STS Markers for Comparative Mapping in Legumes

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Abstract. DNA primers for 37 genes have been developed in pea (*Pisum sativum* L.). Two-thirds of these primers also amplify orthologous sequences in lentil (*Lens culinaris*). The primers were designed to be complementary to highly conserved sequences in exons of known genes. In addition, most of the priming sequences were selected to be 1000 to 3000 bp distant on the genomic DNA and to amplify a fragment that contained at least one intron. Segregating sequence polymorphism in mapping populations of recombinant inbred lines (RILs) derived from wide crosses in *Pisum* was observed by restriction of the amplified fragment with endonucleases recognizing four-base restriction sites. Successful mapping of 36 of these genes in pea demonstrated the utility of these primers for mapping, and it appears likely that the primers should have general utility for comparative mapping in legumes.

The location of genes on a linkage map is a process in which each new locus provides another tool for future studies. The development of DNA technologies has created a nearly limitless supply of molecular markers available in all plant species. Hence, the linkage maps for many plant species have gradually been developed to become powerful tools for genetic studies and crop improvement. In pea, a consensus linkage map with over 1000 markers has been assembled (Weeden et al., 1998). Over 800 of the markers on this map were polymorphic in the population of recombinant inbred lines (RILs) used to establish this map, providing a level of saturation greater than one marker per centiMorgan. Additional linkage maps have been developed for other pea crosses (Dirlewanger et al., 1994; Ellis et al., 1992; Gilpin et al., 1997; Irzykowska et al., 2001; Laucou et al., 1998; McCallum et al., 1997), making available literally thousands of DNA markers in pea.

Unfortunately the majority of these markers have limited utility for comparative mapping studies. Although the various pea maps can be compared with each other through a number of common anchor loci, most of the markers mapped in various crosses are RAPD markers or other markers that are difficult to transport between pea crosses. Furthermore, such markers can rarely be transferred to detect orthologi in crosses of related legume species such as lentil (*Lens culinaris* Medik.) or chickpea (*Cicer arientinum* L.). For such comparative mapping applications, conserved DNA sequences such as those found in the exons of many genes, appear to hold the greatest promise. Restriction fragment length polymorphism (RFLP) mapping of cDNA clones has traditionally provided good anchor markers, but the requirement for large amounts of DNA and the general involvement of radioisotopes complicates the routine application of this tech-

nique. With many DNA sequences now available in GenBank and other databases, DNA primers can be designed to specifically amplify a portion of a previously sequenced gene by means of the polymerase chain reaction (PCR), producing a sequence tagged site (STS) (Olsen et al., 1989). This approach may be more efficient for developing and identifying anchor markers. A convenient method for detecting STS marker sequence polymorphism is through the use of restriction endonucleases, to produce cleaved amplified polymorphic sequence or CAPS markers (Jarvis et al., 1994).

The CAPS approach has been used to locate genes in pea and other crops. Gilpin et al. (1997) designed primers to sequenced cDNAs obtained from etiolated pea seedlings and mapped a number of CAPS loci on the consensus map or linkage maps developed in other crosses. Schneider et al. (1999) obtained gene sequences from GenBank to develop gene specific primers for mapping in *Beta vulgaris*, again primarily by means of CAPS analysis. In these cases, the size of the PCR product was usually under 1000 bp and required a high degree of genetic diversity in the germplasm (or between the two parents) in order to obtain a polymorphism within the PCR product. Such an approach was most effective for wide crosses or for PCR fragments that contained rapidly evolving sequences. Both of these studies demonstrated that amplified products containing introns were more polymorphic than those containing only coding sequences.

We are interested in comparative mapping in the family Fabaceae and in the development of STS markers that potentially can be applied across different genera. Boutin et al. (1995) demonstrated that considerable conserved synteny existed between the linkage maps of *Phaseolus* and *Vigna* species and that some gene-order conservation also existed between the genera *Glycine* and *Phaseolus*. Additional synteny among legume genomes has been suggested by map comparisons between these genera and *Arabidopsis* (Lee et al., 2001) and *Medicago* (D.J. Kim, personal communication). Within the cool season legumes Weeden et al. (1992) estimated that at least 40% of known genes show syntenic locations between pea and lentil, and Simon and

Table 1. Primer Sequences and origin of primer design.

Gene	Symbol	GenBank accession no.[z]	Primer	Primer sequence
Acetohydroxy acid isomeroreductase	*Aair*	AJ251333	F	5' CACACATCCCGTTTCGCTC
			R	5' GAATGCCCCTTCTTCCTCAAG
Actin	*Ac3*	U81046	F	5' GCGATAATGGAACCGGAATGG
			R	5' TTCCTGTGTACTATTGATGG
Ascorbate peroxidase	*Apx1*	M93051	F	5' ATCGCTGAGAAGAAATGC
			R	5' CAAGAGGGCGGAATACAG
Apyrase S-type	*Apy*	AB038554	F	5' GCAATCACTTCCTCCCAATA
			R	5' CAAAATACATCAATCGCTCA
Beta-fructosefuranosidase	*bfruct*	X85328	F	5' ATCACTTTCAACCTCTCAAGAA
			R	5' TTCCCCATACAGCACCTTTAG
Protochlorophyllide reductase	*CipPor*	X63060	F	5' ACTGCTAAGGCTTTGGCTGA
			R	5' AGATTTTGTTAGGCTTGGATCACT
Constitutively photomorphogenic protein	*Cop1*	AJ276592	F	5' CGAACTTCCTGCTCGATAAG
			R	5' CCTTCATTGTCACTTCACAG
Wound-inducible P450 hydroxylase	*CYP82A1*	AF175278	F	5' GTCACGCCCTAAACCAGTC
			R	5' CAGCACACATTCTTCTCCCA
Diminuto	*Dimun*	D86494	F	5' AAGAGGAAGAAGATTTGGGT
			R	5' TCAAAGCCTGCTTCTGGGTA
Disease resistance response protein	*DRR49*	U31669	F	5' ATGGGTGTTTTTAATGTTG
			R	5' AGTTGTAATCAGGATGAG
Fructose bisphosphatase[y]	*Fbpase*	L34806	F	5' CCTTACTCTCCTTCACGTCT
			R	5' CTTTTCAACCTTCTCCACCT
Farnesyltransferase	*Ftase*	L08664	F	5' GGTGGATATGCTGGGGGA
			R	5' AGAATATATTGCTGTAAGGC
T protein of glycine decarboxylase complex	*gdcT*	AJ222771	F	5' AGATGGTTCCTTTTGCTGGT
			R	5' ATCTCAAATCCATCTTCACC
Gibberellin 2 beta-hydroxylase	*Gib2BH*	AF101383	F	5' TCTCCTCACAACCAATCAAGA
			R	5' CAAGCCTATTATCAGCCAACC
Glucose phosphate isomerase[y]	*Gpic*	AB044948	F	5' GAAGCGTGGAAGGATTTG
			R	5' CCATGAACCAGAGCGAA
Glutamine synthetase[w]	*Gsn1, Gsn2*	U28924	F	5' GACCCTGCAAACTTCCTA
			R	5' CCTCTCCATTGATGCCAC
Glutamine synthetase[x]	*Gsp*	U22971	F	5' GACGAATAACGGGGAAGTC
			R	5' ATGGAGCAATAGAGAGTG
Homeodomain protein	*Hop1*	AF063307	F	5' CTTACACTAACCCTTGTTCC
			R	5' CATCACCCACCCATTCTCACT
Heat shock transcription factor	*hsfA*	AJ010644	F	5' CTTCGTTCGTCAACTCAACACC
			R	5' AAAAACCCTTCATTCGCAAAT
Isovaleryl-CoA dehydrogenase	*ivdh*	AJ010946	F	5' CACTTCCTTCCTCTTTGACGAC
			R	5' AACCTGCGTTGCTCTTTCAG
Leghemoglobin	*Lb*	AB009844	F	5' CTTTAGTKAATAGCTCATGGG
			R	5' AGCAAAGCTTCTTTAACCAC
Superoxide dimutase[u]	*Sodmt*	M63003	F	5' GAACATGCCTACTACTTACAG
			R	5' CTCTCTTTCTCATATACTTCAC
Abscisic acid induced protein HVA 22	*P393*	Gilpin et al.	F	5' CTGGTTGGTCCTTCCTT
			R	5' AAAGAGTGACAAGAACC
Glyceraldehyde 3-phosphate dehydrogenase	*P628*	Gilpin et al.	F	5' ATGTGGTCTCAACTGAC
			R	5' AGCTATTGCAAACATGAC
Phenylalanine ammonia lyase	*Paal*1,2	D10002	F	5' TGGAAACAGTAGCAGCAGCC
			R	5' AAGAAATTGGAAGAGGAGCA
Phosphenolpyruvate carboxylase	*Pepcn*	D64037	F	5' GCAAAAGTGAGTGAAGATG
			R	5' GCTTGGAAAACATTAAGGGTTG
Phytochrome apoprotein	*phy*	X14077	F	5' CCTATTTTGGCAGTGGATGTTG
			R	5' ACTTCCGACTGATGTGTAGGCT
ADP-glucose pyrophosphorylase	*Rb*	X96766	F	5' ATGGCTTCTGGTTGTGTGAGCTTG
			R	5' TGCAGTTTCAAGGGAGAGGATATAG
Pectin methylesterase[x]	*rcpme1*	AF081457	F	5' GTTCAAAACTGTGGCTGAGGCT
			R	5' GTGTTCTGGTTTGGGTCTTCTC

Muehlbauer (1997) reported a similar percentage of synteny when comparing the genetic maps of lentil and chickpea.

The availability of complete sequences for many pea genes in GenBank has made it possible to design primers amplifying larger sequences that specifically include one or more introns, thus increasing the likelihood of detecting polymorphisms. Primer design to highly conserved regions in coding sequences also increases the likelihood that the same primers will function for comparative mapping. Such an approach was used by Brunel et al. (1999) to amplify orthologous sequences between *Arabidopsis thaliana* and *Brassica napus* in order to observe polymorphism in the size of the amplified fragment. Here we present primer sequences for 36 pea genes that amplify relatively long (>500 bp) PCR fragments. The fragments display polymorphism when digested with four-base recognition site endonucleases. These genes were successfully located on the pea linkage map using the revealed polymorphism.

Material and Methods

Primer sequences were generally obtained from gene sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/). Exceptions include two cDNA sequence (P393 and P628) reported by Gilpin et al. (1997). In general, we used a pea sequence as the basis for primer design, but determined the conserved nature of this sequence by comparison of the entire gene sequence with the equivalent gene in soybean (or some other legume) and in *Arabidopsis*. In most cases the size of the PCR fragment expected in pea could be predicted from the corresponding sequences, as well as the number and size of the introns. We attempted to choose priming sites that would generate a fragment at least 1200 bp long and to span more than one intron. In certain cases (*bfruct, DRR49, Gsp, Hop*1, *hsfA, rcpme*1 *and Rpl*22) the size of the gene or arrangement of introns precluded the amplification of a fragment >1000 bp. In several cases we were able to design primers that gave much larger PCR fragments. Most of the primers were designed by importing exon sequences from

GenBank into Primerfinder v.0.07 (http://eatworms.swmed.edu/~tim/primerfinder/) and searching for primers that were generally 18 to 22 bp long with annealing temperatures of 55 to 65 °C and minimal self-dimerization and hairpin properties. Exons that were revealed in BLAST searches to show considerable homology among legume genera were preferentially used for primer design when primers with suitable physical properties could be developed for them.

DNA was extracted from young leaves by a standard CTAB miniprep isolation procedure (Torres et al., 1993), and 1 mL of the isolated DNA was used as template for PCR. PCR was performed in 25 μL reactions containing 3 mM MgCl$_2$, 0.25 mM each dNTP and 0.8 mM each of the forward and reverse primers. For fragments with an expected product size <2000 bp, 0.5 units of Taq polymerase (Promega) were used. For longer fragments, 0.05 units of Accutaq LA (Sigma) was used as the thermostable polymerase. The amplification parameters were 94 °C for 2 min for the initial denaturation followed by 35 cycles of 30 s at 94°, 2 min. at the annealing temperature appropriate for the primer pair, and 2 min extension at 72 °C. The final extension was performed for 8 min at 72 °C. For longer fragments the extension time in each cycle was increased by 60 s for every 1000 bp >2000 bp. The amplifications were performed in either an MJ PTC-100 or MJ DNA Engine thermal cycler. Annealing temperatures for primer pairs were optimized individually by testing over a range of temperatures, and for some amplicons for which the expected product sizes were >2500 bp, other parameters were changed to achieve consistent amplification. Five microliters of reaction products was subjected to electrophoresis on a 1% (w/v) agarose gel in 1× TBE buffer to test for successful amplification, and 5 to 10 μL of the remaining PCR product was then digested in 25 μL reactions with restriction endonucleases with four-base recognition sequences to test for polymorphism among the parental lines. The restriction digestions used the buffer provided with each enzyme.

Two populations of recombinant inbred lines (RILs) were used to map the amplified sequences. The JI1794 x Slow F$_2$-

Table 1. Continued.

Gene	Symbol	GenBank accession no.[z]	Primer	Primer sequence
Ribosomal protein 22	*Rpl22*	M60951	F	5' CTCTCTCTTTAGCCATTAAC
			R	5' CTTCCTTGTCAGACTCATC
S-adenosyl-L-homocysteine hydrolase	*SAHH*	L36119	F	5' GATTGGGGTCCTGGTGGTG
			R	5' AAGAGCAGCAACCTTCTCATCAA
Sucrose synthase	*Ssyn*	AJ001071	F	5' GGGAAAGGGATTTTGCAAC
			R	5' AGGCAGGCTGMACGAAAGC
Alpha tubulin	*TubA1 2*	U12589	F	5' GGAAACGCCTGCTGGGAG
			R	5' AACAGTTGGAGGCTGATAAT
Unifoliate	*Uni*	AF010190	F	5' TCTCTCTACTGCACCATCTC
			R	5' CTACATTCCCTCCCGTCCAT
Vicilin J/K	*Vc2*	X67429	F	5' GCTGCTACTCCAATCAAACCG
			R	5' CTTCAGTAACAGTTACTATCAC
Vicilin	*Vc3*	X14076	F	5' ATGGCTGCTACTACAATG
			R	5' AACTGAAGATAGACGATC

[z]http://www.ncbi.nlm.nih.gov.
[y]Cytosol-specific form.
[x]Plastid-specific form.
[w]Nodule-specific form.
[v]After BLAST searching "short nearly identical sequences", sequences AF081457 (pectin methyl esterase) and X67425 (pectinesterase) proved to be more than 99% identical. The primers designed work for both genes.
[u]Mitochondria-specific (Mn-containing).

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Table 2. Annealing temperature, PCR product size, polymorphic enzyme and linkage map position of various STS primers in pea.

Gene symbol	Annealing temp °C	Expected PCR product size	Enzyme	Linkage group
Aair	60	2570	Hinf I, Hae III	VII
ApxI	63	1700	Alu I, Dde I	VII
Apy	62	2000	Dde I	V
bfruct	62	480	Mbo I	III
CipPor	65	1100	Alu I	III
Cop1	60	2000	Hae III	I
CYP82A1	60	1374	---	VI
Dimin	62	1200	Hae III	VII
DRR49	60	600	Hae III	VI
Fbpase	60	1700	Sau 96	V
Ftase	60	900	Hae III	II
gdcT	60	1360	Hinf I	IV
Gib2BH	60	1100	---	IV
Gpic	55	1500/1400	Hae III	VI
Gsn1	60	1350	Ase I	VII
Gsn2	60	1350	Ase I	VI
Gsp	60	900	Hae III	VI
Hop1	60	900	Hae III	VI
hsfA	62	514	Tru9 I	III
ivdh	60	1966	Hinf I	VII
Lb	60	1500/900	Dde I	I
Sodmt	60	1400	Rsa I	III
P393[z]	60	516	Hae III	IV
P628[z]	60	640	Hinf I	IV
Paal 1,2	60	2700	Dde	V
Pepcn	60	3500	Rsa I	III
phy	60	1650	Mbo I	II
Rb	60	3000	Mbo I	III
rcpme1	65	620	Alu I	V
Rpl22	60	900	Rsa I	VI
SAHH	63	1500	Hinf I	VII
Ssym	60	1850	Hae III	IV
TubA1	63	1370	Taq I	III
Uni	60	1250	Rsa I	III
Vc2	60	1612	Rsa I	V
Vc3	58	2364	Mbo I	III

[z]Isolated and designed by Gilpin et al. (1997).

derived RILs constitute the population of 53 lines used to generate the consensus map (Weeden et al., 1998). The MN313 x JI1794 F₂-derived RILs represent a related population of 51 lines that segregate for additional classical markers as well as for several genes influencing tolerance to common root rot (Przyborowski and Weeden, 2000). An initial screen for polymorphism among the amplified PCR products of the three parental lines was performed using the enzymes HinfI, HaeIII, RsaI, and DdeI. If these enzymes failed to reveal polymorphism, the following additional enzymes were included in the screen: AluI, CfoI, MboI, Sau96I, TaqI, and Tru9I. Once a polymorphism was observed between the parents, the RIL populations were scored, and the resulting segregation pattern was compared to those for previously mapped markers using the Quikmap EXCEL macro (N. Weeden and J. Barnard, unpublished). Confirmation that the sequences amplified and mapped were genuinely the gene sequences for which the prim-

ers were designed was achieved by comparing the restriction fragment patterns observed to those predicted from analysis of the GenBank sequences imported into Webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html). For most of the genes mapped, the restriction fragment sizes obtained from several endonucleases were examined In three instances, where gene duplications may have occurred, it was not possible to determine which paralogue was polymorphic and the subject of mapping.
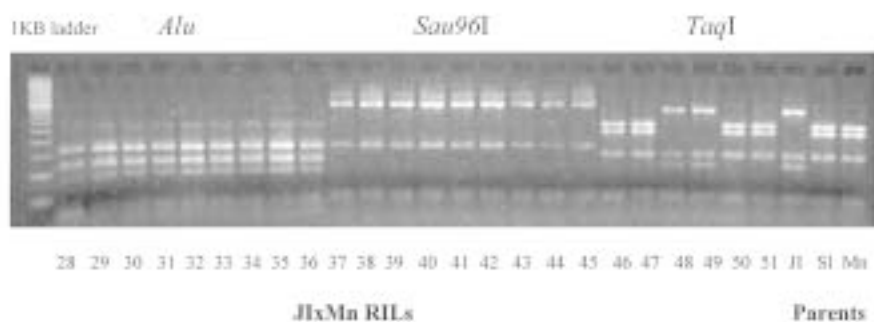
For analysis and mapping of STS markers in lentil we used an F₂-derived F₇ RIL population from the interspecific cross *L. culinaris* x *L. ervoides*. The F₃ generation of this population was used by Weeden et al. (1992) in their comparison of linkage relationships between pea and lentil. Morphological characters were scored again on the F₇ lines. The number of lines in the lentil RIL population (62) was sufficient that in many cases we could locate an STS marker on the map by comparing the F₇ segregation data of the STS marker with that for the homozygous genotypes in the F₃. In critical cases, the isozyme and DNA analysis on the F₇ population was repeated.

Results

The 36 primer sequences that successfully amplified a product of the expected length in pea are shown in Table 1. In most cases the initial primers worked well; however, in several cases (e.g., *CYP82A1*, *DRR49*, *Gib2BH*, *Gsp*, *Paal 1,2*, *Pepcn*, *Rb* and *Uni*) a number of primers had to be tested before an appropriate combination, generating a strong amplification product, was obtained. In several cases (*Gpic*, *Gsn*, *P393*) secondary fragments were observed. For *Gpic* and *P393* a single fragment predominated; however one other fragment displayed polymorphisms when cut with the endonuclease, and both could be mapped. For *Gsn*, the single band observed on 2% (w/v) agarose gels was found to consist of two fragments, both of which displayed polymorphism upon restriction. The annealing temperatures and amplicon sizes in pea for each primer pair are shown in Table 2.

Most primer pairs generated the same sized fragment in the three parental lines (JI1794, Slow, and MN313), at least at the resolving power of 2% (w/v) agarose gels. The identity of amplified products was confirmed by restricting the fragment with one or more endonucleases (Fig. 1) and comparing the size of the resulting subfragments with those expected based on the sequence in GenBank. All amplified fragments, except for that amplified by *ivdh* primers, gave the restriction patterns expected. The DNA fragment amplified by *ivdh* primers gave several

Fig. 1. Restriction fragment sizes of PCR products from primers designed for alpha tubulin (TubA1) match those predicted for the GenBank accession: *Alu*I (169, 231, 238, 327, 327), *Sau96*I (70, 325, 975), and *Taq*I (264, 482, 575). The polymorphism for *Taq*I indicates that JI1794 fragment lacks one *Taq*I site shared by Slow and MN313 and has an additional site.
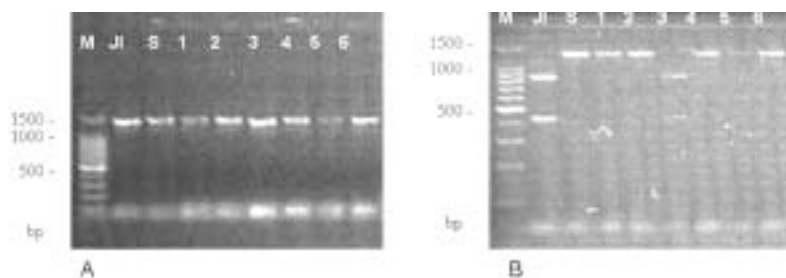

1KB ladder   *Alu*   *Sau96*I   *Taq*I

28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 JI Sl Mn

JIxMn RILs   Parents

Fig. 2. Agarose gels of products from amplification of pea DNA using the SODmt primers. (**A**) Fragments observed directly after amplification. (**B**) Fragments observed after cutting amplified fragments with *Rsa*I. M = molecular weight marker, JI = JI1794 parental line, S = Slow parental line, 1–6 = lines from the JI1794 x Slow RIL population.

additional fragments beyond those expected, suggesting the possibility that the primers have generated more than one over-lapping fragment.

CAPS polymorphisms were observed for most of the STS-PCR products, permitting the mapping of the respective genes in both pea RIL populations. Polymorphism in restriction sites was observed for nearly all fragments amplified from JI1794 DNA as compared to that of MN313 or Slow DNA. Insertion/deletion (indel) polymorphism**s** for *bfruct* and *Gib2BH* were directly observable without restriction digestion. Only the actin STS was monomorphic for all of the restriction enzymes tested. The expected fragment size, restriction enzyme used for mapping and the linkage group on which a given STS locus was placed can be found in Table 2. Figure 2 displays the segregation of a polymor-phism in pea for the sequence encoding Mn-containing superox-ide dismutase. In this case, the sequence in one parent (JI1794) contained a *Rsa*I site, whereas the sequence in the other parent (Slow) was not cut by *Rsa*I. Such a difference is easily resolved on 2% (w/v) agarose gels.

The loci were widely scattered across the linkage map, with at least two genes mapping to each of the linkage groups (Fig. 3). None of the poly-morphisms mapped outside the current ends of the link-age groups. The relative location of specific loci to anchor markers was the same in both RIL popula-tions, although linkage in-tensities between loci oc-casionally varied.

Twenty of the primer pairs were tested using len-til DNA as a template. Thir-teen of these generated amplification products of approximately the size ex-pected (Table 3). Mapping of the *Sodmt* fragment in lentil placed it in a similar region of linkage group III as the *Sodmt* in pea (J.G. Walling and N.F. Weeden, unpublished). Hence, we assume that the fragment generated in lentil was de-

rived from a gene ortholo-gous to that which we mapped in pea. We also examined linkage relationships between two pairs of genes (*Cop1/Lb* and *P363/P628*) in lentil that displayed relatively close linkage in pea. In lentil, *Cop*1 mapped within 5 cM of *Lb* and *P363* mapped ≈10 cM from *P628*. These results clearly establish two areas of linkage conservation between pea and lentil. One of these had been identified previously (Weeden et al., 1992), but the P363/P628 region had not been tested before in lentil.

Discussion

The approach we have used to locate genes on the pea and lentil linkage maps appears to be a method applicable in most species in which segregating populations from wide crosses are available. The 36 genes listed in Table 2 represent about two-thirds of the genes we have attempted to amplify using a CAPS analysis. Our success rate has been close to 100% when a sequence from pea is available. Design of primers from soybean or *Arabidopsis* sequences has proven more challenging, with a success rate of <50%. In certain cases appropriate primers have yet to be designed and the use of degenerate primers, as suggested by Brunel et al. (1999), might be a more satisfactory approach. In other cases (e.g., actin) the entire sequence within the gene appears to be highly conserved, and polymorphism could not be detected with a set of ten endonucleases. However, the large number of gene sequences, now or soon to be available, indicate that this approach will provide a sufficient number of markers to cover the genome at 5 to 10 cM saturation.

Fig. 3. Linkage map of pea based on the mapping populations JI1794 x Slow and MN313 x JI1794. The bar representing each linkage group extends to the most distal markers mapped on the respective group. Standard anchor markers are shown in small type. The STS markers listed in Table 2 are given in larger type, with their positions indicated by the heavier horizontal crossbars.
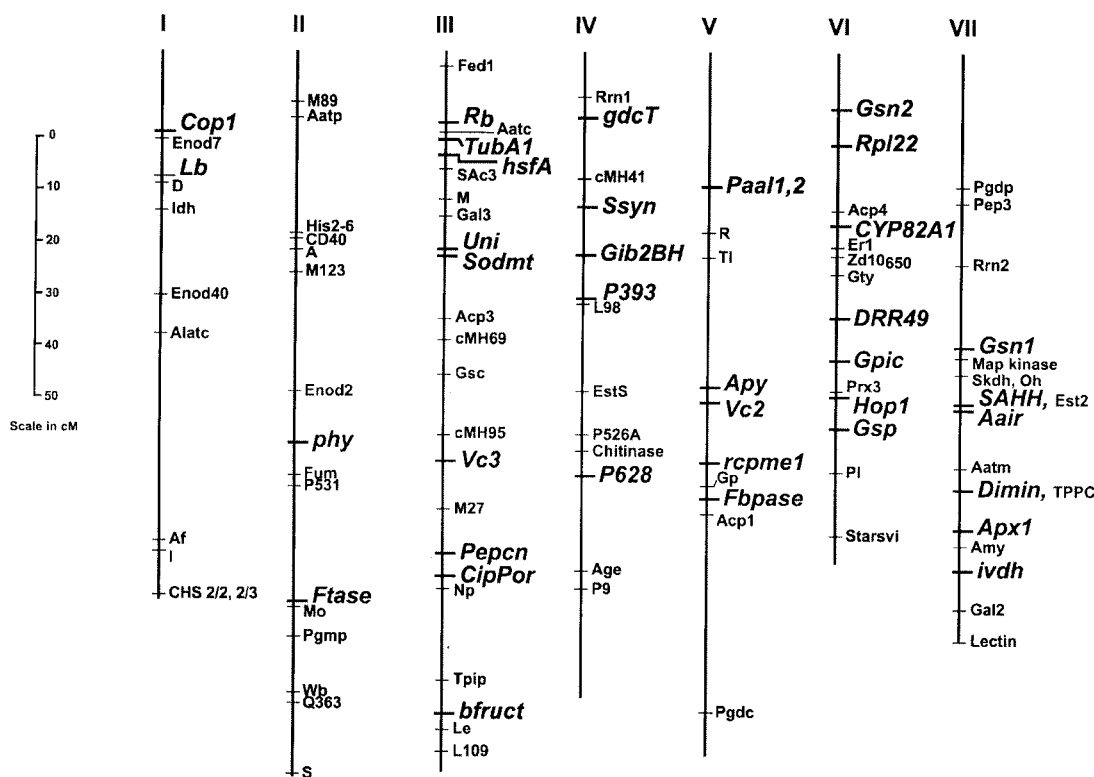
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Table 3. STS primers that successfully amplified products in lentil.

STS primer	Annealing temp (°C)	Fragment size
Cop	60	1000
P628	55	550 + 650
P393	55	550
Fbpp	55	1700
Pepcn	60	1300
MnSOD	55	1500
Paal 1,2	55	1500
Rcpme	60	300
Bfruct	60	500
Apx1	60	800
CipPor	60	1000 + 1100
TubA1	60	900 + 950 + 1500
Rpl22	60	900

For most of the STS amplicons we were able to confirm that the correct fragment had been amplified. The restriction mapping of the product proved to be the most efficient method for confirmation. However, in several cases the position to which the fragment mapped on the pea linkage map also provided evidence. The two glutamine synthetase amplicons mapped to positions on linkage groups VI and VII that matched the positions of *Gsn1* and *Gsn2* that had been previously mapped by RFLP analysis (Walker et al., 1995). Similarly, *Gpic* had been mapped to the same position on linkage group VI by isozyme analysis (Wolko and Weeden, 1990) and *Rb* is a well known morphological mutation mapping to the same position on linkage group III as the *ADP-glucose pyrophosphorylase* fragment (Rochat et al., 1995).

Several primer pairs developed from pea sequences did not generate a fragment of the expected size, or in some cases any fragment, when pea DNA was the template. In most of these cases the expected fragment size was >2000 bp. Apparently our ability to amplify sequences larger than 2000 bp was not reliable even when amplification conditions were modified to optimize for long PCR and commercial polymerases such as Platinum Taq (Gibco) or Accutaq (Sigma) were used. We had hoped to reliably amplify fragments up to 5000 bp in length as the opportunity for detection of polymorphism using restriction fragments would be increased. However, among the primer pairs we developed for fragments >3000 bp, only those for *Paal 1,2* consistently generated the desired fragment.

Two-thirds of the gene specific primers designed for pea amplified lentil sequences directly without further modification. Most of the primers were designed for conserved exon sequences, but in certain instances the conserved sequences did not yield primers with suitable properties and therefore primers that were optimal for pea were designed from less conserved sequences. Some of the sequences we amplified (*vicilin J/K, hsfA*) evolve relatively rapidly, and the priming sites may have undergone significant change since the divergence of pea and lentil. However other genes (*gdcT, Apy*) are relatively conserved but apparently also differed in the primer binding sites that were selected. In these cases, and in applications beyond borders of the tribe Viceae, primers with some degeneracy may work more efficiently. Alternatively, primers could be designed using the specific sequences for *Medicago truncatula, Glycine max*, or other appropriate taxa as more sequences become available. Several of the primer sets also amplified sequences in lupin (Przyborowski and Weeden, unpublished), suggesting that given an appropriate

mapping population in this crop, a test for linkage conservation between pea and lupin would be relatively easy.

Our approach appears to be more efficient at finding polymorphic markers than that used by Gilpin et al. (1997). Of the 18 STSs for which they report primers, nearly half did not show polymorphism or were not appropriate (generated multiple fragments) for comparative mapping. The two loci we used (*P393* and *P628*) are excellent markers, as are two others, *L109* and *Q363* which were not previously mentioned above. The primers reported by Schneider et al. (1999) have not worked particularly well in pea. Presumably the homologous priming sequences in pea contain too many nucleotide substitutions relative to gene sequences from the Brassicaceae to permit direct application of the primer sequences given by Schneider et al. (1999). We conclude that STS primer sets ideally should be developed from gene sequences obtained within the same family of flowering plants. In addition, the level of polymorphism observed using this approach has been somewhat disappointing in narrower crosses (within cultivated germplasm). The line JI1794 is an accession of *Pisum sativum* ssp. *elatius* and exhibits considerable genetic divergence from cultivated types. When different pea cultivars were tested for polymorphism using the STS markers and ten restriction enzymes, we often were unable to observe polymorphism. Thus, the CAPS approach does not appear to be highly applicable to breeding programs. We did not sequence the products from the various breeding lines we tested, and it is possible that there may be substantial sequence polymorphism such as single nucleotide polymorphisms (SNPs) which were not detected by the set of restriction enzymes we used.

With a set of STS markers, it should be possible to rapidly examine the changes that have occurred in the arrangement of genes in related genera. The approach is simple, requiring only PCR and agarose gel electrophoresis capabilities. Radiochemical labeling or denaturing polyacrylamide gels are not required, although considerably more genetic variation could be revealed using such tools. As described above, the technique can be applied in many laboratories with modest equipment and financial resources. Hence, the approach should be useful for the comparison of linkage maps in many minor legume crops such as grass-pea, lentil, field bean, chickpea, red clover, white clover, sweet-clover, bird's foot trefoil, tepary bean, mung bean, cowpea, winged bean, pigeon pea, lablab, indigo, lupin, sainfoin, yam bean, tamarind, bambara goundnut, derris, and guar.

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