Gene-based markers of pea linkage group V for mapping genes related to symbioses

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Introduction

Genetic mapping, is a useful step in the elucidation and understanding of different biological processes, in particular, interactions between plants and microbes during beneficial symbioses. To date, several types of DNA markers, namely RAPD, RFLP, AFLP, SSR, SSAP and gene-specific PCR markers, had been used in pea (*Pisum sativum* L.) for creating linkage maps and mapping the genes of important traits (1, 4, 6, 9, 10, 13, 18, 30). Further steps aimed at coding sequence identification refer to positional cloning, which is rather difficult in pea because of the large size of its genome (8), or candidate gene approach (1), which is now feasible due to advances in genome sequencing of the legume model species *Medicago truncatula* Gaertn. (32).

Comparative mapping studies reported a good conservation of marker order between pea and *Medicago sativa* L., a crop species closely related to *M. truncatula*, for a set of 103 genes (11), and directly between pea and *M. truncatula* for a set of 98 genes (1, 5). The conservation of synteny allows us to use the functional mapping approach in order to clone and sequence pea genes corresponding to phenotypic mutants by taking the following steps:

1. determine a rough location of the gene of interest on the pea genetic map,
2. define the syntenic region in *M. truncatula*,
3. identify *M. truncatula* genes in this region that could be orthologous to pea mutants based on their biochemical function,
4. create pea markers based on selected *M. truncatula* genes,
5. analyze recombination between gene-based markers and the mutation in an extended population, and
6. sequence genes that do not demonstrate recombination with mutant phenotype from corresponding mutant and wild type lines.

In order to exploit the synteny, one has to map pea mutations in relation to gene-based markers and to compare the resulting map with physical map of *M. truncatula*. Initially, we concentrated on EST-derived markers to localize pea genes related to beneficial symbioses.

Materials and methods

Three pea mutant lines induced in the laboratory line SGE (16) were used for gene mapping (Fig. 1). SGEcrt (*curly roots*, *crt*) forms a compact root system when grown in high-density substrate, such as quartz sand (26). SGEapm (*cochleata*, or *coch*) has reduced stipules, abnormal flowers, and sometimes forms roots on the tip of the nodules (27). SGEFix$^-$-7 (*sym*27), defective in nitrogen fixation, forms early senescent, greenish nodules (3).

Segregating populations had been created by crossing mutants SGEcrt, SGEapm and SGEFix$^-$-7 with lines NGB1238, RT9 and 87-18 I-r, respectively, and following propagation of the F_1. For analysis of mutant trait segregation, F_2 plants were grown in growth chambers (Vötsch Industrietechnik VB 1014, Germany) under controlled conditions (day/night – 16/8 hours, temperature 21 °C, relative air humidity 75%). Seeds of F_2 populations were treated with concentrated sulfuric acid for 15 min., washed ten times with distilled water and

planted. F_2 (SGEcrt x NGB1238) (Pop1 – 103 individuals) were grown in quartz sand with full mineral nutrition added (2) and analyzed by phenotype (curled root system) during the 4th week after planting. F_2 (SGEapm x RT9) (Pop2 – 94 individulas) were grown in vermiculite with full mineral nutrition, and formation of stipules was analyzed on the 7th-10th day after planting. F_2 (SGEFix$^-$-7 x 87-18 I-r) (Pop3 – 86 individuals) were also grown in quartz sand, but with mineral nutrition lacking NH_4NO_3 as a source of nitrogen, under inoculation with *Rhizobium leguminosarum* bv. *viciae* CIAM1026 (23) immediately after planting. Four week old plants were scored for the presence or absence of functional nodules by observing their size and color. Because of difficulties in phenotype determination in the F_2, the analysis was repeated in the F_3, which also provided information on F_2 heterozygotes at the *sym*27 locus.

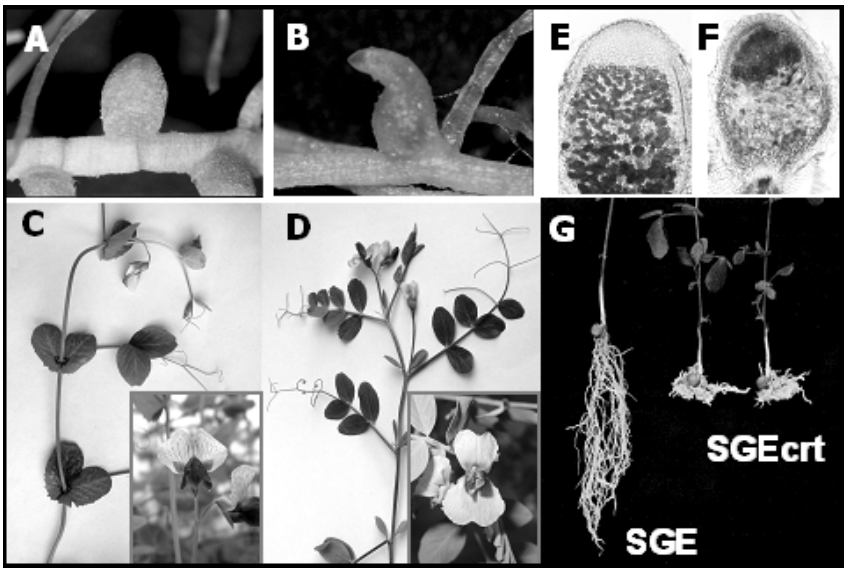

Fig. 1. Phenotypes of crt, coch and sym27 mutants obtained on line SGE. A – nodule of SGE; B – abnormal nodule of SGEapm (coch); C – shoot and flower of SGE; D – shoot and flower of SGEapm (coch): reduced stipules and deformed flower; E – section of nodule of SGE; F – section of early senescent nodule of SGEFix--7 (sym27); G – root system of SGE and SGEcrt (crt) grown in quartz sand (25).

DNA was extracted from leaves of F_2 plants, as well as of parental lines, by a standard CTAB method (21) with slight modifications. PCR amplification of DNA markers was performed in thermocyclers Personal Cycler (Biometra, Germany) and iCycler™ (Bio-Rad, USA). Direct sequencing of PCR products was performed in an automatic sequencer CEQ™ 8000 Genetic Analysis System (Beckman Coulter, USA). Detected SNPs were examined for change in recognition sites of endonucleases with use of web-based program dCAPS Finder 2.0. (20, helix.wustl.edu/dcaps/dcaps.html). Endonucleases for CAPS analysis were supplied by Fermentas (Lithuania) and SibEnzyme (Novosibirsk, Russia). Fractionation of restriction fragments was performed on agarose gels (1 – 3%, depending on size of the fragments). Genes for creating EST-markers were chosen by their location on linkage group V, according to Weeden et al. (30), Brauner et al. (4) and data collected on www.comparative-legumes.org (Table 1). Primers had been designed with help from the web-based program Oligonucleotide Properties Calculator (12, www.basic.northwestern.edu/biotools/ oligocalc.html) and synthesized by Syntol (Moscow, Russia) and Evrogen (Moscow, Russia). Positions of corresponding genes in *M. truncatula* had been detected by CViT-BLAST search on www.medicago.org/ genome/cvit_blast.php (default parameters, BLASTN and/or BLASTX), and the presence of homologous gene sequence had been confirmed by pairwise alignment on NCBI BLAST server (www.ncbi.nlm.nih.gov/blast/ bl2seq/wblast2.cgi) (24).

Genetic maps for each cross were constructed using the program MapL98 (Prof. Yasuo Ukai, Biometrics Laboratory, Graduate School of Agricultural Life Science, the University of Tokyo), (default parameters, LOD > 3.00). Genetic distances between markers were determined by converting the frequency of recombination events into Kosambi units (15).

Results

Genes of interest had been previously localized on linkage group V (LG V) of pea: *crt* in relation to morphological markers *r* and *tl* and the protein marker *Sca* (26), *cochleata* in relation to *gp* and *tl* (19, 31, cited by Rozov et al. (22)), and later in relation to the protein markers *His1* and *Sca* (22), and *sym*27 in relation to morphological markers *gp* and *Ust* (25). Therefore, we have chosen several genes of known position in LG V for

the creation of EST-based markers in order to localize genes of interest more precisely. In several cases pea gene sequences found on NCBI (Table 1) represented only cDNAs, and their exon-intron structure had to be determined using alignments with corresponding homologs in *M. truncatula* and *Arabidopsis thaliana* (L.) Heynh. In addition to known pea markers, a gene for creating the marker *Met*2 was detected in BAC mth2-165g15 of *M. truncatula* located in the middle of syntenic chromosome 7 of *M. truncatula*, and the corresponding sequence of the pea gene was found at NCBI. Also, for primer design for markers *Enol* and *UDPgd* conservative regions of nucleotide sequences of the corresponding genes of *A. thaliana* and *Glycine max* (L.) Merr. were used (17).

Primers were generated on the base of exon sequence to amplify introns as these were considered more likely to contain SNPs. PCR amplifications were performed on DNA of parental lines, and the resulting PCR products (approximately 500 – 2000 bp) were directly sequenced and examined for polymorphism. In the case of length polymorphism (*TI*1 and *Met*2 on Pop3) primer pairs were used directly for segregation analysis on F_2 DNA, as well as in the case of allele-specific amplification (*Apy* on Pop2, *VicJ* on Pop2 and 3). Otherwise, polymorphic

Table 1. Markers used for mapping in *P. sativum*.

Marker name	Accession number	Function assignment	Primers, 5' to 3'	Detection of polymorphism
Rpl24A	*At-AB199790**	*Ribosomal protein L24A*	TGC CGA TTC AGT GGT CMA AG TTC TTS GCT TTC TTC TCA TCC	FspBI (1,2**)
Enol	*At-AY150418* *Gm-AY496909*	*Enolase (2-phospho-D-glycerate hydrolase)*	AGG ATG ACT GGG AGC ACT ATG CCA AGC TCC TCC TCA ATT C	Hpa II (1,2)
Paal2	*D10003*	*Phenylalanine ammonia-lyase*	TGG AAA CAG TAG CAG CAG CC GGT TTC CCT TGC ATA ACT TCA GC	Hae III (1); HindII (2)
Apy	*AB088208*	*Apyrase (ATP diphosphohydrolase)*	GCA ATC ACT TCC TCC CAA T CAA AAT ACA TCA ATC GCT C	Allele-specific PCR (2)
VicJ	*X67428*	*Vicilin J*	GGC TAA CCG AGA TGA CAA CG CTG TGT TGT GGC TCT TGT TCC	Allele-specific PCR (2,3)
TI1	*AJ276900*	*Trypsin/chymotrypsin inhibitor*	TCT ACA GAT GTG CAT TTC GTC CAT GAT ACA TAG TTA TAC TTG CT	AluI (2); length polymorphism (3)
Met2	*AB176565*	*Metallothionein*	AAC TGT GGT TGC GGT ACT AGC TTA TTC TAT AAC TCC AAA AGG GCG	RsaI (2); length polymorphism (3)
Fbpp	*AF378925*	*Fructose-1,6-bisphosphatase*	CCT TAC TCT CCT TCA CGT CT CTT TTC AAC CTT CTC CAC CT	Eco47I (3)
Pme1	*AF081457*	*Pectin methylesterase*	GTT CAA AAC TGT GGC TGA GTG TTC TGG TTT GGG TCT TCT C	MnlI (3)
UDPgd	*At-NM_123294* *Gm-U53418*	*UDP-glucose dehydrogenase*	TGG TGA AGA TTT GCT GCA TTG GTG C TCA TGG ATA GAT CCC TCT GG	HhaI (3)

* Accession numbers relate to pea genes, except those starting with At and Gm (*Arabidopsis thaliana* and *Glycine max*, respectively)

** Numbers in this column indicate the number of segregating population (Pop1, 2 or 3)

sites had been tested on creation or destruction of recognition site of endonuclease, and the corresponding enzyme was used to detect allele specificity of PCR products (Table 1).

Recombination analysis detected genetic distances between markers (Table 2), and genetic maps were constructed for each cross individually (Figs. 2A, B, C). For *coch* and *sym*27 gene-based markers flanking genes

of interest had been determined, while we were not able to find suitable markers flanking *crt* from the top of linkage group V. The segregation of *sym27* and several markers segregating in Pop3 appeared to be distorted

Table 2. Segregation data for adjacent markers in all 3 populations studied.

Gene pair	Number of progeny										Linkage (Haldane cM) ± SE	LOD	Joint χ^2	$P_{(0.5)}$
	PP	PH	PQ	HP	HH	HQ	QP	QH	QQ	Total				
Pop1														
crt - Rpl24A	15	2	0	-	-	-	1	57	24	99	3.3 ± 1.9	15.05	48.11	1.12E-08
Rpl24A - Enol	16	0	0	0	58	1	0	1	23	99	1.0 ± 0.7	37.29	154.07	1.07E-30
Enol - Paal2	15	2	0	1	49	3	0	0	21	91	3.3 ± 1.4	28.61	127.31	4.73E-25
Pop2														
Rpl24A - Enol	22	0	0	1	43	0	0	1	15	82	1.2 ± 0.9	31.74	143.41	1.91E-28
Enol - Paal2	14	1	0	2	27	2	0	1	9	56	5.5 ± 2.3	15.47	74.14	5.77E-14
Paal2 - coch	11	-	5	7	-	22	0	-	11	56	20.2 ± 5.5	3.80	20.04	2.72E-03
coch - Apy	20	-	4	-	-	-	3	-	64	91	7.9 ± 3.0	12.31	60.72	3.21E-11
Apy - VicJ	19	2	1	-	-	-	4	46	21	93	8.8 ± 3.1	11.46	56.30	2.53E-10
VicJ - TI1	22	1	0	1	46	1	0	2	19	92	2.8 ± 1.2	31.59	146.72	3.82E-29
TI1 - Met2	10	9	0	3	37	5	1	5	12	82	16.3 ± 3.3	9.10	40.34	3.91E-07
Pop1														
VicJ* - TI	7	2	0	0	43	1	0	0	24	77	2.0 ± 1.2	27.00	131.42	6.46E-26
*TI1** - Pme1*	5	2	0	6	34	5	1	12	12	77	20.2 ± 3.9	5.84	24.14	4.92E-04
*Pme1 - Met2**	5	8	0	0	38	9	0	0	17	77	11.9 ± 2.9	11.96	59.78	4.99E-11
*Met2** - Fbpp**	5	0	0	0	38	2	0	0	22	67	1.5 ± 1.1	24.39	118.10	4.08E-23
*Fbpp** - sym27**	2	0	0	2	21	4	0	4	14	47	11.4 ± 3.5	8.28	44.28	6.50E-08
sym27 - UDPgd*	5	1	0	1	26	2	0	3	15	53	6.9 ± 2.6	12.94	64.36	5.83E-12

* – segregation ratio of marker deviates from 1:2:1 (P<0.05)
**– segregation ratio of marker deviates from 1:2:1 (P<0.01)
P – allele of 1st marker, Q – allele of 2nd marker, H – presence of both alleles; "-" indicates that marker had been analyzed as dominant.

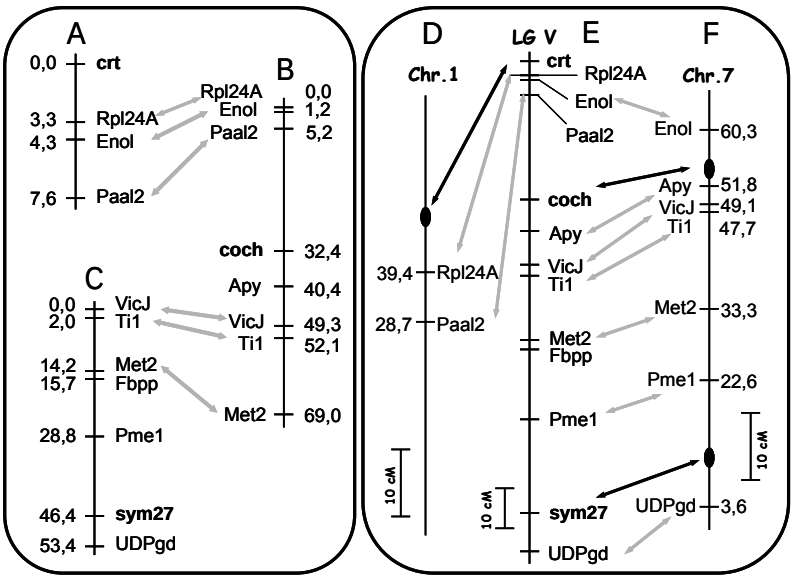

Fig. 2. Genetic maps of pea linkage group V and corresponding parts of genome of M. truncatula (www.medicago.org/genome)
A – genetic map built on the basis of Pop1;
B – genetic map built on the basis of Pop2;
C – genetic map built on the basis of Pop3;
D – part of chromosome 1 of M. truncatula*;*
E – joint map of LG V of pea;
F - part of chromosome 7 of M. truncatula.
Black ovals indicate the positions of presumable orthologs of pea genes of interest.

when compared with a classical 1:2:1 ratio, but linkage between all of them was significant (Table 2).

Genetic maps for all three crosses were compared in order to create a map of pea LG V, corresponding to the karyotype of SGE line (Figure 2E). Also, comparison of the linkage map of pea LG V with physical maps of chromosomes 1 and 7 of *M. truncatula* was performed. *M. truncatula* BACs containing corresponding homologs of pea genes used as markers were detected by CViT-BLAST search against pseudochromosomes of *M. truncatula* (available at www.medicago.org/genome), and the genetic positions of identified BACs were used for creating comparative maps of pea LG V and chromosome 7 and part of chromosome 1 of *M. truncatula* (Figs. 2D, E, F; 3), on which the positions of probable orthologs of the mutated pea genes were detected.

Table 3. Positions of homologues of pea markers in *M.truncatula* genome (according to data deposited on www.medicago.org/genome).

Name of pea marker	*M.truncatula* BAC containing homologue	Accession number of *M. truncatula* BAC	Chromosome of *M.truncatula*	Position on *M.truncatula* genetic map, cM
Rpl24A	mth2-11a6	AC146788	1	39.4
*Enol**	mth2-21l10	AC148219	6	60.3
Paal2	mth2-17f11	AC146719	1	28.7
Apy	mtab-58m19	AC145753	7	51.8
VicJ	mth2-36p20	AC148289	7	47.8 – 49.1
TI1	mth2-27l9	AC135311	7	47.7
Met2	mth2-165g15	AC147202	7	33.3
Fbpp	Not known	Not known	Not known	Not known
Pme1	mth2-33b23	AC122166	7	AC122166
UDPgd	mth2-23c14	AC119411	7	AC119411

BAC containing homologue of *Enolase* is placed in chromosome 6, whereas results of genetic mapping of *Enolase* place it in chromosome 7, at the position 60.3 cM from the top.

Discussion

In pea, a long history of genetic mapping with use of morphological and molecular markers resulted in a genetic map containing positions of numerous mutations (7, 28). In our work we performed the genetic mapping of three pea mutations in relation to gene-based markers, in order to exploit the synteny of pea and *M. truncatula* genomes for cloning and sequencing the mutated pea genes. Mutants obtained in the line SGE were crossed with genetically remote lines, expecting to get a high level of polymorphism in segregating populations. Indeed, the sequences of tested markers were polymorphic, and the most remote line 87-18 I-r exhibited length polymorphism of *TI1* and *Met2*. Nevertheless, this line appeared to carry the JI1794 allele of *Sym*22 leading to formation of a decreased number of nodules, and this made the analysis of symbiotic traits difficult in this particular case.

The use of several mutants obtained in the same line, SGE, as well as the use of the same markers, let us combine the results of three independent crosses and calculate the resulting map of LG V. The order of markers used, as well as the genetic distances between them, is in good agreement with previously published pea maps (www.comparative-legumes.org). However, the new results on mapping *crt* are in contradiction with those described in the article of Kuznetsova et al. (17), even though they are obtained on the same segregating population. We attribute this discrepancy to the fact that most of the markers used by Kuznetsova et al (17) were dominant markers, and when we took into consideration additional co-dominant markers (and excluded dominant morphological markers *r* and *tl*), the genetic distances between markers changed noticeably. We therefore consider these results on *crt* mapping as an update of the previous ones.

The comparison of relative positions of markers on pea and *M. truncatula* maps confirmed the high level of macrosynteny between pea LG V and chromosome 7 of *M. truncatula* and revealed a small region of synteny between the top of pea LG V and the middle part of chromosome 1 of *M. truncatula*. The phenomenon of

synteny makes it possible to define the position of probable orthologs of pea genes of interest in the genome of *M. truncatula*. Based on localization of *cochleata*, the position of *M. truncatula* orthologous gene was calculated to be around 54.0 cM on the map presented on www.medicago.org. A *Tnt*1-disrupted gene of *M. truncatula* with a similar phenotypic manifestation is localized exactly in this site and, therefore, is probably orthologous to *coch* (P. Ratet, unpub.). The positions of orthologs of *sym*27 and *crt*, are not detected so accurately, for two reasons. First, the saturation of the *M. truncatula* physical map is not high enough throughout, and the regions probably syntenic to those of *crt* and *sym*27 still need to be sequenced properly. Second, the homologs of the markers *Rpl*24A and *Paal*2 (that are close to *crt*) lie not in chromosome 7 (as the homolog of *Enol* does), but in chromosome 1, suggesting the existence of synteny between pea LG V and chromosome 1 of *M. truncatula* and making uncertain the position of the orthologue of *crt*. Therefore, some additional markers need to be designed based on sequences of *M. truncatula* genes localized in "syntenic regions", paying particular attention to genes of transcriptional regulators and membrane proteins as the most probable candidates to be *crt* and *sym*27, respectively.

Conclusions

Genetic mapping of pea genes makes possible gene cloning based on homology with known genes of model objects *M. truncatula*, *A. thaliana* and *Lotus japonicus* (Regel.) Larsen, and the exploitation of syntenic relationships between legume plants rapidly facilitates the work. To date, eight pea symbiotic genes have been cloned using this approach (3), and precise mapping of *crt*, *coch* and *sym*27 with the use of cross-species gene-based markers will draw us near to the cloning and sequencing of these genes. The set of developed gene-based markers of pea LG V is also planned to be used for mapping the symbiotic pea genes *sym*16 and *sym*38, previously localized in pea LG V (14, 29). In general, functional mapping seems to be a fruitful approach for cloning pea genes related to symbioses.

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