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A linkage map of the pea (*Pisum sativum* L.) genome containing cloned sequences of known function and expressed sequence tags (ESTs)

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Abstract A linkage map of the pea (*Pisum sativum* L.) genome is presented which is based on F_2 plants produced by crossing the marrowfat cultivar 'Primo' and the blue-pea breeding line 'OSU442-15'. This linkage map consists of 209 markers and covers 1330 cM (Kosambi units) and includes RFLP, RAPD and AFLP markers. By mapping a number of anchor loci, the 'Primo' × 'OSU442-15' map has been related to other pea linkage maps. A feature of the map is the incorporation of 29 loci representing genes of known function, obtained from other laboratories. The map also contains RFLP loci detected using sequence-characterized cDNA clones developed in our laboratory. The putative identities of 38 of these cDNA clones were assigned by examining public-sequence databases for protein or nucleotide-sequence similarities. The conversion of sequence-characterized pea cDNAs into PCR-amplifiable and polymorphic sequence-tagged sites (STSs) was investigated using 18 pairs of primers designed for single-copy sequences. Eleven polymorphic STSs were developed.

Key words *Pisum sativum* · Linkage mapping · DNA markers · Expressed sequence tags · Sequence-tagged sites

Introduction

The garden pea (*Pisum sativum* L.) is an important agricultural plant with a rich history of genetic research. The wide morphological variation which *P. sativum* ($2n = 2x = 14$) exhibits has provided genetic markers for pioneering geneticists and formed the basis for earlier linkage maps (Blixt 1974). Linkage maps of the pea genome containing molecular markers have also been published (Ellis et al. 1992, 1993; Dirlewanger et al. 1994; Weeden et al. 1996).

Genetic linkage maps containing DNA markers are powerful tools for plant genetic research and for plant breeding. Linkage maps have assisted in the identification of DNA markers linked to single genes of major agronomic importance, such as many disease-resistance genes, and have permitted the identification of tightly linked DNA tags for use as diagnostic tools in plant breeding. Through the use of linkage maps, characterization of quantitatively inherited traits has been facilitated, including identifying the genomic regions containing contributing loci, postulating the types of gene action that may be involved, and determining the role of epistatic effects in specifying phenotype (Tanksley 1993). Using the linkage map described in this article, our research group has mapped and characterized quantitative trait loci (QTLs) for seed weight and green seed color by interval mapping (Timmerman-Vaughan et al. 1996; McCallum et al. 1997). Linkage maps based on molecular markers also have the potential to bridge the gap between our understanding of phenotype based on genetics and our understanding of organismal biochemistry and physiology. Map-based cloning is one approach to determining the biochemical basis of an inherited phenotype. The "candidate gene" approach is a second means of relating a phenotype to its underlying biochemical basis by demonstrating that "candidate genes" (sequences displaying an activity which could explain the phenotype) are tightly linked to, or co-segregate with, the genetic locus of interest.

In this paper, we present a linkage map of the pea genome based on a cross between two cultivated types. The map consists of 209 markers, the majority of which

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detect DNA polymorphisms directly. Many of the DNA markers are characterized with regard to their product, because they are either cloned sequences of known function or expressed sequence tags (ESTs). In addition, the map contains a large number of PCR-based markers. Through the use of anchor loci, the map has been related to the "standard map" of the pea genome (Weeden et al. 1996) which contains both molecular and morphological markers.

Materials and methods

Plant material

An F_2 population of 102 individuals was developed by crossing two *P. sativum* genotypes, 'Primo' (Cebeco, Lelystad, The Netherlands) and 'OSU442-15' (Baggett and Hampton 1977). Details of this population were published previously (Timmerman-Vaughan et al. 1996; McCallum et al. 1997). A reference mapping population of 51 single-seed-descent recombinant inbred lines (RILs), resulting from a cross between *P. sativum* ssp. *humile* accession 'JI1794' (John Innes Institute, Norwich, UK) and *P. sativum* ssp. *sativum* accession 'Slow' (N. Weeden, Cornell University, Geneva N.Y., USA), was also used (Weeden et al. 1993 b).

Disease-resistance markers

Segregation of the disease-resistance loci *mo* (recessive gene for resistance to bean yellow mosaic virus, BYMV) and *sbm-1* (recessive gene for resistance to pea seed-borne mosaic virus, PSbMV) was scored in the 'Primo' (susceptible) × 'OSU442-15' (resistant to both viruses) mapping population. Five F_3 plants descended from each F_2 individual were challenged by mechanical inoculation, as described previously (Timmerman et al. 1993). Susceptibility to BYMV was scored by observing the appearance of bright yellow mosaic symptoms on leaves. Susceptibility to PSbMV was scored by observing the appearance of leaf curl and mild mosaic symptoms on leaves, and by Western-blot analysis as described previously (Timmerman et al. 1993).

DNA-marker methods

DNA was isolated, and RAPDs and RFLPs were analysed, as described by Timmerman et al. (1993). DNA for RAPD analyses was isolated from individual F_2 plants, while DNA for RFLP and AFLP analyses was isolated from young leaves pooled from five F_3 descendants. RAPDs were amplified using primers obtained from Operon Technologies (Alameda, Calif., USA), kits A through Z. The RAPDs which were scored on the F_2 progeny set were only some of those that produced scorable polymorphisms using the 520 Operon primers. RAPD markers were named as in the example Y02_610; where Y02 indicates that Operon primer Y02 was used, and the polymorphic band is estimated to be 610 bp in length. AFLPs were analysed as described by Timmerman-Vaughan et al. (1996) and by Pickering et al. (1995) using methodology based on the European Patent Application of Zabeau (1992). AFLP markers are designated as AFP(number)(letter). Using the example AFP1e, this locus was detected using primer *Pst*P1 (all AFLPs presented in this map used *Mse*P2 as the second primer; Zabeau 1992), and was the fifth polymorphic band down from the top of the autoradiograph.

Linkage-map construction

Linkage maps for markers segregating in the 'Primo' × 'OSU442-15' F_2 population were computed using MAPMAKER/EXP version 3.0 (Lincoln et al. 1992). LOD threshold values for assigning markers to linkage groups were set at LOD $\geq$ 3.0. Pairwise segregation of polymorphic loci in the 'JI1794' × 'Slow' RILs was also computed using MAPMAKER/EXP version 3.0. Marker-segregation data for the 'JI1794' × 'Slow' RILs were generously provided by Dr. N. Weeden (Cornell University, personal communication).

Random cDNA clone characterisation

A pea cDNA library was constructed using poly (A^+) RNA isolated from etiolated seedlings of the *P. sativum* cultivar Whero (New Zealand Institute for Crop and Food Research Ltd, Lincoln, New Zealand). The cDNA was cloned into λZAPII using a ZAP-cDNA synthesis kit (Stratagene), following the manufacturer's instructions. cDNA clones likely to label high-copy number sequences were eliminated by hybridizing dot blots of PCR-amplified insert with ^{32}P-labelled total pea DNA.

Plasmids (pBluescript-SK(−)) containing each insert were excised from phage according to the manufacturer's instructions (Stratagene). Plasmid DNA for sequencing was purified using Recovery Midi Preps (Hybaid). Automated nucleotide sequencing was performed at the University of Otago (Dunedin, New Zealand) using an ABI373 sequenator (Applied Biosystems). Manual sequencing was performed on plasmid DNA by the dideoxy nucleotide chain-termination method using a Sequenase kit (United States Biochemicals). The predicted protein sequences were compared against the non-redundant combined databases (Release date May 12, 1997) using the BLASTX program (Altschul et al. 1990), provided by the NCBI e-mail server (blast@ncbi.nlm.nih.gov). Protein alignments returning BLASTX scores of at least 80 were considered (Newman et al. 1994). Sequences without significant alignments were compared against the dbEST database (Boguski et al. 1993) using the TBLASTX program, and against the non-redundant combined databases using the BLASTN program.

Development of STS PCR assays

Primers were designed to amplify products from 20 single-copy cDNA clones using the program Primer3 (Rozen and Skaletsky 1996) with the following selection criteria: primer length of 24 nt, Tm of 60°C, minimal complementarity of primers and minimal internal duplex formation.

PCR assays were carried out in a final volume of 25 µl in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatine, 200 µM of each of the four dNTPs, 0.625 U of *Taq* polymerase (Boehringer-Mannheim), 1.0 µM of each primer and 20 ng of total pea DNA. Reactions were capped with a drop of mineral oil. Standard amplification conditions were 40 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 8 min. Amplification products were analysed by electrophoresis through 1% agarose, 1% NuSieve agarose (FMC) in 1 × TBE, then visualized by ethidium bromide staining.

To discover polymorphisms, PCR products amplified from 'Primo' and 'OSU442-15' DNA were digested with a range of restriction endonucleases which recognize 4- and 5-base sequences: *Alu*I, *Bst*UI, *Hae*III, *Hha*I, *Hpa*II, *Taq*I, *Rsa*I, *Sty*I, *Scr*FI, *Hin*fI, *Fok*I, *Ase*I, *Ban*I, *Hin*cII and *Sau*96. Some pairs of PCR products were also digested with *Bfa*I, *Mse*I, *Nla*III, and *Sau*3A. Two units of restriction enzyme were added to 15 µl of PCR reaction, and the digestions were incubated either for 2 h or overnight at the recommended temperature. $MgCl_2$ was added to reactions to 5 or 10 mM depending on the manufacturer's recommended Mg^{++} concentration.

Polymorphisms were analysed by agarose/NuSieve agarose-gel electrophoresis, as described above. Once restriction enzymes able to distinguish between the 'Primo' and 'OSU442-15' genotypes were identified, individual F_2 progeny were analysed.

Results and discussion

The linkage map of the pea genome constructed using progeny of the cross 'Primo' × 'OSU442-15' (P × 4) is shown in Fig. 1. The map consists of 14 linkage groups, containing 207 markers detected by DNA polymorphisms and two disease-resistance loci. Linkage groups corresponding to the known linkage groups of pea were identified. The roman numeral linkage groups designations adopted for the P × 4 map relate to the "standard genetic map" of Weeden et al. (1996). In addition, there were four unidentified linkage groups, designated A through D. The map length is 1330 cM (Kosambi units). Three classes of DNA markers were used: RFLPs, RAPDs and AFLPs. RFLPs were detected using: (1) anonymous cDNA probes from a library constructed in our laboratory, (2) anonymous pea, lentil or mungbean cDNA probes obtained from other researchers, and (3) cloned pea sequences of known function obtained from other researchers. In addition to the 209 mapped markers, there were six which failed to demonstrate linkage to any other markers (Fig. 1), although these six displayed expected segregation ratios.

Assignment of linkage groups

The relationship of the P × 4 map to other published maps of the pea genome was determined with the aim of increasing the general usefulness of the P × 4 map. A "standard map" of the pea genome, which contains molecular markers as well as many morphological traits, has been prepared (Weeden et al. 1996). Anchor loci from a number of sources were used to identify the linkage groups making up the P × 4 map: "standard reference markers", as described by Weeden et al. (1993 b), were mapped in the P × 4 cross; markers from the Ellis et al. (1992, 1993) map were mapped in the P × 4 cross; and markers from the P × 4 map were mapped in RILs from the cross 'JI1794' × 'Slow' (Weeden et al. 1993 b), a population segregating for many "standard reference markers". Markers from the partial linkage map of Dirlewanger et al. (1994) have not been analyzed in the P × 4 cross.

The markers used to compare the P × 4 map with the "standard genetic map" (Weeden et al. 1996) and the Ellis et al. (1992, 1993) map are listed in Table 1. In addition, the linkage relationships of markers co-segregating in both the P × 4 cross and 'JI1794' × 'Slow' cross are presented in Table 2. Comments on the evidence for some linkage-group assignments are made below. In particular, the linkage groups designated IA/II and III/IVB contain markers that have been assigned to distinct linkage groups in previous maps (Ellis et al. 1992, 1993; Weeden et al. 1993 a).

Linkage group IA

This short linkage group has been assigned to group IA because the RFLP locus M89 and the standard reference locus *Aat-p* co-segregate in the 'JI1794' × 'Slow' RILs (Table 2).

Linkage group IA/II

This linkage group contains markers which were previously assigned to separate linkage groups (Blixt 1974; Weeden et al. 1993 a; Ellis et al. 1993). On previous maps, *Lg-j* was assigned to group IA (Weeden et al. 1993 a) or to group 1 (Ellis et al. 1993), while *Cvc* and *mo* were assigned to group II (Weeden et al. 1993 a), and *Cvc* and c243 were assigned to group 2 of the Ellis et al. (1993) map. In addition, the RFLP marker P531 is linked to *Fum*, another group II marker, in the 'JI1794' × 'Slow' cross (Table 2). Paruvangada et al. (1995) previously reported the linkage between group IA and II markers for two crosses, 'JI1794' × 'Slow' and 'Sparkle' × 'WL1238', and suggested that these markers may reside on the same chromosome. This suggestion is supported by analyzing the P × 4 cross. As further support for these findings, Kosterin (1993) and Kosterin and Rozov (1993) presented data which indicated that linkage groups IA and IB may not reside on the same chromosome.

Linkage group IB

This linkage group was identified using probes c44 and c267 from group 1 (bottom) of the Ellis et al. map (1992, 1993). The group 1 (bottom) linkage group contains the *i*-locus and the leghemoglobin locus, providing references to the standard map. Comparison of group IB from the P × 4 map with group 1 (bottom) of the Ellis et al. (1992, 1993) map suggests that a significant portion of group IB, possibly including the region containing the *i*-locus, remains unidentified in the P × 4 map.

Linkage groups III and III/IVB

The linkage group on the P × 4 map designated as III was identified because it contains the major *Adh-1* locus, a reference locus on both the Weeden et al. (1996) and Ellis et al. (1992, 1993) maps, and because it also contains HSP21, an RFLP locus which shows linkage to the reference locus *M* in the 'JI1794' × 'Slow' RILs (Table 2).

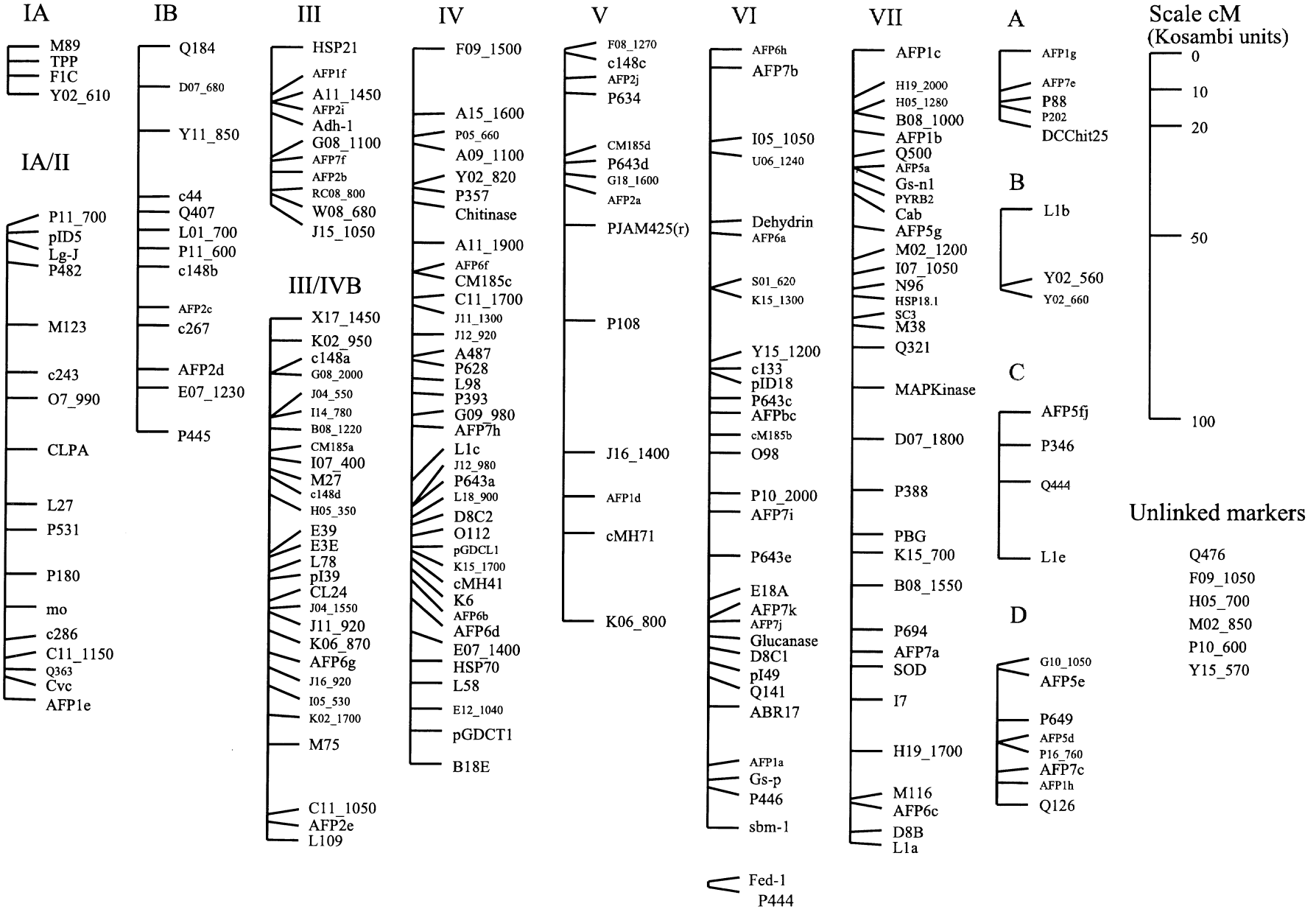
IA
M89
TPP
F1C
Y02_610
IA/II
P11_700
pID5
Lg-J
P482
M123
c243
O7_990
CLPA
L27
P531
P180
mo
c286
C11_1150
Q363
Cvc
AFP1e
IB
Q184
D07_680
Y11_850
c44
Q407
L01_700
P11_600
c148b
AFP2c
c267
AFP2d
E07_1230
P445
III
HSP21
AFP1f
A11_1450
AFP2i
Adh-1
G08_1100
AFP7f
AFP2b
RC08_800
W08_680
J15_1050
III/IVB
X17_1450
K02_950
c148a
G08_2000
J04_550
I14_780
B08_1220
CM185a
I07_400
M27
c148d
H05_350
E39
E3E
L78
pI39
CL24
J04_1550
J11_920
K06_870
AFP6g
J16_920
I05_530
K02_1700
M75
C11_1050
AFP2e
L109
IV
F09_1500
A15_1600
P05_660
A09_1100
Y02_820
P357
Chitinase
A11_1900
AFP6f
CM185c
C11_1700
J11_1300
J12_920
A487
P628
L98
P393
G09_980
AFP7h
L1c
J12_980
P643a
L18_900
D8C2
O112
pGDCL1
K15_1700
cMH41
K6
AFP6b
AFP6d
E07_1400
HSP70
L58
E12_1040
pGDCT1
B18E
V
F08_1270
c148c
AFP2j
P634
CM185d
P643d
G18_1600
AFP2a
PJAM425(r)
P108
J16_1400
AFP1d
cMH71
K06_800
VI
AFP6h
AFP7b
I05_1050
U06_1240
Dehydrin
AFP6a
S01_620
K15_1300
Y15_1200
c133
pID18
P643c
AFPbc
cM185b
O98
P10_2000
AFP7i
P643e
E18A
AFP7k
AFP7j
Glucanase
D8C1
pI49
Q141
ABR17
AFP1a
Gs-p
P446
sbm-1
Fed-1
P444
VII
AFP1c
H19_2000
H05_1280
B08_1000
AFP1b
Q500
AFP5a
Gs-n1
PYRB2
Cab
AFP5g
M02_1200
I07_1050
N96
HSP18.1
SC3
M38
Q321
MAPKinase
D07_1800
P388
PBG
K15_700
B08_1550
P694
AFP7a
SOD
I7
H19_1700
M116
AFP6c
D8B
L1a
A
AFP1g
AFP7e
P88
P202
DCChit25
B
L1b
Y02_560
Y02_660
C
AFP5fj
P346
Q444
L1e
D
G10_1050
AFP5e
P649
AFP5d
P16_760
AFP7c
AFP1h
Q126
Scale cM
(Kosambi units)
0
10
20
50
100
Unlinked markers
Q476
F09_1050
H05_700
M02_850
P10_600
Y15_570

Table 1 Loci used to cross reference linkage groups in the 'Primo' × 'OSU442-15' map with other linkage maps of the pea genome

Linkage group	Locus	Reference linkage group	Literature citation
IA/II	*Lg-j*	I (top)	Weeden et al. 1996
		1	Ellis et al. 1993
IA/II	c243	2	Ellis et al. 1993
IA/II	*mo*	II	Weeden et al. 1996
			Marx and Provvidenti 1979
IA/II	Cvc	II	Weeden et al. 1996
		2	Ellis et al. 1993
IB	c44	1 (bottom)	Ellis et al. 1993
IB	c267	1 (bottom)	Ellis et al. 1993
III	Adh-1 (bright band)	III	Weeden et al. 1996
		3	Ellis et al. 1993
III/IV	M27	III	Table 2, this study
III/IV	M75	IVB	Table 2, this study
III/IV	L109	IVB	Table 2, this study
IV	gdcL	7 (bottom)	Ellis et al. 1993
IV	cMH41	IV	Weeden et al. 1996
IV	gdcT	7 (bottom)	Ellis et al. 1993
V	*r* (pJAM425)	V	Weeden et al. 1996
		7 (top)	Ellis et al. 1993
V	cMH71	V	Weeden et al. 1996
VI	pID18	VI	Timmerman et al. 1994
VI	c133	VI	Ellis et al. 1993
VI	pI49	VI	Weeden et al. 1996
			Timmerman et al. 1993
VI	GS-p (GS185)	VI	Weeden et al. 1996
			Timmerman et al. 1993
VI	*sbm-1*	VI	Weeden et al. 1996
			Timmerman et al. 1993
VII	AFP1c	VII (top)	Table 2, this study
VII	Cab	VII (bottom)	Weeden et al. 1996
		4 (top)	Ellis et al. 1993
VII	GS-n1	VII (bottom)	Weeden et al. 1996
		4 (top)	Ellis et al. 1993
VII	SOD	VII (bottom)	Weeden et al. 1996

Linkage group III/IVB has been so designated since it contains M27 and B08_1220, markers which are linked to a linkage group III reference marker (cMH95) in the 'JI1794' × 'Slow' RILs (Table 2), and M75 and L109, markers which are linked to the linkage group IVB reference markers *Tpi-p* and *le*, respectively, in the 'JI1794' × 'Slow' RILs (Table 2). The linkage of M75 and *Tpi-p* in the 'JI1794' × 'Slow' RILs is strengthened by two RAPD markers which map between M75 and *Tpi-p* [*Tpi-p*-(6.7 cM, LOD 6.2)-B156b-(1.0 cM, LOD 11.4)-B566c-(0.0 cM, LOD 5.1)-M75]. There is uncertainty about the assignment of the linkage group designated IVB, containing *Np*, *Lox*, *le* and *Tpi-p*, to satellite chromosome 4 (Folkeson 1990; Weeden et al. 1993 a). Therefore, it is unclear whether the linkage relationships we detected between group III and group IVB markers in the P × 4 cross represent the usual alignment of markers or a translocation. A translocation involving linkage groups III and IVB was described by Lamm and Miravelle (1959); however, these authors placed their translocation breakpoint in a different region of linkage group III than the junction between the group III and IVB markers that we observed in the P × 4 cross. No segregation distortion was observed in the P × 4 cross for the group III and group IVB markers.

Fig. 1 Linkage map of the pea genome produced by analyzing F_2 progeny of the cross 'Primo' × 'OSU442-15', calculated using MapMaker. The relative orders of loci with *large type* are significant (LOD ≥ 2.0), while the loci in *small type* are presented in the best order but are less significant (LOD ≤ 2.0)

Linkage group VII

This linkage group was identified using a number of anchor loci, including Cab and SOD (Table 1). The probe used to map Cab, 0.9MI, was obtained from Dr. Noel Ellis (John Innes Institute), who reported that Cab sequences detected using this probe mapped to linkage groups 4 and 6 (Ellis et al. 1993). Cab was originally mapped by Polans et al. (1985), and assigned to linkage group VII. Using the 0.9MI probe, we mapped Cab sequences to the same region as Polans et al. (1985). The linkage group VII assignment was supported by co-segregation of RFLP markers from the P × 4 map with group VII isozyme markers in the 'JI1794' × 'Slow' cross (Table 2).

Table 2 Supporting evidence for identification of 'Primo' × 'OSU442-15' map linkage groups based on co-segregation of loci from the 'Primo' × 'OSU442-15' map with loci in RILs from the cross 'JI1794' × 'Slow'

Linkage group[a]	Test locus[b]	Anchor locus[c]	cM[d]	Pairwise LOD
IA	M89	*Aat-p*	7.6	5.0
IA\II	P531	*Fum*	6.4	6.5
IA\II	C11_1200	*wb*	10.4	4.2
III	HSP21	*M*	11.6	4.0
III	B08_1250	cMH95	7.6	5.0
III	M27	cMH95	7.6	5.0
IVB	M75	*Tpi*	7.2	2.5[e]
IVB	L109	*le*	4.2	7.1
IV	L98	*Rrn1*	9.4	3.6
V	P108	*tl*	7.0	5.7
V	P649	*Pgd-c*	7.2	5.5
VII	AFP1c	*Pgp-p*	3.9	7.9
VII	AFP1c	*Pep-3*	5.8	4.5
VII	AFP1c	*Rrn2*	4.4	6.6
VII	AFP5g	*Skdh*	0.0	12.3
VII	MAPKinase	*Skdh*	1.2	10.6
VII	Q500	*Skdh*	2.4	9.6

[a] Linkage groups as identified in the "standard map" of the pea genome (Weeden et al. 1996)
[b] Locus segregating in both 'Primo' and 'OSU442-15', and in 'JI1794' and 'Slow'
[c] Anchor loci segregating in 'JI1794' and 'Slow'
[d] Genetic distance (Kosambi units) determined by multipoint mapping
[e] The significance of this linkage is supported by two RAPD loci which map within the interval

SOD was mapped previously as an isozyme (Weeden et al. 1993 a). We mapped SOD as an RFLP (Table 3) using a Cu/Zn SOD sequence.

Linkage mapping of genes of known or putative function

The P × 4 linkage map contains 103 loci detected as RFLPs using either cloned sequences of known function or anonymous cDNA probes. Although the map locations of a number of these probes have already been reported (Weeden et al. 1996; Ellis et al. 1992, 1993; Timmerman et al. 1993, 1994), 78 of these sequences were not mapped previously. Fifty seven of these loci were detected using anonymous cDNAs from our library. To improve the biological relevance of the P × 4 linkage map, many of these cDNAs were sequenced and their putative identities were determined by searching public-sequence databases.

Mapping of cloned sequences of known function

A collection of 79 cloned pea sequences of known function were obtained from other researchers and screened for polymorphisms in 'Primo' and 'OSU442-15'. From this collection, 29 probes (37%) detected RFLPs and were mapped. These mapped loci are listed in Table 3. The copy number detected using each of

Table 3 Cloned sequences of known function, obtained from researchers in other laboratories, which have been mapped using the 'Primo' × 'OSU442-15' cross

Group	Locus	Function/activity	Reference
IA	TPP	Thiol protease	Granell et al. 1992
IA/II	pID5	Granule-bound starch synthase I	Dry et al. 1992
IA/II	Lg-J	Legumin J	Gatehouse et al. 1988
IA/II	CLPA	Chloroplast envelope protein	Moore and Keegstra 1993
IA/II	Cvc	Convicillin	Newbigin et al. 1990
III	HSP21	Heat-shock protein	Vierling et al. 1988
III	Adh-1	Alcohol dehydrogenase	Llewellyn et al. 1987
III/IV	pI39	Disease-response protein	Chiang and Hadwiger 1991
III/IV	CL24	Chloroplast ribosomal protein	Gantt 1988
IV	Chitinase	Leaf chitinase	Vad et al. 1993
IV	pGDCL1	Glycine decarboxylase L subunit	Turner et al. 1992
IV	HSP70	Heat-shock protein	Marshall and Keegstra 1992
IV	pGDT1	Glycine decarboxylase T subunit	Kopriva et al. 1995
V	PJAM425(r)	Starch branching enzyme	Burton et al. 1995
VI	Dehydrin	Dehydration stress protein	Robertson and Chandler 1992
VI	pID18	Granule-bound starch synthase II	Dry et al. 1992
VI	Glucanase	β 1-3 glucanase	Chang et al. 1993
VI	pI49	Disease response protein	Culley et al. 1995
VI	ABR17	ABA responsive protein	Iturriaga et al. 1994
VI	Gs-p	Plastid glutamine synthase	Tingey et al. 1987
VI	Fed-1	Ferredoxin	Elliott et al. 1989
VII	Gs-n1	Nuclear glutamine synthase	Tingey et al. 1987
VII	PYRB2	Aspartate transcarbamoylase	Williamson and Slocum 1993
VII	Cab	Chlorophyll a/b binding protein	Ellis et al. 1993
VII	HSP18.1	Heat-shock protein	Lauzon et al. 1990
VII	MAPKinase	MAP kinase	Stafstrom et al. 1993
VII	PBG	Porphobilinogen deaminase	Witty et al. 1993
VII	SOD	Cu/Zn super oxide dismutase	White and Zilinskas 1991
A	DCChit25	Inducible chitinase	Chang et al. 1995

these probes was estimated by examining the number of bands appearing on blots of parental DNA digested with 6–8 different restriction endonucleases. Many of the loci (14 out of 29) appeared to represent single-copy sequences. The remaining 15 probes apparently detected two or more gene copies, but only one copy was polymorphic in this cross, and could therefore be mapped. For four sequences, the polymorphic bands had the strongest hybridization signal (Table 3), and for the remaining sequences the mapped bands were approximately equal in signal intensity to other non-polymorphic bands. No minor hybridizing bands were mapped.

Mapping and sequence-characterization of anonymous cDNA clones

A total of 336 anonymous cDNA clones from our cDNA library were used to probe parental Southern blots containing total 'Primo' and 'OSU442-15' DNA. Of these probes, 71 (21%) detected polymorphisms but only 51 probes produced signals that could be scored accurately on Southern blots containing suitably digested F_2 progeny DNA. Using these 51 probes, 57 loci were mapped. The locus detected by one random cDNA probe, Q476, remains unlinked to other markers.

To generate expressed sequence tags (ESTs), 53 of the anonymous clones from the Whero cDNA library produced in our laboratory were sequenced. Not all of the sequenced clones were mapped, and not all of the mapped clones were sequenced. The sequences of these 53 cDNA clones have been deposited with the dbEST database (Boguski et al. 1993). The majority of the clones appeared to be partial cDNAs, which may reflect the quality of the cDNA library.

Forty four of the cDNA sequences showed similarity to sequences in public databases using BLAST software (Table 4). Putative identities were assigned for 38 of the cloned sequences based on inferred protein sequences, using the BLASTX program to search the non-redundant combined databases. One additional cDNA, D8B, was identified based on nucleotide-sequence similarity with the 3′-end of a pea transcript. Therefore, the rate of sequence identification was 74% (39 out of 53 sequences). Five sequences showed significant similarity with sequences in the dbEST database, which was determined using the TBLASTX program. There were 34 matches to genes from higher plants, including three matches to genes from pea; and there were ten matches to genes from outside the plant kingdom, including genes from mammals, *Caenorhabditis*, bacteria and cyanobacteria (Table 4).

Mapping of sequences not yet fixed in copy number or genome location

During the development of the $P \times 4$ map it became obvious that rare sequences (four cDNAs out of > 400 used to probe parental Southern blots) were present in 'Primo' and 'OSU442-15' in different copy numbers. The four cDNAs were: c148 (obtained from Noel Ellis, John Innes Institute), CM185 (a mungbean clone obtained from Nevin Young, University of Minnesota), and L1 and P643 (cDNAs cloned in our laboratory). The individual polymorphic bands for each of these four probes segregated independently and were dominantly inherited. Monomorphic bands were also observed for all four of these probes. The inheritance and segregation patterns observed indicate that each band-present locus in one parent is matched by a null-allele in the other parent; therefore, the copy number for these sequences is not yet fixed within *P. sativum* germplasm. Ellis et al. (1992) reported examples of three cDNA clones (including c148) which detected different numbers of copies in different pea lines. Similar observations have been made in rice (McCouch et al. 1988) and lentil (Weeden et al. 1992).

The loci detected by each of these sequences are dispersed throughout the pea genome. Polymorphisms detected by the four cDNAs listed above mapped to linkage groups IB (c148b), III/IVB (c148a, CM185a, c148d), IV (CM185c, L1c, P643a), V (c148c, CM185d, P643d), VI (P643c, CM185b, P643e), VII (L1a), C (L1b) and D (L1e). Surprisingly, some clustering of unrelated sequences was observed, with 8 of these 16 loci occurring as four linked pairs. The paired loci were: CM185a and c148d on group III/IVB; L1c and P643a on IV; CM185d and P643d on V; and P643c and CM185b on VI. This observation suggests that there may be preferred sites within the genome for the insertion of the duplicate copies of these sequences. Our results, combined with those of Ellis et al. (1992), indicate that the use of some cDNA sequences as "anchor loci" may result in ambiguous or incorrect linkage-group assignment when constructing maps of the pea genome.

Conversion of ESTs to polymorphic sequence-tagged sites (STSs)

Eighteen mapped, single-copy, ESTs were chosen to initiate the development of polymorphic STSs. The sequences of the primer pairs designed for each EST and results of the polymorphic STS development are summarized in Table 5. Sixteen of the primer pairs amplified a single band in both 'Primo' and 'OSU442-15' using standard conditions. One primer pair failed to amplify a PCR product (for cDNA Q184) whilst the primer pair designed using P88 sequences amplified many bands. For 15 of the primer pairs, the amplification products from 'Primo' and 'OSU442-15' DNA were not distinguishable on the basis of electrophoretic mobility in a 1% agarose, 1% NuSieve agarose, $1\times$ TBE gel. The PCR products amplified by the primers for sequence Q500 were different in size. Ten of the primer pairs directed amplification of products larger

Table 4 List of pea cDNA clones with significant similarity to entries in sequence databases

Group	Clone	Putative identification/function	Organism	Score[a]	Accession[b]
IA/II	P482	Auxin regulated protein AUX88	*Glycine max*	261	AA427337
IA/II	M123	Glutamine synthetase	*Staphylococcus aureus*	93	AA427338
IA/II	L27	DNA photolyase	*Synechocystis* sp.	107	AA427339
IA/II	P180	40*s* ribosomal protein S18	*Arabidopsis thaliana*	258	AA430902
IA/II	Q363	Sugar starvation-induced protein	*Zea mays*	179	AA430903
IB	Q184	Expressed sequence tag	*Arabidopsis thaliana*	171	AA430904
IB	Q407	Probable ligand-binding protein	*Rattus rattus*	104	AA430905
IB	P445	Ca-dependent protein kinase	*Vigna radiata*	323	AA430906
III/IVB	M27	Prunasin hydrolase precursor	*Prunus serotina*	124	AA430907
III/IVB	M75	Brain protein homolog	*Vicia faba*	154	AA430908
III/IVB	L78	Expressed sequence tag	*Arabidopsis thaliana*	120	AA430950
III/IVB	L109	Unknown protein	*Ricinus communis*	281	AA430909
IV	P628	Glyceraldehyde-3-phosphate dehydrogenase	*Pisum sativum*	305	AA430910
IV	L98	apaG protein	*Escherichia coli*	145	AA430911
IV	P393	Abscisic acid induced protein HVA22	*Hordeum vulgare*	125	AA430912
IV, VII C, D	L1	Aldehyde dehydrogenase	*Synechocystis* sp.	347	AA430951
IV, V, VI	P643	NADH-ubiquinone dehydrogenase 24-kDa subunit	*Caenorhabditis elegans*	201	AA430913
IV, VI	D8C	GTP-binding protein	*Arabidopsis thaliana*	294	AA430914
IV	L58	Protein kinase SNF1 homolog	*Triticum aestivum*	134	AA430915
V	P108	Thioredoxin	*Nicotiana tabacum*	390	AA430916
VI	O98	Glucose-1-dehydrogenase	*Bacillus megaterium*	88	AA430917
VI	Q141	Disease-resistance-response protein	*Pisum sativum*	245	AA430918
VII	Q500	Expressed sequence tag	*Arabidopsis thaliana*	374	AA430919
VII	N96	Ubiquitin-like protein 12	*Arabidopsis thaliana*	974	AA430920
VII	P694	Endo 1-3 β-glucosidase	*Glycine max*	245	AA430921
VII	I7	Cold-acclimatization protein	*Triticum aestivum*	270	AA430922
VII	D8B	Metallothionein-like protein	*Pisum sativum*	512-N	AA430923
A	P88	NADH-ubiquinone oxidoreductase B22 subunit	*Bos taurus*	152	AA430924
A	P202	Folate biosynthesis protein 1	*Bacillus subtilis*	127	AA430925
C	Q444	S-phase-specific protein	*Arabidopsis thaliana*	175	AA430926
Unmapped	K35	60*s* ribosomal protein L23A (L25)	*Nicotiana tabacum*	445	AA430927
Unmapped	K37	Invertase (β-fructofuranosidase)	*Vica faba*	386	AA430928
Unmapped	M32	Glutamine-ammonia lyase	*Rhizobium leguminosarum*	175	AA430929
Unmapped	N24	Ribosomal protein L32	*Arabidopsis thaliana*	469	AA430930
Unmapped	O17	DNA-binding protein ACBF	*Nicotiana tabacum*	220	AA430931
Unmapped	P9	Remorin	*Solanum tuberosum*	196	AA430932
Unmapped	P116	Acyl-CoA-binding protein	*Brassica napus*	97	AA430933
Unmapped	P240	Chalcone-flavanone isomerase 1	*Medicago sativa*	1006	AA430934
Unmapped	P446	Expressed sequence tag	*Arabidopsis thaliana*	104	AA430935
Unmapped	P524	Cyclin	*Medicago sativa*	166	AA430936
Unmapped	P627	Expressed sequence tag E2E2T7	*Arabidopsis thaliana*	307	AA430937
Unmapped	P695	Endo-1,3-β-glucosidase	*Lycopersicon esculentum*	191	AA430938
Unmapped	Q306	60*s* ribosomal protein L32	*Arabidopsis thaliana*	298	AA430939
Unmapped	Q354	Cytochrome c oxidase subunit Vb	*Oryza sativa*	400	AA430940

[a] All are BlastX scores or TBlastX scores except for D8B, which is a BlastN score
[b] Accession number assigned by the GenBank dbEST database

than the size predicted by the cDNA sequence, suggesting the presence of introns.

To discover polymorphisms, the products amplified using 'Primo' and 'OSU442-15' DNA were digested with a collection of restriction endonucleases. Restriction endonuclease digestion revealed polymorphism between 'Primo' and 'OSU442-15'amplification products for 10 of the 16 primer pairs tested (62%) (Table 5). In general, polymorphisms were more likely to be detected from amplification products which might contain probable intron sequences.

Finally, to demonstrate that the polymorphic STSs mapped to the same genomic locations as the RFLPs, the segregation of each polymorphic STS was scored in F_2 progeny of the $P \times 4$ cross. The polymorphic STSs demonstrated co-dominant inheritance. In all cases, there was a complete correspondence between the RFLP genotype and polymorphic STS genotype (data not shown).

The success rate for converting cDNA sequences into polymorphic STSs was 62%. Once developed, this type of marker has a number of advantages over RFLPs, RAPDs and AFLPs. The polymorphic STS markers are co-dominant, unlike the RAPDs and most AFLPs. The polymorphic STSs are detected by a PCR assay followed by restriction-endonuclease digestion, therefore they use little template DNA, and are rapid and relative inexpensive to assay, an improvement on

Table 5 Summary of the primer sequences and restriction endonucleases used to convert RFLP markers into polymorphic STSs

cDNA	Primer sequences	Expected length	Observed length	Polymorphism detection
P482	GGTGTTTGATGAAACTGAACTGAG TAGGTCTATTTGGCGAAGATAAGG	415	629	*Rsa*I, *Hpa*II, *Mse*I
P531	ACGAGTAAAGATGGTCCTGCTATG AGGGTAAAAGTTGTTCCAAATGTC	227	227	None
Q363	TCGAGACTTTAAACCCGAATCTAC TCCACTGTCTTTGTCTTTAGGTTG	440	671	*Alu*I, *Mse*I, *Scr*F1, *Hinf*I
Q184	CCACTCTTGTGCACCTTTTAGTAG TACATTAAGAATCATGGTTGCCAC	461	No product amplified	
M27	TATGTAAGAGGATGAGCAAACCTG TACTTGTGTTGTCATAGGCTCACC	522	522	*Rsa*I, *Hinf*I
L109	GTTCCATGACAGTAGAAGCATTTG GTAGCATACATTTCCTTGCATCTG	346	346	*Taq*I, *Sau*3A
P628	ATGTGGTCTCAACTGACTTTATTG AGCTATTGCAAACATGACTAAAC	420	640	*Hinf*I
P393	CTGGTTGGTCCTTCCTTATTTTAC AACGGATAAAGAGTGACAAGAACC	418	516	*Rsa*I, *Hha*I, *Sty*I
D8C	AAGACGAGCTACGATTTCATCTG AATTATTTAAGTTTCTGCCAAGCC	368	368	None
K6	AAACTAGCACCTCAGTAGCGTTG TTCATAATTCATAATTCCTTGGGC	808	907	*Alu*I
L58	GTTTCTGGTGGTGGAAGTGAAG AGGAATTTCAAGAGTCCAATTACG	406	406	None
P108	GAAGAAAGAGAAAAATGGCAGAAG GCAGAAACACTCTTCATGCATATC	455	643	*Taq*I, *Ase*I
P446	TTTTTCTAGAGCAATTCATCCTCC AGCCATACAATTATTGCCTAAAGG	371	371	None
Q500	CTTTGTTTAGGTTGAAGGAAAACG CACCAATTTAGTGCAGTACAAACC	473	2409[a] 2190[b]	*Alu*I, *Hae*III, *Hha*I, *Hpa*I, *Rsa*I, *Scr*FI, *Hinf*I, *Ase*I, *Sau*96
I7	CTGATCATGTTCTTAAACTCGGTG CCAGATAGGGTAAACCAACAGAAG	485	1367	None
P88	CGCTCTTAAAGACACTCTCAACTG AAATATCTTCCACATCATCAGCAC	440	Many bands amplified	
P202	CTTGTTTATTCGCTTTTCAACTCC TATGACATCACTTTTACATCTCGC	592	1382	*Alu*I, *Taq*I
Q126	ATAATAACAAAGCGGCAAGTTCAG ACAATGCACAAAGGAGAATTTAAG	365	505	None

[a] Fragment size observed in 'Primo' DNA
[b] Fragment size observed in 'OSU442-15' DNA

RFLPs. The polymorphic STSs we have developed produce clear, strong signals which are easy to score in segregating progeny, in contrast to some RFLPs and RAPDs. Since the polymorphic STSs we have developed are based on cDNA sequences, they are likely to be of value in many pea pedigrees, although this has not been tested. In our experience, the majority of RAPDs and AFLPs are pedigree specific.

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