective primer annealing temperatures and products separated in 2% agarose gels. STS markers were named with the AFLP marker name preceded by the abbreviation 'STS'.

Sequence-specified AFLP primers were generated by extending the original selective AFLP primer 3'-end with 2–5 addtional nucleotides from the respective clone sequence. If necessary, the 5'-end of the primer was shortened by 1–4 nucleotides to optimize the primer annealing temperature. Amplification reactions were optimized according to the respective primer annealing temperatures

and the results separated in 2% agarose gels. Sequence-specified AFLP markers were named with the AFLP marker name and the prefix 'ss' (sequence specified).

### Linkage analysis

Bulked segregant analysis (BSA) was used to limit detectable DNA polymorphisms to the region around the gene of interest (Michelmore et al. 1991). Two *def* wild-type and two *def* mutant bulks, each prepared with equal amounts of DNA of ten different homozygous F2 plants, were used for the BSA. AFLP loci that showed linkage to the *def* gene were further analysed in F2 single plants. Linkage analysis was performed using MAPMAKER 3.0 (Lander et al. 1987) with a minimum LOD of 3.0. The recombination fraction was calculated with the Kosambi (1944) function presupposing positive crossing-over interference in distal chromosome regions (Esch and Weber 2002).

## Results

### Plant crossing and segregation of the *def* locus

Handcrossing of the wild-type DGV and the mutant PF led to successful pod production in 50% of the crosses, with a strongly reduced number of seeds per handpollinated flower. F1 plants showed the wild-type phenotype. In the F2, the *def* phenotype segregated 355:121 (wild-type:mutant) following a ratio of 3:1 ($\chi^2 = 0.045$, $df = 1$, $P = 0.8323$). F3 analysis of the 355 F2 plants showing the wild-type phenotype revealed 115 homozygous and 240 heterozygous *def* genotypes. The genotypic segregation 115:240:121 (homozygous wild-type:heterozygous: homozygous mutant) followed a 1:2:1 segregation ($\chi^2 = 0.185$, $df = 2$, $P = 0.9117$). The mutant phenotype was restricted to the funiculus and did not influence other visible phenotypic characteristics (Fig. 1).

### AFLP analysis

In an initial step the level of polymorphism between DGV and PF was investigated using 64 primer combinations (see Fig. 2). The average number of fragments amplified

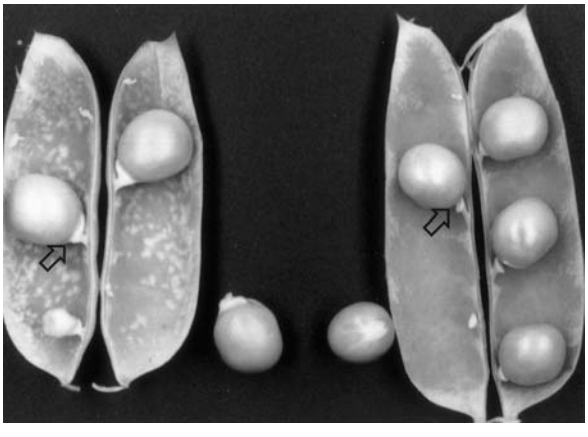

**Fig. 1** The *def* mutation. Opened fruits of a *def* mutant (PF, left) and a *def* wild-type (DGV, right) plant. The hilum abscission zone is lost in the mutant but clearly visible in the wild-type (*arrows*)

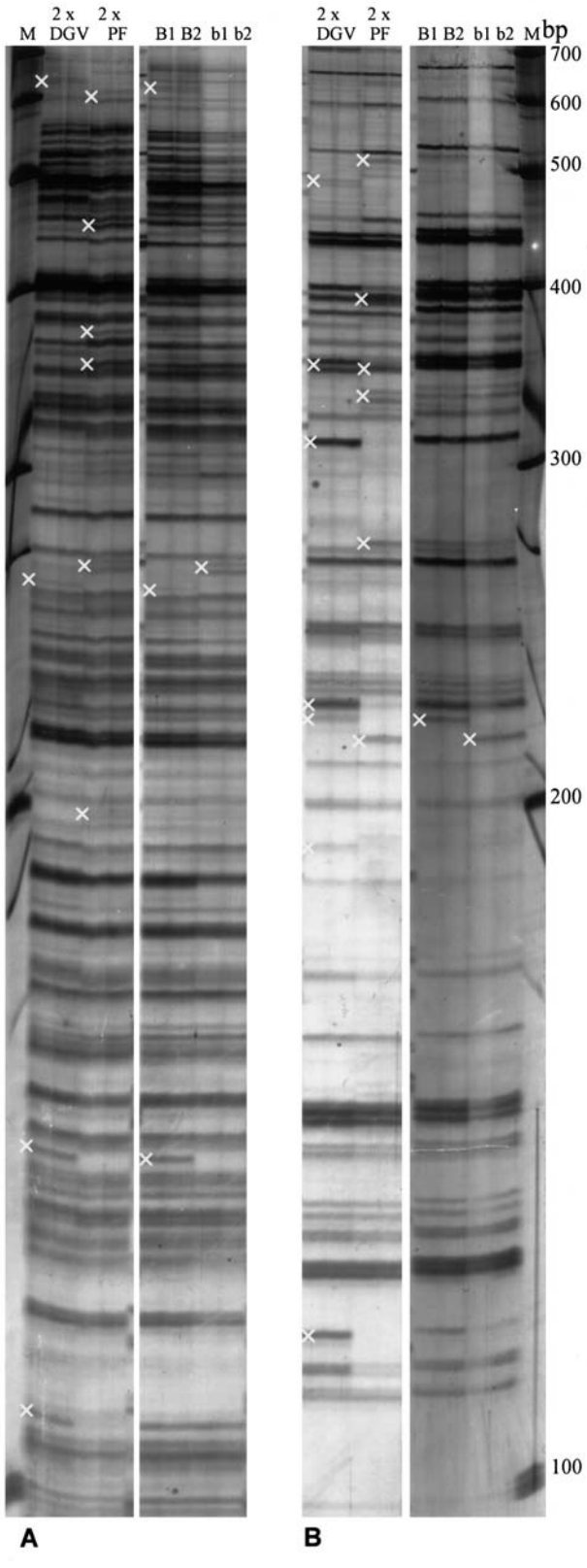


**Fig. 2A, B** Representative AFLP analysis of the parental lines DGV and PF in parallel to the bulked segregants. **A** primer E-AAC × M-CTT; **B** primer E-ACT × M-CTG; 2 × DGV and 2 × PF (four left lanes) are loaded in parallel to the four bulks (four right lanes). B1/B2 are the dominant bulks; b1/b2 are the recessive bulks. The *white* x's indicate polymorphic bands between either the parents (left lanes) or the bulked segregants (right lanes). *M*: 100-bp ladder

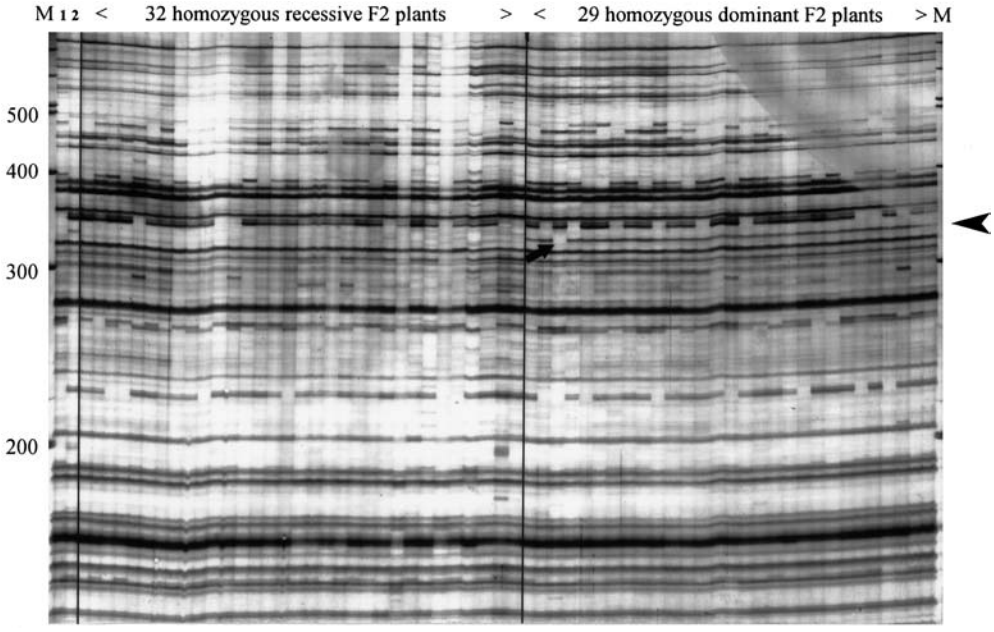


**Fig. 3** F2 plant segregation analysis of the AFLP marker CGAT 330. Analysis of the parental lines PF (*lane 1*) and DGV (*lane 2*), of 32 homozygous recessive F2 plants and 29 homozygous dominant F2 plants. The marker CGAT 330 (*arrow on right*) co-segregates except in one plant (*arrow in middle*). *M*: 100-bp ladder

**Table 1** Dominant AFLP markers with a pairwise map distance to the *def* gene less than 5 cM. Upper-case letters indicate association of loci with *def* wild-type allele, lower-case letters indicate association of loci with the *def* mutant allele

| AFLP-marker | Pairwise map distance to the *def* locus in cM (Kosambi) | AFLP-marker | Pairwise map distance to the *def* locus in cM (Kosambi) |
|---|---|---|---|
| CTTG 220 | 0,8 | CAAA 090 | 3,0 |
| GCAT 080 | 0,9 | CAAA 290 | 4,0 |
| CAAC 570 | 1,2 | ACTT 630 | 4,3 |
| AGTG 150 | 1,6 | CGAG 230 | 4,5 |
| actt 270 | 1,8 | CGAC 350 | 4,5 |
| GGTT 270 | 2,2 | cctg 410 | 4,9 |
| gcta 350 | 2,9 | ACTT 130 | 5,0 |
| CGAT 330 | 2,9 | | |

**Table 2** Dominant STS marker derived from AFLPs with coupling phase and pairwise map distance to the *def* gene

| STS marker | Primer sequences (5'–3') | In coupling to the *def* DGV or PF allele | Pairwise map distance to the *def* locus in cM (Kosambi) |
|---|---|---|---|
| STS-CGAC-350 | L: GAATTCACGTAGTTGGTTTTAC<br>R: TTAACACAGTTTTTTATAAAGTAACTC | DGV | 4.5 |
| STS-CGAG-230 | L: GAATTCACGGTTGGAACTG<br>R: TTAACAGTCATGTCTGCTC | DGV | 4.5 |
| STS-gcta-350 | L: GAATTCAGCAGCAGAGAAA<br>R: TTAACTATTCAAAATATTGCAAA | PF | 2.9 |

per primer combination was approximately 60. Each AFLP banding pattern revealed a minimum of four, a maximum of 16 and an average of eight polymorphic bands. This corresponds to >13% average sequence polymorphism between DGV and PF, as detectable with the AFLP method. BSA was used with 64 primer combinations to identify AFLPs linked to the gene of interest (Fig. 2). These AFLPs were further analysed in F2 plants (Fig. 3). For the F2 analysis only the homozygous wild-type and the homozygous mutant-type plants were selected due to their higher information content concerning the gene of interest. Also individuals included in the bulks were confirmed by single-plant analysis. Linkage of 38 AFLPs to the *def* locus was proven in an analysis of 60 F2 single plants. Markers that at this point showed no recombination were further analysed in up to 208 homozygous F2 plants. Fifteen AFLP loci have pairwise map distances to the *def* gene less than 5 cM, five less than 2 cM and two less than 1 cM (Table 1).

**Table 3** Dominant sequence specified AFLP markers with coupling phase and pairwise map distance to the def gene

| SsAFLP marker | Primer sequences (5'–3'), additional specifying bases underlined | In coupling to the *def* DGV or PF allele | Pairwise map distance to the *def* locus in cM (Kosambi) |
|---|---|---|---|
| ssACTT-130 | L: GCGTACCAATTCAACAAAC<br>R: GAGTCCTGAGTAACTTGTA | DGV | 5.0 |
| ssAGTG-150 | L: TGCGTACCAATTCAAGAAG<br>R: TGAGTCCTGAGTAACTGGC | DGV | 1.6 |
| ssCAAC-570 | L: TGCGTACCAATTCACACCT<br>R: TGAGTCCTGAGTAACACAG | DGV | 1.2 |
| ssactt-270 | L: TGCGTACCAATTCAACGTC<br>R: TGAGTCCTGAGTAACTTGG | PF | 1.8 |
| sscttg-210 | L: CGTACCAATTCACTAAATC<br>R: GAGTCCTGAGTAACTGGA | PF | 5.6 |

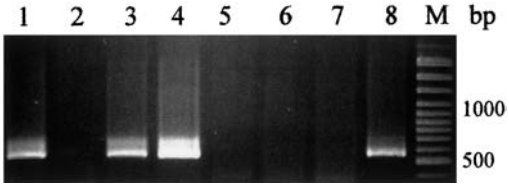


**Fig. 4** Sequence specified AFLP analysis of ssCAAC 570. PCR of the parental lines DGV (*lane 1*) and PF (*lane 2*), the dominant bulks B1 (*lane 3*) B2 (*lane 4*), the recessive bulks b1 (*lane 5*) b2 (*lane 6*) and two recombinant F2 plants *148* (*lane 7*) and *98* (*lane 8*); *M*: 100-bp ladder

## STS markers and sequence-specified AFLPs

Twelve AFLP bands were further analysed to convert them into STS markers. Up to ten clones per band were sequenced and 1–5 different clones were detected from one band. Only three PCRs with sequence-specific primer pairs directly reproduced the initial AFLPs, consequently converting them into a polymorphic STS (Table 2). PCRs with specific primers derived from the clones from the other nine analysed AFLP bands led to amplification products of the same size in both parental plants. Firstly, this can be due to a misleading cloning and specification of background fragments co-migrating with the original polymorphic band. Secondly, the primer extensions can lead to a mispriming with the initial DNA polymorphism, if it is caused by a point mutation within or at one of one of the flanking AFLP restriction sites. To overcome the latter limitation, we used the sequence information of the clones to generate primers corresponding to the original AFLP selective primer sequence with two to five additional selective sequence specific bases and we performed a PCR with the pre-amplified diluted AFLP template following the AFLP procedure. Interestingly, another five AFLPs could be specified to single PCR products which showed the primary polymorphism and which could easily be analysed in normal agarose gel electrophoresis (Fig. 4, Table 3). We called this marker a 'sequence specified AFLP' (ssAFLP). The STS marker as well as the ssAFLP marker behaved as dominant markers and followed the segregation of their underlying AFLPs.

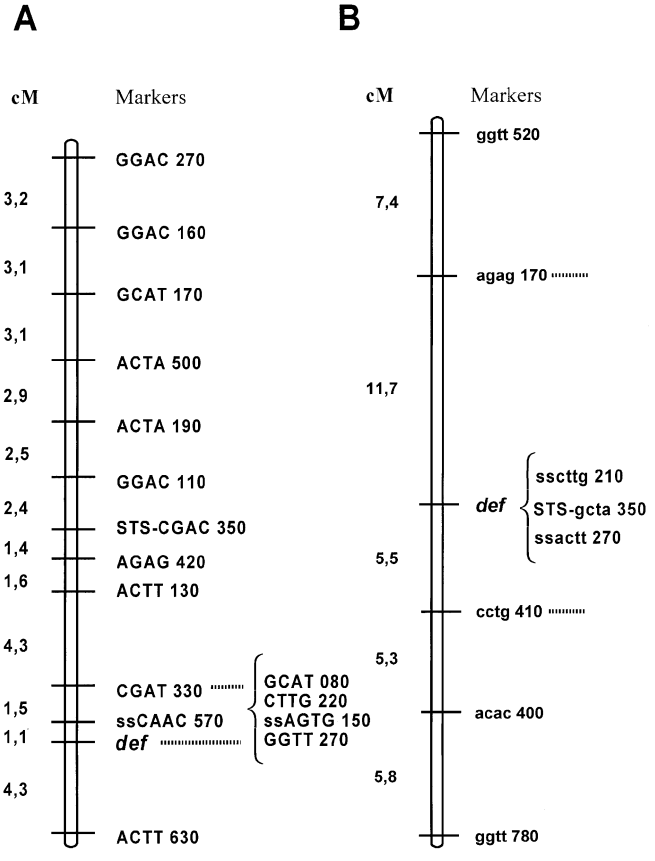


**Fig. 5A, B** Multi-point linkage maps of the region around the *def* gene. **A** Map of the markers associated with the DGV/wild-type *def* allele. **B** Map of the markers associated with the PF/mutant *def* allele. Marker loci after the brackets are placed in a close interval marked by the *dash lines*. Note that the orientation of both maps to each other is random

## Linkage analysis

Thirty eight AFLPs, ssAFLPs and STS loci where scored in a multi-point linkage analysis with MAPMAKER 3.0. The difficulties of mapping dominant markers in a coupling and repulsion phase (Säll and Nilsson 1994; Knapp et al. 1995) were circumvented by separating the markers into one group coming from the *def* wild-type

parent DGV, and a second group coming from the *def* mutant parent PF.

This led to two independent local maps of the region around the *def* locus (Fig. 5A, B). Twelve markers from the wild-type parent were successfully integrated into a genetic linkage map with the *def* locus spanning about 31 cM. In a close interval between the *def* locus and the locus CGAT 330 a group of additional four markers could be placed (Fig. 5A).

Five AFLPs from the mutant parent could successfully be integrated with the *def* locus in a second map spanning 36 cM. An additional three markers could be placed in the interval between the neighboring AFLP loci to the *def* gene (Fig. 5B).

## Discussion

In this study we successfully used the AFLP technique together with silver staining to detect closely linked markers to the *def* gene in pea.

Combining the AFLP technique with the DNA silver-staining procedure from Bassam et al. (1991) has already been described previously, also with pea (Chalhoub et al. 1997; Tiwari et al. 1999; Coyne et al. 2000) and with a number of other species (Zhang and Stommel 2000; Mano et al. 2001). This combination is of interest particularly for smaller laboratories lacking the resources for automated sequencing or the use of radioactivity. One strong advantage of the silver staining is the ease of cloning interesting AFLP bands, which can be cut out directly and exactly due to their *in gel staining*. We increased the amount of PCR product loaded on the gel by reducing the amount of added denaturating loading buffer from half to one-fifth of the final volume without any changes in the separation quality.

In this work we identified and analysed 38 AFLP loci which are linked to the *def* gene. Fifteen of the markers showed less than 0.05, five less than 0.02 and two less than 0.01 recombination fractions to the *def* gene. This results in a good marker saturation of the region around the *def* locus. We established two genetic maps of this region, which in one map combine the markers in coupling to the wild-type *def* allele, and in another with the markers in coupling to the mutant *def* allele. The maximum length of the two maps is 31 cM and 36 cM, respectively, indicating that the BSA delimits detectable polymorphisms to the so-called 'genetic window' with a maximum size of not more than 40 cM around the gene of interest.

The relation of genetic to physical distance is of predominant importance concerning the success of a map-based cloning approach. Recent reports showed evidence for the fact that genes are hot spots for recombination events (Schnable et al. 1998). It is suggested that in gene-rich regions the relation of basepairs to cM, with less than 200 kbp per 1 cM, is similar among all plants, regardless of their overall genome size (Künzel et al. 2000). These regions are proposed to be equally accessible to map-based cloning (Gill et al. 1996). Furthermore, high recombination rates in distal chromosome regions have been demonstrated (Pedersen et al.1995; Künzel et al. 2000; Petes 2001). Summarizing this, we assume a good chance for an advantageous relation of cM to bp around the *def* locus for map-based cloning, although pea with about $3.8–4.8 \times 10^9$ bp (Ellis 1993) has a comparably big genome. Besides this we consider the possibility for a inter-species positional cloning strategy based on microsynteny between pea and the model legume *Medicago truncatula*, as shown recently (Gualtieri et al. 2002).

To speed up and to simplify the ongoing segregation analysis we developed sequence tagged site (STS) markers from the AFLPs tightly linked to the *def* gene. Thereby we faced the same difficulties to convert AFLPs into STS markers that have already been stated by various authors (e.g. Reamon-Büttner et al. 1998; Shan et al. 1999; Prins et al. 2001; Meksem et al. 2001). In our study we mainly had to deal with the loss of the original polymorphism after the specification process, due to wrongly analysed contaminating DNA fragments comigrating with the interesting polymorphic band, or due to point mutations at or within the restriction sites causing the AFLP. Therefore we could only convert 3 of 12 analysed AFLPs into STS markers.

Different strategies have been proposed to distinguish background fragments from the polymorphic sequence including Southern hybridization (Reamon-Büttner et al. 1998). Inverse PCR (Bradeen and Simon 1998; Wen et al. 2002), *Taq*Man analysis and BAC sequencing (Meksem et al. 2001) have been used to capture point mutations within or at the restriction sites for the design of STS primers.

We hereby report a simplified procedure to convert AFLP bands into single PCR products. If the direct way to convert an AFLP into a STS marker failed, we used primers corresponding to the original AFLP selective primers with an additional two up to five selective bases from the sequenced clone. The theoretical concept behind this is the following: each additional selective base will reduce the group of amplified fragments on average by 75%. If in total six selective bases (three on each original AFLP selective primer) lead to a maximum of 150 amplified bands just four additional selective bases more should reduce the number of amplified bands to a single band. The restriction-site polymorphism which is captured in the pre-amplification template can now be displayed as a single locus.

We named this as single-loci 'sequence-specified AFLPs' (ssAFLPs). The ssAFLP method allowed us to assign five additional clones to the initial AFLP bands and to analyse these interesting markers as single bands in normal agarose gels, instead of the time-consuming and costly AFLP polyacrylamide gels.

Any further analysis to convert ambiguous clones into STS markers requires time-consuming efforts without predictable results. Of course this may be reasonable in cases where the general importance of a marker locus is ensured, but as long as a marker locus is useful only in a limited field one has to find a balance in the expenditure and the benefit of an extended analysis. In our opinion the

ssAFLP offers a reliable and most of all a rather simple way to focus on the interesting polymorphism.

**Acknowledgements** We thank the plant breeding company Pajbjergfonden for kindly supplying the pea breeding line 'Pajbjergfonden 42403'.

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