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Identification of DNA amplification fingerprinting (DAF) markers close to the symbiosis-ineffective *sym31* mutation of pea (*Pisum sativum* L.)

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Abstract We demonstrate efficient genome mapping through a combination of bulked segregant analysis (BSA) with DNA amplification fingerprinting (DAF). Two sets of 64 octamer DAF primers, along with two PCR programs of low- and high-annealing temperatures (30°C and 55°C, respectively), appeared to be enough to locate molecular markers within 2–5 cM of a gene of interest. This approach allowed the rapid identification of four BSA markers linked to the pea (*Pisum sativum* L.) *Sym31* gene, which is responsible for bacteroid and symbiosome differentiation. Three of these markers are shown to be tightly linked to the *sym31* mutation. Two markers flanking the *Sym31* gene, A21-310 and B1-277, cover a 4–5 cM interval of pea linkage group 3. Both markers were converted to sequence-characterized amplified regions (SCARs). The flanking markers may be potential tools for marker-assisted selection or for positional cloning of the *Sym31* gene.

Key words DNA amplification fingerprinting · Pea · Symbiosis

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Introduction

Nitrogen-fixing symbiosis between legume plants and soil bacteria of the genera *Rhizobium* or *Bradyrhizobium* results in the formation of a new specific plant organ, the root nodule. Starting with the exchange of soluble signal molecules, the plant-microbe dialog continues via bacterial root colonization, infection, and penetration into the inner root layers, accompanied by an initiation of the nodule meristem and nodule differentiation. During the formation of the nodule, numerous plant and bacterial genes are differentially expressed, providing the complicated machinery for symbiosis establishment (Spaink 1995; Long 1996). Host-plant genes involved in nodulation are divided into two groups depending on their method of identification. The first group consists of *sym*-genes. These genes are known only by their mutation phenotypes and mode of inheritance; they are usually identified after experimental mutagenesis or by the screening of naturally occurring plant populations for genotypes with symbiosis abnormalities, followed by genetic analysis (Caetano-Anollés and Gresshoff 1991; Phillips and Teuber 1992; Brewin et al. 1993). Mutational study of the *sym*-genes allows for mapping and complementation tests and provides genetic data for the further molecular analysis. The other group of symbiosis genes are the nodulin genes (Van Kammen 1984), identified by methods detecting differential levels of mRNA in the presence or absence of bacterial infection and nodulation (Perlick and Pühler 1993; Vijn et al. 1993; Szczyglowski et al. 1997).

The cloning of *sym* loci has not yet been published, despite the isolation of a large number of symbiosis-deficient mutants in different legume species (Phillips and Teuber 1992; Gresshoff 1995). The most powerful method for the isolation of genes from complex genomes is positional, or map-based, cloning (Collins 1995). As an initial step for positional cloning, accurate

and detailed mapping of a gene of interest has to be performed. Several PCR-based techniques are currently being used for molecular mapping in plants. These methods use primers with: (1) arbitrary (RAPD, Williams et al. 1990; DAF, Caetano-Anollés et al. 1991), (2) semi-arbitrary (AFLP, Vos et al. 1995; S-SAP, Waugh et al. 1997) or (3) specific (microsatellite, Röder et al. 1995) structure. The BSA method was recently developed to search for linked markers by comparing PCR-products of pooled DNA from specific subsets of a segregating population (Michelmore et al. 1991). Bulked DNA from several plants homozygous for a given recessive mutation is compared to DNA from several phenotypically wild-type (homozygous wild-type and heterozygous) plants from the same population (Michelmore et al. 1991). After the BSA markers are found, the PCR-fragments are cloned and used to reveal standard RFLPs and/or converted to specific PCR-probes, SCARs (Paran and Michelmore 1993). These SCARs provide anchorage sites for gene cloning via the screening of high-molecular-weight DNA libraries and the generation of overlapping contigs for chromosome walking or landing (Tanksley et al. 1995).

Sym-mutations have been mapped in several legumes, including the soybean *nts-1* supernodulation mutation (Kolchinsky et al. 1997), the *in6* locus responsible for ineffective (non-fixing) symbiosis in alfalfa (Kiss et al. 1995), and several loci in common bean (Nodari et al. 1993). The largest number of mapped *sym*-mutations is available for pea (Brewin et al. 1993; Kneen et al. 1994; Kozik et al. 1995, 1996; Temnykh et al. 1995). While most pea *sym*-mutations are dispersed randomly throughout the genome, there is at least one region representing a cluster of a large number of symbiosis-related genes. This genomic region, named the *sym*-cluster, is located in the first linkage group of pea and contains the *sym*-genes *Sym-1*, *Sym-2* and *Nod-3*, as well as nodulin genes *Enod7*, *Enod40* and the leghaemoglobin gene (Weeden and Wolko 1990; Kozik et al. 1995, 1996; Temnykh et al. 1995). About 700 RFLP and AFLP markers, mapped in five recombinant inbred populations, are now available in pea (Hall et al. 1997); however, these molecular maps, do not contain any of the *sym*-mutations mapped elsewhere, except for the loci placed within the *sym*-cluster.

A single recessive Mendelian mutation in gene *Sym31* was discovered by Borisov et al. (1992) after screening ethyl methane sulfonate (EMS)-treated seeds of the pea laboratory line "Sprint-2". This mutant forms white root nodules lacking nitrogen fixation activity when inoculated by effective strains of *Rhizobium leguminosarum* bv *viciae* (Borisov et al. 1992). Ultrastructural analysis of these nodules showed abnormal symbiosome development with no bacteroid differentiation. Joint genetic analysis of *sym31* and *sym-13*, another mutation responsible for premature bacteroid degradation (Kneen et al. 1990; Borisov et al. 1997), revealed the primary role of the *sym31* mutation in this two-gene cascade, as developmental epistasis was observed (Borisov et al. 1997). One of the advantages of the *sym31* mutation for genetic and physiological studies is its clear and distinctive phenotype: the mutant forms relatively large white nodules in contrast to wild-type pink nodules. The *Sym31* gene was mapped in the 3rd linkage group, about 30 cM from the morphological marker *M* and 15 cM from the locus *uni*^{tac} (Rozov et al. 1995). Hence, a primary goal for the positional cloning of *Sym31* is the availability of more-detailed map information for this genomic region. Here we describe the effective coupling of BSA and DAF with the identification of flanking markers for the *Sym31* gene.

Materials and methods

Pea-plant culture and determination of the *Sym31* phenotype

The Sprint-Fix$^-$2 line, homozygous for the *sym31* mutation, was crossed with the tester wild-type line NGB 101238 (this line is identical to line JI73, John Innes Centre, Norwich, UK). Plants were grown in the greenhouse and the F_1 generation was self-pollinated to obtain the F_2 population. For inoculation a commercially effective *R. leguminosarum* bv *viciae* strain, CIAM 1026, was employed. Seeds were inoculated at planting by a bacterial suspension in water (10^7–10^8 cells per plant). The *sym31* mutant phenotype was determined as described by Borisov et al. (1992). The F_2 population contained 167 plants and segregated 128 (Fix$^+$):39 (Fix$^-$). Several leaves from every characterized F_2 plant were either frozen and stored at -70°C, or lyophilized and kept at room temperature. These leaves were used for DNA isolation.

Genomic DNA isolation and preparation of bulks

Plant DNA was isolated as described by Fulton et al. (1995). After dissolving the DNA in water, the concentration was determined using a TKO 100 fluorimeter (Hoeffer, San Francisco, Calif.). For DAF analysis, F_2 DNA was diluted to a concentration of 2.5 μg/ml. For preparation of the positive DNA pool, eight Fix$^+$ F_2 segregants were tested in the F_3. Since only 3–5 seeds were available for verification in some cases, there was a probability that some of those F_2 plants determined as wild-type homozygotes were actually heterozygotes. For this reason, our BSA screening was mostly aimed at the identification of markers linked in *cis* to *Sym31*. For each negative DNA pool, 12 homozygous recessive F_2 DNAs were mixed.

DNA amplification fingerprinting (DAF)

DAF oligos were synthesized by Research Genetics (Huntsville, Ala.). DAF analysis was performed as described by Caetano-Anollés and Gresshoff (1994). Briefly, for octamer primer screening, 10 μl of PCR mixture contained DAF reaction buffer [4 mM $MgCl_2$, 1 μl of 10 × Stoffel buffer (Perkin-Elmer, Norwalk, Conn.)], 200 μM of each dNTP (United States Biochemical, Cleveland, Ohio, USA), 3 μM of octamer primer, 5 ng (2 μl) of the bulked DNA and 2U of the Stoffel fragment of *Taq* DNA Polymerase (Perkin-Elmer, Norwalk, Conn.).

For mini-hairpin primer screening, the DAF reaction buffer consisted of 4 mM $MgSO_4$, 10 mM KCl, 4 mM $(NH_4)_2SO_4$, 0.1% Triton X-100 and 20 mM Tris-HCl, pH 8.3.

Low-annealing-temperature DAF (DAF-3 program) was performed in an Ericomp thermocycler with a heating-cooling transition speed of 14C°/min. The PCR profile consisted of 1 s at 96°C and 1 s at 30°C (no extension step was employed). After 35 cycles the DAF mixture was extended for 7 min at 72°C. High-annealing-temperature DAF (DAF-15 program developed originally by F. Ghassemi, University of Tennessee, Knoxville) was performed in a MJR thermocycler (MJ Research, Inc., Watertown, Mass.) using the following conditions: 3-min denaturation at 95°C, then 35 cycles of 30 s at 95°C, 2 min at 55°C and 30 s 72°C.

Two microliters of DAF products were separated in polyester-backed 10% polyacrylamide gels containing 7 M urea. Mini-Protean gel chambers (BioRad, Inc., Richmond, Calif.) were used for separation. Gels were run for 40 min. at 300 V and then silver stained as described by Bassam et al. (1991). After staining, the gels were placed in a solution containing 10% acetic acid, 35% ethanol and 1% glycerol for 10 min to prevent cracking, then they were dried and stored at room temperature.

Genetic-linkage analysis

Recombination frequencies were calculated for 90–100 F_2 segregants using MAPMAKER version 1.0 (Lander et al. 1987) or the Map Manager program (Manly et al. 1995) for a Macintosh computer. For-genetic linkage analysis a LOD of 3.0 was used, except for the hpA26-350 marker (because of the repulsion-phase status for this marker, only a LOD of 2.87 was acceptable for linkage calculations).

Isolation and cloning of DAF products

Each band of interest was excised from a still-wet polyester-backed polyacrylamide gel and incubated in 20 µl of TE at 95°C for 20 min. Two microliters of the eluate were employed for the subsequent amplification in a final volume of 10 µl using the original DAF primer. Three microliters of the DAF re-amplification mixture were used directly for cloning into vector TA pCR2.1 (Invitrogen, Carlsbad, Calif.). The 10-µl ligation mixture was diluted five times and dialyzed overnight against TE. Two microliters of the mixture were used for the transformation of competent *Escherichia coli* DH10B cells (Gibco-BRL, USA) by electroporation (PULSER equipment, BioRad Inc., Richmond, Calif.). Transformed cells were grown overnight on LB plates containing ampicillin, X-gal and IPTG in standard concentrations (Sambrook et al. 1989).

DNA sequencing and SCAR-primer construction

Cloned DAF markers were sequenced using the 373A Sequencer and the ABI Prism Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.). For every cloned DAF marker at least eight different recombinant clones were sequenced. Specific SCAR primers were chosen using the Primer Selection program of the University of Minnesota (http://alces.med.umn.edu/rawprimer.html) and synthesized by Integrated DNA Technologies (Coralville, Iowa). The SCAR primers for B1-277 were CCCATGATCCCTT GGAAGAAA (forward) and GCAGGCCCAATATTTTCTAAC-CAT (reverse). The SCAR primers for A21-310 were CGTTCCAT-GAGTCATAAAGCGAAT (forward) and CGTTCGATGGGAAT-GCAATTAGAG (reverse).

Results

A coupling of DAF and BSA

For the bulked segregant analysis of the region containing the *Sym31* gene, four octamer DAF oligonucleotide sets were used. Each set contained 64 primers with the following 5-nucleotide core sequences at the 5′-end: GCCCG (set *A*), GCAGG (set *B*), CCGAG (set *C*) and GATCG (set *D*). Sixty four 3′-end trinucleotide combinations were used to compile each corresponding 64 primer set. Two of these sets (*A* and *B*) were found to give reproducible and informative DAF patterns for approximately 55 primers each, whereas only about 20 primers from the *C* and *D* sets yielded informative DAF profiles (data not shown). This difference in amplification efficiency seems to be related to primer structure, as we obtained the same result for other legume (soybean and *Lotus japonicus*) and non-legume (potato and turfgrass) DNAs (data not shown).

Two thermocycler programs are currently employed in our laboratory for DAF, namely DAF-3 with a 30°C annealing temperature and DAF-15 with a 55°C annealing temperature. The profiles generated by DAF-3 and DAF-15 are shown in Figs. 1 and 2, respectively. Both programs yield 40–50 PCR bands (150–1000-bp in size) per gel lane when resolved by polyacrylamide gels and visualized by silver staining. Since the difference in annealing temperatures between these programs is 25°C, the resultant PCR products and the gel profiles produced differ dramatically. Assuming that changing the PCR program results in the amplification of different loci, the use of two programs instead of one should nearly double the number of screened genomic regions. The above combination of 128 primers, along with the two DAF programs, gives about 10 000 screened genomic loci. Given the average pea map length as 2000 cM (Hall et al. 1997), and assuming a uniform distribution of DAF markers within the genome, such screening should allow the location of a marker within a distance of 5 cM from any gene of interest.

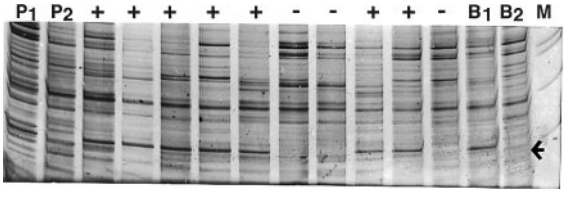

Fig. 1 DAF pattern for marker A21-310 (primer GCCCGTTTC) linked in *cis* to the pea *Sym31* gene. A low-annealing-temperature program (DAF-3) was employed. Marker location is shown by an *arrow*. DNA in lanes: P_1 wild-type parent; P_2 mutant parent; (+) Fix^+ F_2 segregants; (−) Fix^- (*sym31*/*sym31*) F_2 segregants; B_1 positive F_2 DNA pool; B_2 negative F_2 DNA pool; *M* Biomarker (BioVentures, Inc., USA)

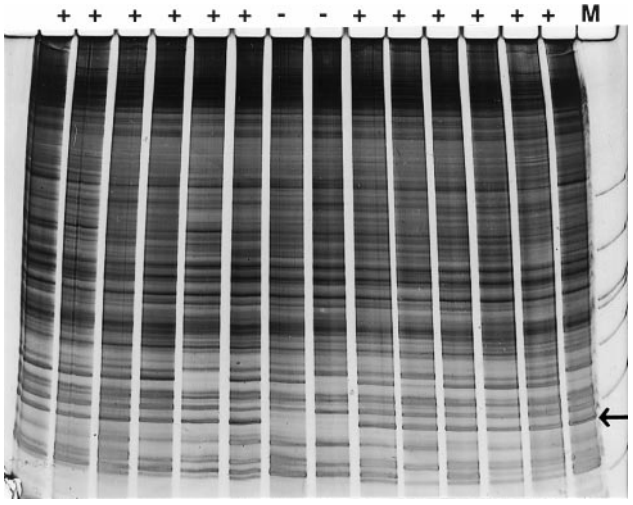

Fig. 2 Mapping of marker B1-277 (primer GCAGGCCC) in F_2 individuals. A high-annealing-temperature program (DAF-15) was used. Marker position is shown by an *arrow*. DNA in lanes: (+) Fix^+ F_2 segregants; (−) Fix^- (*sym31*/*sym31*) F_2 segregants. *M* Biomarker

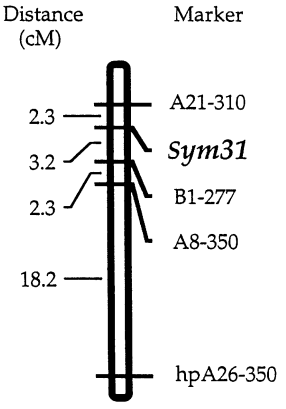

Fig. 3 Marker arrangement in the vicinity of the *Sym31* locus of pea constructed by MAPMAKER version 1.0 (Lander et al. 1987)

Identification of new markers linked to the *Sym31* locus

Homozygous F_2 DNA pools, prepared according to *Sym31* wild-type and mutant phenotypes, were pre-screened with the octamer primer set *A* (GCCCG-NNN sequence). As an alternative, the same pools were also pre-screened with the 60 mini-hairpin 11-mer primers recently suggested for DAF analysis (Caetano-Anollés and Gresshoff 1994). The mini-hairpin oligonucleotides increase the number of PCR-amplified bands by the formation of a terminal (5′) loop structure within the primer, which results in reduced annealing-specificity (Caetano-Anollés and Gresshoff 1994). The DAF-3 PCR program was used for comparative pre-screening. The mini-hairpin primer screening enabled us to obtain one marker, hpA26-350 (a 350-bp product obtained with primer hpA26, GCGAAAGCTGT), linked in *trans* to *Sym31* (data not shown). The linkage between the hpA26-350 marker and *Sym31* was determined to be 21 cM (LOD 2.87), and the marker was included in the final *Sym31* map (Fig. 3). Screening of the same F_2 DNA pools with octamer primer set *A* yielded a marker obtained with primer A21 (GCCCGTTTC). This marker, A21-310 (a 310-bp product), linked in *cis* to *Sym31* (Fig. 1), was placed 2.3 cM from the *Sym31* locus on the opposite side of *Sym31* to hpA26-350. Based on this comparative screening using mini-hairpins/octamers, only octamer oligonucleotides were employed in further experiments.

To identify markers located between *Sym31* and hpA26-350, new F_2 DNA pools were constructed. The new positive pool contained the *Sym31* wild-type DNA with the "no-band" phenotype for hpA26-350. The negative pool consisted of mutant *sym31* segregants possessing the hpA26-350 band. These two pools were screened with the *A* and *B* octamer sets using the DAF-3 program. In this analysis only markers linked in *cis* were analyzed. Screening identified the A8-350 marker (primer GCCCGCTA), which was placed 3.5 cM from *Sym31*. Since A21-310 and A8-350 appeared to flank the *Sym31* gene, the third set of F_2 pools was constructed according to the A21-310/A8-350/*Sym31* wild-type and mutant phenotypes. The pools, which theoretically differed only in the genomic region within the genetic window between A21-310 and A8-350, were compared using the same *A* and *B* primer sets and the DAF-15 program (high-annealing-temperature DAF). The screening resulted in the detection of marker B1-277 (primer GCAGGCCC), located in *cis* to the *Sym31* gene (Fig. 2).

The four identified DAF markers were mapped in 90–100 individual F_2 plants. The final genetic map of the vicinity of the *Sym31* locus, constructed by "MAPMAKER" (Lander et al. 1987) and confirmed by the Map Manager program (Manly et al. 1995), is shown in Fig. 3. In order to obtain SCARs from markers A8-350, A21-310 and B1-277, the corresponding bands were isolated from polyacrylamide gels, re-amplified and cloned using an improved DAF marker-cloning protocol (Men and Gresshoff 1998). Markers B1-277 and A21-310 flanking the *Sym31* locus were sequenced (GenBank Accession Nos. AF042741 and AF042742, respectively), and specific PCR primers to these sequences were synthesized. SCAR fragments were amplified from parental Sprint-2 Fix^- (*sym31* mutant) and NGB 101238 (wild-type) genomic DNAs. The B1-277 SCAR polymorphism revealed between the parents, as well as between Fix^- and Fix^+ F_2 segregants, is shown in Fig. 4. This SCAR represents a pseudo-codominant marker: only the 277-bp PCR product amplified from the wild-type parent is tightly linked to the *Sym31* locus. A 350-bp band amplified from the mutant parent did not show linkage to *sym31* (Fig. 4). Sequence analysis of the B1-277 and A21-310 sequences did not reveal significant homology to any sequence from the EMBL Data Library. Southern

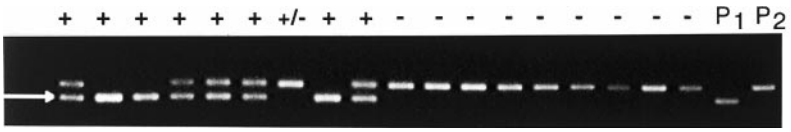

Fig. 4 SCAR PCR analysis of the B1-277 marker. The 272-bp band is shown by an *arrow*. The larger product (approximately 350-bp long) is not linked to the *Sym31* gene. DNA on the lanes: (+) Fix^+ F_2 segregants; (−) Fix^- (*sym31*/*sym31*) F_2 segregants; (+/−) recombinant F_2 plant (Fix^+ phenotype) lacking the 272-bp band; P_1 wild-type parent; P_2 mutant parent

hybridization of the A21-310 probe to the original DAF gel revealed four products of different size (data not shown), although the A21-310 SCAR amplified a single PCR product from both parents. Parental SCAR products were confirmed to be identical to the A21-310 DAF marker by direct PCR band sequencing (data not shown).

Discussion

We have demonstrated an efficient combination of BSA and DAF. BSA has proved to be a very effective strategy for plant genome mapping when coupled with AFLP and RAPD techniques, and has been applied to the identification of DNA markers linked with agronomically important genes in many plant species, including tomato (Thomas et al. 1995), potato (Ballvora et al. 1995), apples (Cheng et al. 1996), rice (Zhang et al. 1996), beet (Scholten et al. 1997) and soybean (Kasuga et al. 1997). The DAF method, developed by Caetano-Anollés et al. (1991) as an efficient and inexpensive PCR-based technology, shares general similarities with the RAPD technique (Williams et al. 1991). Furthermore, DAF allows one to obtain 6–10 times more PCR products per reaction than the RAPD method, giving 30–80 bands per primer with an average reproducibility of about 80%. DAF was shown to be a very effective technique for phylogenetic analysis, the genotyping of organisms, and the determination of genetic relationships (Baum et al. 1994; Prabhu et al. 1997). Many papers describe the utility of BSA for RAPDs. Since the effectiveness of BSA depends on the number of PCR bands produced per primer, DAF seemed to be an effective option to use with BSA. For large mapping-projects it is also important that DAF requires template DNA quantities as low as 1 ng per PCR mixture (usually larger amounts are needed for other PCR-based technologies).

We screened four octamer DAF oligonucleotide sets, containing 64 primers each. Two of the sets, *A* (GCCCG-NNN) and *B* (GCAGG-NNN), turned out to be the most effective using two DAF PCR programs, DAF-3 (30°C annealing temperature) and DAF-15 (55°C annealing temperature). This strategy allowed the identification of three markers, A21-310, A8-350 and B1-277, tightly linked in *cis* to the pea *Sym31* locus. A correlation between the number of scorable PCR bands and the probability of finding a linked marker was estimated by Martin et al. (1991). In our case 30–50 DAF bands per primer were scorable, resulting in the identification of one marker linked within 2–4 cM for every 70 tested arbitrary primers. This result is in close correlation with theoretical speculations (Martin et al. 1991). We have also used the same 128 octamers along with the DAF-3 and DAF-15 programs for a BSA in soybean, and have identified a DAF marker tightly linked to the supernodulation *nts-1* locus (Men and Gresshoff, in preparation). The data obtained shows that the DAF/BSA combination is generally applicable for plant genome mapping.

The *sym31* mutation blocks symbiosome development at early stages (Borisov et al. 1997; Sherrier et al. 1997; Dahiya et al. 1998). The *Sym31* gene was mapped by morphological markers in the 3rd pea linkage group (Rozov et al. 1995). Two other symbiosis-related loci were also mapped on the same linkage group; namely, the non-nodulating mutation *sym-7* (Kneen et al. 1994) and the early nodulin gene *Enod12* (Men et al. 1993). Both of these, however, are located on the other arm of the chromosome. In the present work we have identified DAF markers linked to the *Sym31* locus. The A21-310 and B1-277 markers flank a 5.5 cM genomic interval harboring the *Sym31* gene. These markers were converted into SCAR markers making them useful for other pea accessions. Furthermore, they can be used as anchoring probes for the physical mapping of the region, for further molecular characterization of the *Sym31* locus by BSA, as well as for effective selection for *Sym31* wild-type and mutant phenotypes without *Rhizobium* inoculation. The availability of a RFLP and AFLP map for pea (Hall et al. 1997) makes it possible to find additional tightly linked markers, decreasing the genetic "window" which contains the *Sym31* gene. Since the relationship between recombination (map units) and physical distance in this region of the pea genome has not yet been determined, it is difficult to predict the utility of the identified markers for positional cloning of the wild-type copy of *Sym31*. For instance, in tomato (950 Mb genome), the map-unit relationship varies from 100 to 2000 kb per cM (Paterson and Wing 1993). Recently published data have shown that, in a genomic region representing a hotspot for recombination, 1 cM may correspond to as low as 24 kb of tomato DNA (Gorman et al. 1996). In contrast, even for the small genome of *Arabidopsis* (140 Mb), some regions possessing suppressed

recombination may represent 400 kb/cM (Hardtke and Berleth 1996).

The ultimate goal of our work is the isolation of the *Sym31* gene by positional cloning. This strategy was first successfully applied to genes in humans (Collins 1995). Recently several plant genes, primarily involved in plant disease resistance, have been isolated by positional cloning (e.g., Martin et al. 1993; Song et al. 1995; Cai et al. 1997). The deduced protein sequences of the identified plant resistance genes share significant homology, such as leucine-rich repeats, nucleotide binding sites or serine/threonine domains (for a review see Staskawicz et al. 1995). Positional cloning of plant symbiosis-related genes (*sym*-genes) might reveal new classes of DNA sequences, which should provide a further understanding of fundamental developmental processes in plants, as well as symbiotic rather than pathogen-based plant-microbe communication. A large number of mapped and phenotypically characterized *sym*-mutations, along with an informative molecular map, make pea a suitable organism for the positional cloning of *sym*-genes, despite the fact that pea possesses a large genome and a relatively low transformation efficiency (Schroeder et al. 1993). The markers for the *Sym31* locus described here increase the possible candidature of pea for further molecular analysis of *sym* mutations. Another possible candidate region for the positional cloning of *sym*-genes in pea is the *sym*-cluster (the 1st linkage group) characterized by a set of molecular markers (Kozik et al. 1996).

The alternative strategy of using the model legumes *Lotus japonicus* and *Medicago truncatula* was recently suggested for the analysis of symbiosis- and mycorrhiza-related plant genes which are absent in the model plant *Arabidopsis thaliana* (Gresshoff 1995; Cook et al. 1997). These model legumes, with small genomes and high transformation capabilities (Stiller et al. 1997), should be very effective for the positional cloning of *sym*-genes equivalent to those found in legumes with low transformation frequencies (e.g., pea and soybean). For pea, the closest model seems to be *M. truncatula*, as it possesses the same type of indeterminate nodulation (Cook et al. 1997; Doyle et al. 1997). Therefore, an alternative strategy for the positional cloning of pea *sym*-genes lies in a comparative phenotypic and physiological analysis of mutants identified in pea (Borisov et al. 1992; Kneen et al. 1994; Temnykh et al. 1995) and *M. truncatula* (Benaben et al. 1995; Sagan et al. 1995; Penmetsa and Cook 1997), in order to establish homologous symbiosis mutations. For the *Sym31* gene, syntenic co-mapping of *M. truncatula* mutations along with the here-identified A8-350, A21-310 and B1-277 markers could lead to the discovery of a *Sym31* analog in this model legume.

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