



Genetic Resources

Amplification and Detection of Polymorphic Sequence-Tagged Sites in *Lathyrus sativus*

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Abstract. A simple procedure was developed to convert *Lathyrus sativus* defence-related expressed sequence tags (ESTs) into mappable genetic markers by using PCR. Twenty-nine STS primer pairs were generated on the basis of sequence information from an *L. sativus* cDNA library. These primers were used to screen for polymorphisms between 2 *L. sativus* accessions, ATC 80878 and ATC 80407, resistant and susceptible, respectively, to *Mycosphaerella pinodes* infection. All 29 primer pairs amplified PCR products in both accessions, 11 of which amplified multiple RAPD-like products. The remaining 18 primer pairs amplified single monomorphic products. Following cloning, sequencing, and database searches, 17 of 18 PCR products were confirmed to have amplified the targeted genome region. Ten of these 17 STS primer pairs revealed polymorphisms between ATC 80878 and ATC 80407 when PCR products were digested with a range of restriction endonucleases. These results suggest that the STS-based PCR analysis will be useful for generating informative molecular markers in *L. sativus* for future genome mapping experiments.

Key words: ascochyta blight, defence-related ESTs, grasspea, molecular markers, *Mycosphaerella pinodes*, sequence-tagged sites

Abbreviations: CAB, chlorophyll a/b binding; CAPS, cleaved amplified polymorphic sequence; EST, expressed sequence tag; SSR, simple sequence repeat; STS, sequence-tagged sites.

Introduction

Conventional detection of RFLP markers for genetic map construction by Southern hybridisation analysis is a slow, costly, and labour-intensive procedure. In recent years, marker technology has progressed from hybridisation-based RFLP markers to PCR-based markers of random amplification of polymorphic DNA (RAPD) (Williams et al., 1990), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), and sequence-tagged sites (STS) (Olson et al., 1989), which are economical to generate and quick and easy to assay.

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The large number of gene discovery programs conducted for a range of species has recently led to a dramatic increase in the number of expressed sequence tags (ESTs) deposited in public databases. EST databases are a potentially valuable source of genetic markers for constructing genome linkage maps. Because ESTs are derived from coding DNA, which generally has a high degree of sequence conservation, EST markers are more likely to be transferable across species boundaries compared with markers derived from nonexpressed sequences, such as AFLPs, RAPDs, and simple sequence repeat (SSR) markers.

Earlier methods for mapping ESTs involved RFLP analysis by using ESTs as hybridisation probes. A more efficient approach is to use ESTs to design specific PCR-based primers to amplify sequence-tagged sites (STSs), followed by restriction endonuclease analysis to develop cleaved amplified polymorphic sequence (CAPS) markers, also known as PCR-RFLP markers. Such markers have been successfully developed for Sugi (*Cryptomeria japonica*) (Iwata et al., 2001), field pea (Gilpin et al., 1997), *Pinus* (Cato et al., 2001), and barley (Mano et al., 1999). The advantages of STS or CAPS markers over other marker systems are that they are quick and easy to assay and that they require little DNA. They are robust, as an amplified product is always obtained, and they represent real functional genes because they are developed from STS primers designed from cDNA/ESTs. CAPS markers are also codominantly expressed, unlike RAPDs, AFLPs, and some SSRs, which are dominant markers.

A major limitation in developing STS or CAPS markers is the cost and labour of extensive sequence information required to synthesize appropriate gene-specific primers and identify potential polymorphic restriction sites. However, from the construction and partial characterisation of the *L. sativus* cDNA library (Skiba, 2003), the sequence information needed to develop primers to amplify *L. sativus* STSs already exists.

L. sativus L., commonly known as grasspea or chickling pea, is an important pulse crop grown as a rich protein source for stock feed and human consumption in drought-prone areas of Africa and Asia (Campbell et al., 1994). *L. sativus* is a hardy plant, tolerant to drought, flood, and moderate salinity, and it assists in maintaining soil fertility by fixing atmospheric nitrogen (Campbell et al., 1994). *L. sativus* is reportedly resistant to ascochyta blight, which is primarily caused by *Mycosphaerella pinodes* (Berk. and Blox.) Vestergren (Weimer, 1947; Gurung et al., 2002). Given the close genetic relationship, *L. sativus* may serve as a potential source of resistance genes to ascochyta blight resistance in field pea (*Pisum sativus* L.) breeding programs.

In this report, sequences of 28 defence-related ESTs and 1 chlorophyll a/b-binding (CAB) EST identified from an *L. sativus* cDNA library were used to develop STS primer pairs to amplify STSs in genomic DNA from 2 *L. sativus* accessions, ATC 80878 and ATC 80407, resistant and susceptible to *M. pinodes* infection, respectively. Polymorphisms were detected between amplified products following restriction endonuclease digestion. This demonstrated the usefulness of STS-based PCR analysis for generating informative molecular markers in *L. sativus* for future genome mapping studies.

Material and Methods

Solutions

- *Taq* polymerase (Invitrogen, Australia)
- 1 × TBE: 10.8 g Tris, 5.5 g boric acid, 0.93 g EDTA per litre
- LB/ampicillin broth: 10 g tryptone, 5 g yeast extract, 5 g NaCl per litre, pH to 7.0 with NaOH, 100 µg/mL ampicillin
- ABI Prism BigDye Terminator Sequencing Ready Reaction Mix (PE Biosystems, USA)
- Restriction endonucleases
- Loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose
- 2% agarose gel: 2 g agarose, 100 mL 1 × TBE

DNA isolation

Seeds of resistant and susceptible *L. sativus* (accession ATC 80878 and ATC 80407, respectively) were obtained from the Australian Temperate Field Crop Collection (ATFCC), Horsham, Victoria, Australia. Accessions were subjected to several generations of inbreeding to produce homozygous breeding accessions prior to experimental use. Leaf and stem tissues were collected from inbred plants of both accessions from which genomic DNA was extracted by using a modified CTAB method (Taylor et al., 1995).

STS primer design

Primer pairs were designed on the basis of the sequences of 28 defence-related clones and 1 CAB cDNA clone from an *L. sativus* library (Skiba, 2003) by using the program Primer3 (Rozen and Skaletsky, 1998). Designed primers were 18-27 bp long, possessing minimal complementarity and minimal internal duplex formation, and they were synthesised by GeneWorks Pty Ltd. (Australia) (Table 1).

Amplification of genomic DNA fragments by using STS primer pairs

Genomic DNA extracted from both *L. sativus* accessions were used as templates for PCR amplification by using the STS primer pairs (Table 1). PCR amplifications were performed in 50-µL reactions containing 1 U of *Taq* polymerase, 5 µL 10 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1 mg/mL gelatin [pH 8.3]), 0.24 mM of each dNTP (Promega, USA), 0.4 µM of each of the forward and reverse primers, 2.5 mM MgCl₂, and 60 ng of genomic DNA template. A negative control containing no DNA was used in every set of reactions to test for contamination.

PCR reactions were performed in a PCRExpress thermal cycler (Thermo Hybaid). The cycling parameters for amplifying DNA regions with the STS primers were 1 cycle of initial denaturation at 94°C for 1 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 41-65°C for 1 min, and extension at 72°C for 1 min; and a final extension cycle at 72°C for 5 min. The optimal annealing temperature for each primer pair was based on the ability to amplify a single fragment/product from the genomic DNA. For primers that did not amplify a

Table 1. Sequences and sources of the 28 defence-related and 1 chlorophyll *a/b*-binding (CAB) EST primer pairs.

Primer Name	Sequence (5' → 3')	EST
2F	CTG AGC TGG TTG GTG TGA	LS0002: Subtilisin inhibitor
2R	ATT GAA GGG AAA AGA AAA GAC A	
24F	CTC ACC ACC ACC GCA CAT AC	LS0024: Disease resistance response protein DRRG49 C
24R	GAA ACC AAC CTT CAA AGT TCA GA	
58F	TTG CCC CAT CCA AGT GTT	LS0058: Putative chitinase
58R	GGG GGA CAA CAA AAA TG	
59F	CAA ACA CAC ATA GCA TAT TAA GTG AA	LS0059: Disease resistance response protein 39 precursor
59R	CCA TAA ATG AGA AAG AAA ATG GA	
81F	GGT GAC AAA TAC TGC AAC TGG	LS0081: Pathogenesis-related protein 4A
81R	ACG AAA TGA TAT GCC TTG TTT T	
159F	GCT TGA AGG GTT TTG ATG GT	LS0159: β -glucan-binding protein
159R	ACA GAG GTT TAT CGT CAT TTT TCT C	
185F	CAA AGG AGA TGC TAA GCC C	LS0185: Pathogenesis-related protein
185R	ACT AAA TTC AAC AAA ACA AAA GCA	
246F	TCA TCT TCA ACT TGT TTG GGG	LS0246: Putative β -1,3-glucanase
246R	AGA AAA GGC GAT TCG TTT GA	
304F	TCC GAA AAA CAA TGG AAG GA	LS0304: Cutinase negative acting protein
304R	TCT CCG ATT CAG GGT AGC A	
342F	TCA GAT GCA TTT ATG AAC ACT ATT TTA	LS0342: Glutathione peroxidase
342R	CCA GGA TCC AGG GAG AAA	
351F	GGG ACC AAA CAA AAC CAA AA	LS0231: Lipid transfer protein
351R	TCA GTA AGT AGC CAA GCC AAT C	
465F	CAG CGG GTC AGG GAG AAA	LS0465: Protein kinase-like (serine/threonine kinase PBS1)
465R	ACT ATA AAA TGA TGT AGG AGA AAA CCA	

Table 1 (continued).

Primer Name	Sequence (5' → 3')	EST
467F	GCA GTT GAA GGT TAC CGT ATT G	LS0467: Chlorophyll <i>a/b</i> -binding (CAB) protein
467R	CCA TGC AIT GTT GTT GAC TG	
524F	GAG GGC CAT TGT GCA AGT	LS0524: S-adenosylmethionine synthetase 2
524R	TCC CAT TTA AGA GGC TTC ACC	
574F	AAA CAT CTG ATC CAT GAT TAC AAA A	LS0574: Cf-9 resistance gene cluster
574R	CAG AGT ACG CCG GTG GAG	
612F	AAC CGC CGA TGT GCT TTT	LS0612: Laccase-like protein
612R	TTT TCC CTT GGT GAT TTT GG	
616F	GGG CTC TTA CAC CGG AAT CT	LS0616: Putative auxin repressed protein
616R	CAT CCC GCC AAG CAA AAC	
674F	GGG ATG ATG AGG CTG TTC TG	LS0674: Putative WD-repeat protein
674R	AAA AGG CCT GGG GAT CAT T	
753F	CTG ATG AGA AGT TCA CTC GTT TG	LS0753: TMV resistance protein homolog
753R	CTC CAG CAC CAA ATC CAT AA	
761F	GAT GCT TCA GTG TTG TTT GGT	LS0761: Polygalacturonase inhibitor protein
761R	ATA CAT TTT TAT TTT ATG GTA GAT GCC	
786F	TCC TAC TGG GAT TGA CAG TGA	LS0786: Multiresistance protein
786R	TTG GAA AAA GAA AGA AAC CG	
787F	TCC TTC TAT GAT GCC TGG TG	LS0787: 6-Phosphogluconate dehydrogenase
787R	GAA AAC CAA AAT CCC AGA AAT	
792F	TCA TCT GGA ATT CAT AAA AAT GG	LS0792: EREBP-4
792R	AAG GTA GGC TTG GGA TAA GTT C	
834F	CAC AGT TCA AGA AAA CTG AAA AAT ACA	LS0834: Seven in absentia-like protein
834R	TGC TTT GGC CCA ATA TTT CT	

Table 1 (concluded).

Primer Name	Sequence (5' → 3')	EST
896F	TCC CCA CAA GGG GTA CAA	LS0896: PRIA precursor
896R	GGA GCC CAA TCG GAG AGA	
923F	CTG TGA CGA TCA CTG CAA GA	LS0923: Defence-related peptide 1 (PSD1)
923R	AAG ATA AAA GTG CAT AAC CAG TAG G	
942F	CCA ACC AAC CAA ACC AAG A	LS0942: Calmodulin-binding protein/ER66
942R	AGA CGG TCA TCT GAA TTG ATA TTT T	
993F	CAC GTA CAA TTC AGA AAC AAC ACA	LS0993: Disease resistance response protein 230 precursor
993R	GCA GTG GTA ACA ACG CAG AG	
1005F	ACC TTG TTC TCC CAG CTC TC	LS1005: Chalcone reductase
1005R	GGC CAA CTG CCT TAT TCA AA	

single product, lower annealing temperatures were used so that RAPD-like polymorphic bands between the resistant and susceptible accessions could be identified. Aliquots of 5 μ L of each PCR amplification were analysed by means of electrophoresis through 2% agarose gels in 1 \times TBE and then stained with ethidium bromide. Products were visualised with UV light. Each PCR reaction was repeated to verify reproducibility of amplification profiles.

Cloning and sequencing of PCR products

Single monomorphic products, amplified from ATC 80878 and ATC 80407 genomic DNA, were cloned into pGEM-T Easy Vectors (Promega, USA) and transformed into *Escherichia coli* JM109 Competent Cells. Transformed cells were cultured in LB/ampicillin broth, from which plasmid DNA was isolated by using the QIAprep Miniprep Kit (QIAGEN, Australia). Subsequently, plasmid DNA containing inserts was used as a template for sequencing. To eliminate cloning and sequencing artefacts, a qualitative control measure was employed that involved sequencing 2 duplicate clones representing each PCR product. Each clone was sequenced twice, once from the 5' end (using the T7 promoter primer) and once from the 3' end (using the SP6 promoter primer) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Protocol (PE Biosystems, USA) and an ABI Prism 377 DNA Sequencer (Griffith University DNA Sequencing Facility, Queensland, Australia).

Sequence analysis of STS products

To obtain an accurate sequence for each product amplified from the ATC 80878 and ATC 80407 plants, the forward and reverse reads of each duplicated product were compared by using the Reverse (Genetics Computer Group Inc., 1998) and PileUp (Genetics Computer Group Inc., 1998) programs available in the BioManager software portal (www.biomanager.angis.org.au). To ensure that the correct product was amplified and cloned for the STS primer used, clones of each product were identified by using Identify an Unknown DNA Sequence (Altschul et al., 1997). The PileUp program was used to align the sequences of the amplified products from ATC 80878 and ATC 80407 for each STS primer pair to determine if any sequence variations existed, which may be exploited to detect polymorphisms between the 2 accessions.

Detection of polymorphisms

To detect polymorphisms between ATC 80878 and ATC 80407, single monomorphic products amplified by STS primers were digested with a range of restriction endonucleases: *Acc* I, *Alu* I, *Apa* I, *Apo* I, *Bam*H I, *Bcl* I, *Bgl* I, *Bsa*J I, *Bsr* I, *Bst*X I, *Dde* I, *Dpn* I, *Eco*R I, *Hae* III, *Hha* I, *Hind* III, *Hinf* I, *Hpa* I, *Kpn* I, *Mbo* I, *Mlu* I, *Mnl* I, *Msp* I, *Nar* I, *Nci* I, *Nco* I, *Nla* IV, *Not* I, *Pst* I, *Pvu* I, *Pvu* II, *Rsa* I, *Sal* I, *Taq* I, *Xba* I, and *Xho* I. Restriction digest reactions were performed in 0.5-mL thin-walled PCR tubes containing 5 μ L of the PCR STS amplified product, 1 μ L restriction enzyme buffer, 1 U restriction enzyme, and Milli-Q water to a final reaction volume of 10 μ L. Where applicable, BSA was added to the restriction digest reaction to a final concentration of 0.1 mg/mL. Digestion

Table 2. STS amplicons produced from ATC 80878 (R) and ATC 80407 (S) by using the 29 STS primer pairs.

STS Primer Pair Number	Optimal Annealing Temperature, °C	Expected Product Size, bp	Amplified Product Size, bp	Nucleotide/Function Match	Accession No. ^b	E Value
2	64	326	~320	Subtilisin inhibitor	ICIS_VICFA	R ^c : 4e-08 S ^d : 1e-07
24	41	664	MP ^a bands	-	-	-
58	54.4	400	MP bands	-	-	-
59	58.3	470	~600	Disease resistance response protein 39	PR39_PEA	R: 2e-26 S: 2e-26
81	56.4	249	~250	Pathogenesis-related protein 4A	Q9M7D9	R: 6e-23 S: 1e-22
159	58.3	279	~300	β -glucan-binding protein	Q9FST0	R: 0.0003 S: 0.0002
185	58.3	316	~320	Pathogenesis-related protein	Q39450	R: 2e-13 S: 2e-10
246	58.3	617	~800	Putative β -1,3-glucanase	Q9ZQG9	R: 8e-05 S: 7e-05
304	43	575	MP bands	-	-	-
342	43	595	MP bands	-	-	-
351	56	584	~1100	Lipid transfer protein	O22110	R: 4e-06 S: 4e-08
465	58.3	300	~300	Protein serine/threonine kinase PBS1	Q9FE20	R: 2e-06 S: 2e-09
467	60.3	258	~260	Chlorophyll <i>a/b</i> -binding protein	CB2D_LYCES	R: 2e-44 S: 1e-38
524	62.6	267	~270	S-adenosylmethionine synthetase 2	Q9FPL6	R: 2e-43 S: 3e-36

Table 2 (concluded).

STS Primer Pair Number	Optimal Annealing Temperature, °C	Expected Product Size, bp	Amplified Product Size, bp	Nucleotide/Function Match	Accession No. ^b	E Value
574	41	236	MP bands	-	-	-
612	54	559	~560	Laccase-like protein	Q9LFD2	R: 3e-38 S: 4e-34
616	50	544	MP bands	-	-	-
674	58	616	MP bands	-	-	-
753	58.3 - 64	600	~600	TMV resistance protein	Q9FHX1	R: 0.0001 S: 0.0002
761	58.3	480	~500	Polygalacturonase inhibitor protein	Q9XHD6	R: 2e-35 S: 2e-38
786	42	506	MP bands	-	-	-
787	56	1200	~1100	6-phosphogluconate dehydrogenase	Q4O311	R: 3e-55 S: 2e-42
792	41	555	MP bands	-	-	-
834	58.3 - 62.3	494	~500	Seven in absentia-like protein	Q9M359	R: 5e-42 S: 2e-49
896	60	514	MP bands	-	-	-
923	58.3	230	MP bands	-	-	-
942	56	600	~600	Calmodulin-binding protein	Q9FPR9	R: 1e-05 S: 3e-13
993	64	367	~800	Xylose isomerase	XYLA_HORVU	R: 1e-17 S: 2e-20
1005	64	900	~1100	Chalcone reductase	Q41399	R: 3e-06 S: 3e-08

^aMP indicates multiple polymorphic bands.^bAccession number assigned by GenBank, SwissProt, or SpTtEMBL database.^cR indicates resistant parental sequence (ATC 80878).^dS indicates susceptible parental sequence (ATC 80407).

Table 3. Results from restriction endonuclease digestions of products amplified by STS primers from ATC 80878 and ATC 80407.

STS Primer Pair Used	Enzymes That Did Not Digest PCR Products	Enzymes That Digested PCR Products	Enzymes That Produced Polymorphic Banding Profile
2	<i>Hae</i> III, <i>Msp</i> I, <i>Xba</i> I, <i>Mlu</i> I GQ, <i>Nl</i> IV, <i>Mnl</i> , <i>Nci</i> I	<i>Bsr</i> I, <i>Hinf</i> I, <i>Hpa</i> I, <i>Taq</i> I, <i>Apo</i> I	<i>Apo</i> I
59	<i>Hae</i> III, <i>Msp</i> I	<i>Hinf</i> I, <i>Dde</i> I	<i>Hinf</i> I
81	-	<i>Hae</i> III	<i>Hae</i> III
159	<i>Msp</i> I	<i>Hae</i> III, <i>Alu</i> I	<i>Hae</i> III
185	<i>Rsa</i> I, <i>Nar</i> I, <i>Hind</i> III, <i>Dpn</i> I, <i>Apa</i> I, <i>Bgl</i> I, <i>Nco</i> I, <i>Hinf</i> I, <i>Hha</i> I, <i>Hpa</i> I, <i>EcoR</i> I, <i>Nci</i> I, <i>Bsa</i> I, <i>Apo</i> I	<i>Hae</i> III, <i>Bcl</i> I, <i>Mnl</i> I, <i>Mbo</i> I, <i>Dde</i> I	NA
246	<i>Bam</i> H I, <i>Kpn</i> I, <i>Dpn</i> I, <i>Sal</i> I, <i>Nla</i> IV, <i>Alu</i> I, <i>Taq</i> I, <i>Not</i> I, <i>Pvu</i> I, <i>Nci</i> I, <i>Xba</i> I, <i>Hha</i> I, <i>Pst</i> I, <i>Apa</i> I	<i>EcoR</i> I, <i>Rsa</i> I, <i>Msp</i> I, <i>Nco</i> I, <i>Hae</i> III, <i>Apo</i> I, <i>Bsa</i> I, <i>Hinf</i> I, <i>Dde</i> I, <i>Hpa</i> I, <i>Mnl</i> I	NA
351	<i>Acc</i> I, <i>Hae</i> III	<i>Hinf</i> I, <i>Dde</i> I	<i>Dde</i> I
465	<i>Bst</i> XI, <i>Nci</i> I, <i>Bsa</i> I, <i>Nla</i> IV	<i>Msp</i> I, <i>Mni</i> I, <i>Hinf</i> I	NA
467	<i>Dpn</i> I	<i>Bsa</i> I, <i>Nla</i> IV, <i>Dde</i> I, <i>Hind</i> III	NA
524	<i>Apa</i> I, <i>Nla</i> IV	<i>Hae</i> III, <i>Mnl</i> I	<i>Mnl</i> I
612	<i>Msp</i> I, <i>Nci</i> I, <i>Hha</i> I	<i>Kpn</i> I, <i>Rsa</i> I, <i>Hinf</i> I, <i>Xho</i> I, <i>Taq</i> I, <i>Nla</i> IV	<i>Kpn</i> I, <i>Rsa</i> I, <i>Xho</i> I, <i>Taq</i> I
753	-	<i>Rsa</i> I	<i>Rsa</i> I
761	<i>Kpn</i> I	<i>Hinf</i> I, <i>Dde</i> I, <i>Taq</i> I, <i>Hha</i> I, <i>Mnl</i> I	NA
787	<i>Hind</i> III, <i>EcoR</i> I, <i>Nci</i> I, <i>Hha</i> I, <i>Apo</i> I, <i>Bsa</i> I	<i>Bam</i> H I, <i>Dde</i> I, <i>EcoR</i> V, <i>Rsa</i> I, <i>Acc</i> I, <i>Msp</i> I, <i>Pvu</i> II, <i>Kpn</i> I, <i>Mnl</i> I, <i>Sal</i> I, <i>Nla</i> IV	<i>Mnl</i> I
834	<i>Xba</i> I, <i>Msp</i> I, <i>Dpn</i> I, <i>Nco</i> I, <i>Hae</i> III, <i>EcoR</i> I, <i>Kpn</i> I, <i>Hha</i> I, <i>Bsa</i> I, <i>Nci</i> I, <i>Apo</i> I	<i>Mnl</i> I	NA
942	<i>Msp</i> I, <i>Nci</i> I	<i>Dde</i> I, <i>Rsa</i> I, <i>Nla</i> IV, <i>Mnl</i> I, <i>Bsa</i> I	NA
1005	<i>Dpn</i> I	<i>Rsa</i> I, <i>Dde</i> I, <i>Hae</i> III	<i>Rsa</i> I

reactions were incubated for 2 h at the manufacturer's recommended temperature. Restriction profiles were analysed on 2% agarose-gels, as previously described. Restriction digests that produced polymorphisms were repeated to verify reproducibility of digestion profiles.

Results and Discussion

Amplification of STSs from ATC 80878 and ATC 80407 genomic DNA

Of the 29 STS primer pairs assessed, 18 amplified single monomorphic bands, in

both ATC 80878 and ATC 80407 (Table 2). Amplicons ranged in size from 250-1200 bp. Suitable PCR conditions were obtained by varying the annealing temperature. The remaining 11 primer pairs amplified multiple bands (Table 2), even under the most stringent amplification parameters. However, some primer pairs produced amplification products that were polymorphic between ATC 80878 and ATC 80407. Although primers were designed from specific sequences, multiple copies of these genes or closely related sequences may exist in the *L. sativus* genome, which may explain the amplification of multiple products. However, such polymorphisms observed between ATC 80878 and ATC 80407 by using these primer pairs should be scored only in a dominant manner in a mapping population, i.e., the presence or absence of an amplified product, similar to the amplicons obtained from RAPD primers. Iwata et al. (2001) also observed that 53% of the polymorphisms generated from STS primers tested on Sugi (*C. japonica*) showed dominant banding patterns in the screening.

Sequence analysis of STSs amplified from ATC 80878 and ATC 80407

To confirm that the targeted genome region had been amplified, monomorphic bands from ATC 80878 and ATC 80407 were sequenced. Forward and reverse sequencing reads of each monomorphic band were compared to ensure sequencing accuracy. No nonspecific PCR products were obtained following database searches, except for that amplified with primer pair 993, which was expected to amplify a gene coding for a disease resistance response protein 230 precursor (Table 2). Instead, the amplified product was homologous to a nucleic sequence coding for xylose isomerase. This indicated that the primers designed to amplify this gene lacked specificity. Four of the STS primer pairs amplified products larger than the size predicted by the cDNA sequence, suggesting the presence of introns (Table 2). For example, following alignment analysis (data not shown), primer pair 59 amplified a product from genomic DNA that was approximately 140 bp longer than the cDNA sequence at the 5' end. The amplicon produced using primer pair 246 was approximately 200 bp longer at the 3' end than the cDNA sequence.

Sequence variations were also observed among STSs amplified from ATC 80878 and ATC 80407, following alignment analysis. STSs amplified by primer pairs 465 and 524 possessed only a single nucleotide difference between ATC 80878 and ATC 80407, and products generated by primer pair 761 possessed two nucleotide differences. Several nucleotide differences were observed in all the STSs amplified by all the other primer pairs, with insertions of varying sizes being identified in products amplified by primer pairs 59, 159, 185, 246, 351, and 787 (data not shown).

Screening for polymorphisms in STSs from ATC 80878 and ATC 80407 via restriction digestion

The single monomorphic products amplified by 17 STS primers from both ATC 80878 and ATC 80407 genomic DNAs could not be distinguished from each other until they were digested with restriction endonucleases. Further analysis was not conducted on STS primer 993 because it amplified a nontarget product. Of the 17 primer pairs tested, 10 produced products that generated polymorphic

banding patterns between ATC 80878 and ATC 80407 after restriction endonuclease digestion. The remaining 7 primer pairs failed to reveal polymorphisms between the two accessions (Table 3) even after digestion. Therefore, the success rate for converting cDNA/EST sequences into polymorphic STS was 58.8%. Most polymorphisms observed in this study appeared to involve base substitutions or perhaps insertions or deletions of only a few bases, rather than length variations in the amplified DNA fragments, resulting in the gain or loss of a restriction site. The extent of sequence variation between ATC 80878 and ATC 80407 was relatively low given that polymorphisms were detected only after many different restriction endonucleases were tested. Products amplified by primer pairs 81 and 159 displayed polymorphic banding patterns between ATC 80878 and ATC 80407 only when digested with *Hae* III. Restriction endonuclease *Rsa* I digested and revealed polymorphic profiles for products amplified by primer pairs 612, 753, and 1005. STS products amplified with primer pairs 524 and 787 displayed polymorphisms when digested with *Mnl* I. Restriction endonucleases *Apo* I, *Hinf* I, and *Dde* I were also useful in detecting polymorphisms between the two accessions for products amplified by STS primers 2, 59, and 351, respectively.

Results from this study supported the general utility of the STS approach for genome analysis. This PCR-based STS analysis will be useful for generating informative molecular markers in *L. sativus* for potential use in future genome mapping experiments. One disadvantage of STS PCR primers is the need for sequence analysis before primers can be designed, which can be a costly procedure. However, this must be accomplished only once, after which STS primer sequences may be a useful source of genetic markers for *L. sativus* mapping and potentially for mapping in other *Viciae* species. Once developed, this type of marker has a number of advantages over RFLPs, RAPDs, and AFLPs. Polymorphic STS markers are codominant, unlike RAPDs and most AFLPs. Furthermore, because they are detected by means of PCR assay followed by restriction endonuclease digestion, they use little template DNA and are rapid and relatively inexpensive to assay, compared with RFLPs. Polymorphic STSs developed in this study produced clear, strong, reproducible signals, which should be easy to score in a segregating mapping population. Because ESTs are derived from coding DNA, which generally has a high degree of sequence conservation, EST markers/ polymorphic STSs may be more likely to be transportable across species boundaries, thereby facilitating comparative mapping (Lyons et al., 1997). Tsumura et al. (1997) reported some success in using the cDNA-based STS markers in several conifer species for comparative mapping. Therefore, the polymorphic *L. sativus* STSs may be useful as markers to the more closely related members of the *Viciae* tribe and to serve as anchors for comparative mapping with field pea.

This is the first report of the use of nucleotide sequences from cDNA clones from an *L. sativus* cDNA library to design primers to amplify STSs from *L. sativus* genomic DNA. This method has the potential for developing codominant molecular markers useful for *L. sativus* linkage map construction and comparative mapping with related species.

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