# Sequences of major Genes in the Pisum Genus

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## Summary of project

Partial sequences are being obtained from 22 major genes in a wide diversity of *Pisum* germplasm with the intent of investigating phylogenic relationships among the accessions and determine if there was sufficient variation to permit the unambiguous identification of most commercial varieties. The genes selected either controlled traits of interest to breeders or were tightly linked to such genes. The genes were also selected to be widely distributed across the pea genetic map, with at least two on each linkage group. In order to maximize sequence polymorphism, intronic regions were targeted by using primers orthologous to coding sequences flanking larger (500-1000 bp) introns in the gene or two adjacent introns separated by a short exon. Preliminary studies on commercial varieties showed that not all large introns displayed sequence polymorphism, and data for several genes were discarded due to low polymorphism, or other regions of the gene showing greater sequence variation were selected for the larger study. The final list of genes selected, primers used, location of gene on linkage map and size of amplicon are given in Table 1. These included genes/markers for BYMV resistance (*mo*), PEMV resistance (TI-en), SBMV resistance (*sbm1*), Fusarium wilt race 1 resistance (NTFY), powdery mildew resistance (*er1*), two root rot QTLs, several seed starch or protein genes, two flowering time genes, a stem branching gene, and several genes influencing leaf morphology.

Table 1.	Genes studied, primer sequences, ar	d location of gene on genetic map				
<u>Gene</u> symbol	Protein encoded	Phenotypes affected	Forward primer	Reverse primer	<u>LG</u>	<u>Length of</u> amplicon
Pur	myb transcription factor	Purple pod	GAACATAAGAAGTTCCTTGAAGC	CCAATATCTGAACTTCCACGC	Ι	1040
Alatc	cytolsolic alanine aminotransferase	marker in middle of LG I	CTGACCTTCCCTCGCCAG	CCTTGCTCGCACCATCAGT	Ι	770
1	staygreen	cotyledon color	GATGTCTCTACATGTTCACTGTCAC	AGAGGACCCCAACATTCTACCTTG	Ι	1000
CvcA	convicilin A	seed protein	CACCATCTTCCTTCCTCAACAC	TGTGGCAACATTAGAGCTCCC	Ш	850
Мо	eukaryotic initiation factor 4E	virus resistance	AAATGCCCTCTCAGAGCCA	TATCGAGCCTTTGCACCTC	Ш	800
Rb	ADP-glucose pyrophosphorylase	starch synthesis	GATTGTACAATCCAACACTCC	CTATCTGCTTCTTGAACGCC	Ш	470
Rms1	carotenoid oxygenase	basal branch number	CAAAATCATTCGACCGTCGC	CTTAGATTACAGAACCTTCGCTC	Ш	840
Tl-en	Marker for En gene	pea enation virus resistance	AGTCTATCGCCGTTGAAGCC	GGATTGAACATCTTCCTTTCCTC	Ш	500
SIn	gibberellin 2-oxidase	stem length	CCAACTTCAGAACAATACACC	CTGAAATCCAGCTACCATCTC	Ш	740
Ntfy	nuclear transcription factor Y	marker for Fusarium wilt race 1 resist	CAGAATGCCTTTGCCCCT	CCTCTTGCATCTTGTCGC	Ш	900
Ko1	ent-kaurene oxidase	stem length	CACAAAGATGGCTCAGAAATATGG	CATTGGCTCCAAGAACACTTGC	Ш	560
GlyOH	glucan endo-1,3-beta-glucosidase	marker for upper portion of LG IV	TGATCACTACCACGGATCCCA	ACCTGCAGAAGTATTCCCCAC	IV	800
Peam5	MADS box transcription factor 5	control of inflorescence	GAGTTGGTACCAAGAAGTGTCT	GGTATGAAGGCTGCTGAAAGG	IV	800
Fro	ferric chelate reductase	iron metabolism	AGAACTCCAACACAAGATAAACA	CCCTTAGAGAAGTTGAGGTTCAACGG	IV	900
VicJ/K	Vicilin J/K	seed protein	ATCCGATCAAGAGAACCCC	AGAACCTTTTCTATCTCCTCG	V	1400
Fbpase	Plastid fructose 1,6-bisphosphatase	starch synthesis	CATCCAATCTTGATGCTGCAG	CTTTTCAACCTTCTCCACCTC	V	1200
SulTr	sulfate anionic transporter	sulfate metabolism (marker for lowe	GCAAAATTGGCTGGACTTCAACC	ACGCACTCCAGTACCCCAACC	V	1100
Er1	Mlo family protein	powdery mildew resistance	AGCTATGTGACTTTGCCTCT	AGTCTTGCATTTTCATACCC	VI	1100
Gpic	cytosolic glucose phosphate isomeras	carbohydrate metabolism	GCATCTCGCACAAAAGGAC	TAGTTGGAGGATCAGCTGG	VI	450
Sbm1	eukaryotic initiation factor 4E	seedborne mosaic virus resistance	GAAATGGACTGCGAACTATCCG	GAAACTCCTTCCACTGTTTTCC	VI	1400
Pgmc	cytolsolic phosphoglucomutase	carbohydrate metabolism	TGTACTGGTGATGGTCGTT	AGAGACTGCAGGAGTTGAA	VII	630
Skdh	quinate/shikimate 5-dehydrogenase	pathway to aromatic compounds	GGTGGTAAGTATGATGGTGATG	CCCATAACAAGTCCAATGAATGG	VII	1000
Pao	pheophorbide a oxygenase	marker for daylength sensitivity	GCTTTTCGGGAGCTAATGAAGGG	GTGTGAAGGTGAGGCTTGTGTAC	VII	500

## **Notes on Specific Genes**

#### Er1 Fend (long) or Fend2 (short)

There are two datasets for *Er1*, the gene which confers powdery mildew resistance in pea, and three primer sets were used. In the case where the ER1 FEND and REND primers were used, there is polymorphism; however, most of the polymorphism (including a very nice SSR sequence) is in the portion of the gene that was sequenced when the Er1-FEND primer was being used. As the Er1-FEND priming site is nearly 2000 bases from the Er1-REND priming site, the sequences obtained using this combination (noted as 'long') sometimes did not overlap. Because of this, the Er1-FEND2/Er1-REND combination (noted as 'short' sequences) was used for most of the variety sequencing. There is little polymorphism among varieties in this region. Future work should obtain the Er1-FEND sequence for at least a sample of these varieties (some susceptible and some resistant to powdery mildew).All the lines gave product with the Er1-FEND2 and Er1-REND combination. As was the case for a Er1-F4/Er1-RF1 combination, there is no DNA polymorphism in this region that corresponds to susceptibility/resistance to powdery mildew.

Location of primers for the ER1 gene. The sequence for Er1-FEND and Er1-REND combination includes the priming site Er1-FEND2 and all of the Er1-FEND2/Er1-REND sequence, the green highlighting is broken in the Er1-FEND2 region to permit yellow highlighting of this priming site).



TCAACCCCAACACGCCATGTCTCCTGTTCACCTGCTCCATAGACACAC TGCTGGAAACAGCGACAGTCTACAAACTTCTCCCGGAAAAGTCTGATTATA AAAATGAACAGTGGGATATTGAAGGAGAAGGACCAACTTCCCTAAGAAAC GATCAAACAGGGCAACATGAGATTCAAATAGCGGGTGTCGAGTCATTTTC GTCAACCGAATTGCCGGTTAGAATTAGACATGAAAGCACCTCTGGTTCAA AAGATTTTTCTTTCGAGAAGCGCCACTTAGGGAGCAATTAGAATTGTAGA TATTGATAACCAGTTCAATGTATACCAATTAGGTACATTCTTGCAGATAA AGATAGAGGAACTCCTTCTAAGAATGGAGTGTAAATTTGTTGAGGTAGCA GCTTGATTTGTGGATATAATCATAGGGTATGAAAATGCAAGACTATATTT Er1-Rend

TGTGAATTTTGTTTCCTGTGCAAATGATTAATGCTTAGTTTG

#### Fbpase Fructose bisphosphatase

Attached is the next data set (for a section of the plastid-specific fructose bisphosphatase gene). It is not very polymorphic, giving only 2 major alleles in the varieties I screen. Thus, I did not pursue this gene extensively. The schism between varietal groups did follow that between two standard wild accessions (JI 1794 and JI 261), suggesting that the ancestry of the two alleles goes back quite a ways. JI 1794 is considered one of the nearest ancestors of domesticated pea, so those varieties with the other allele (more similar to that in JI 261) may contain a section of LG V that was introgressed from a different wild source for some special trait (which is unknown to me). May be worth letting Rebecca look it over.

#### FRO (Ferric chelatase reductase)

Attached is the sequence data for ferric chelatase reductase (FRO). The gene from the landrace 'Slow' was sequenced by Mike Grusak a while ago and is given in complete form with primer sites marked in yellow and the sequenced portion highlighted in green. The gene is interesting because it marks a portion of the pea genome thought to contain a QTL for resistance to fusarium wilt race 2. The portion sequenced turned out to be reasonably polymorphic and grouped varieties into interesting assemblages (most of the winter pea types formed a clear group). Thus, the gene may be important for variety ID. The primer sequences are not precisely homologous to the sequence obtained for 'Slow'. It may be that better primer sequences can be generated, but the Table will contain those I used.

#### Fructose biphosphatase

Attached are the sequences for plastid-specific fructose bisphosphatase. There isn't much variation within the varieties (2 major alleles), but the alleles are quite different. I didn't do enough of the elatius accessions to be able to pick out the sources of the two alleles, but there is some variation among the elatius I did examine.

#### Gib2betaOH-2. Gibberelin 2 Beta Hydroxylase

I thought I was amplifying a sequence on LG IV (primers were designed to the medicago homolog of a gene on LG IV) and then thought based on initial mapping data that it might be the SLENDER locus on LG III. However, now that the pea genomic sequence is out, it shows closest homology to a sequence of the other gibberellin 2 oxidase on LG VI (best referred to as gibberellin 2-beta hydroxylase). I still intend to confirm the LG VI linkage, but for now I will accept that it is a homolog of SLENDER on LG VI (Jim Reid

reported both genes). I am calling the gene 'Fo' in the data set, but it probably should be referred to as 'Gib2betaOH-2.' Despite these issues, the primers amplify it specifically, and there are 5 alleles in the cultivated germplasm, making it very useful for distinguishing varieties.

#### *Gpic* The Glucose Phosphate Isomerase Gene

Here are the sequences for the end of the cytosolic glucose phosphate isomerase gene (Gpic). There wasn't a lot of polymorphism in this sequence, so sequences were not generated on the more recently released cultivars. However, a number of P. fulvum accessions obtained from Shahal Abbo in Israel were sequenced. He has published on these accessions, so there is passport data available on each. There are a couple of longer sequences (JI 261, WL808, Majoret) for which the primer Gpic-FendA was used instead of Gpic-Fend. However, there wasn't much additional polymorphism revealed, so the consistently good Gpic-Fend/Rend combination was used.

#### Мо

This gene controls resistance to several viruses, including bean yellow mosaic virus. The part sequenced is relatively short and not particularly polymorphic with only 2 major alleles. Thus, sequencing wasn't expanded to most of the wild accessions.

#### Pao

Hi again, Jamin. Here are the data for PAO, a short gene segment with reasonable variability, including a number of indels. This gene is right next to the primary gene affecting flowering sensitivity to daylength (Sn) and should be an excellent marker for breeders wanting to follow daylength sensitivity in crosses. I tried to work on the Sn gene directly, but it is too short and there was little sequence variability.

## *Pgmc* The cytosolic phosphoglucomutase gene

The section sequenced is relatively short (~600 nucleotides) and spans just one intron. However, it did show considerable polymorphism in the general Pisum germplasm and split the commercial varieties into three major sections. As before, the *M. truncatula* gene genomic sequence is included with the exons marked in yellow as well as the approximate priming sites in blue. A cDNA sequence from pea is also included, with the priming sites highlighted in blue. The sequences are very good, with few missed calls.

#### Pur/MybPur (Purple Pod)

Attached are the sequences for a portion of the MybPur (purple pod, Pur) gene on LG I of pea. The sequences are of variable length because several primers were used (the two main pairs, MybPurF1/R1 and MybPurFseq/Rseq, are highlighted on the genomic sequence for the gene from Cameor at the top of the file). I started with the F1/R1 primer pair, but found that in some cases the forward and reverse sequences did not overlap sufficiently to give complete confirmation. The Fseq/Rseq produced a shorter product that gave complete sequence confirmation, so I switched to this pair in the latter part of the

study; however, when I compared F1/R1 sequences and Fseq/Rseq sequences for a number of accessions, the F1R1 sequences proved highly reliable. Hence, I am very confident about the accuracy of the longer sequences in the data set. The portion of the gene I sequenced shows a good level of polymorphism, so it should be useful for distinguishing varieties. The gene is also relatively closely linked (<10 cM) to a cluster of genes involved in nodule formation (Lghb, sym2, Nod3) and may be useful in breeding programs focused on improving nitrogen fixation. Let me know if you have any questions.

## RB gene

Allele designations are as follows:
A-1: The large group containing Almota, Amarillo, Arvika.
A-1a: PI261624 (only differs from A-1 by one base, which could be due to a PCR error)
A-2: Mexique, PI 343338, Admiral
A-3: JI 261 (a wild P. sativum with some fulvum in it)
A-4: St Maurin
B-1: the large group containing Ambassador, Atlas, Darien, etc.
B-2: JI 12794 and Cruiser
B-3: Early Freezer
B-4: PI 220174

B-5: Primo B-6: WL808 (abyssinicum) B-7: P. fulvum B-8: VIR 6070 B-8a: VIR 6071 (again differs from 6070 by only one base)

## RMS1

RMS1 isn't very polymorphic, with most varieties possessing the same allele. I didn't do many elatius samples, and even some of these had an allele very similar to the common one found in domesticated lines. The part sequenced was only the last third of the gene, so maybe other sections are more variable.

## SKDH

Attached is the data set for SKDH. This was a fairly large (>1000 nt) fragment and the forward primer didn't work particularly well, so the 5' end is occasionally missing. However, there is significant variation, even within cultivated lines. The variation within cultivars appears to be primarily due to introgression off this region of LG VII from different wild forms rather than diversification post cultivation. This region of LG VII is not known to possess highly important genes for breeders, so perhaps that is why a number of alleles from elatius types are present in the cultivated material.

## SGR Staygreen; (Mendels green/yellow cotyledon gene)

These are sequences for an intron (plus flanking coding regions) in Mendel's green/yellow cotyledon gene. This is (by far) the most polymorphic of the gene segments that I have sequenced. I almost eliminated this choice when I was prescreening possible genes because many of the varieties do not give

a product (I think because even the priming sites occasionally mutate). For the varieties that gave product, at least 4 alleles were present, so it is useful for variety fingerprinting. However, I think its real value will be for discriminating among wild accessions and possibly landraces. Virtually every P. fulvum and P. sativum elatius accession tested possessed a different allele, and most differed for several indels, not just SNPs. This sequence can serve as a built-in bar code for identifying accessions (both distinguishing among and identifying duplicates). I will get sequences for all the remaining fulvum and 'elatius' PI's as well as a significant set of landraces. The wild accessions should all be distinguishable, and any 'unknown' or mislabeled accessions should be able to be assigned to the correct original collection. I suspect that W6-15019 and Wt 109 are derived from the same original accession if you don't have passport data showing that already. The landraces display less diversity, and their inclusion in the additional screening would be more to understand how useful the SGR sequence might be in categorizing them. Note that most of this variation is in the yellow cotyledon type. I haven't really analyzed the green cotyledon varieties extensively, but I think they would be much more limited in their polymorphism.

#### *SulTr* The Sulfate Anionic Transporter Gene on LG V.

This gene fragment had limited polymorphism within cultivars, and I did not do many of the landraces or wild accessions, although the five elatius and fulvum accessions for which I obtained data all had unique alleles.

#### Tl-en

On the medicago sequence the upper highlighted region corresponds to the F1R1 primers (priming sites in yellow, approximate amplicon in green). The second highlighted region reflects the F2R2 primers. Primers were switched after the first plate of sequencing because the results were strange. The F1/R1 primers gave a shorter fragment than I expected, but the sequences continued beyond the reverse primer, indicating there might have been a second priming site. I didn't trust the sequence beyond the reverse primer (edited out in the data I sent), and because there was so little polymorphism, I switched to the F2/R2 combination (which amplified the second section highlighted in green). Although these primers gave the predicted larger fragment, there was also little polymorphism, so I did not do many *Pisum elatius* samples. Each of the sequences in the data is labeled either F1R1 or F2R2 so that you can quickly separate these into two groups.

#### SBM1

Here are a batch of Sbm1 sequences. This is a very interesting gene because not only is it an important virus resistance gene and there is lots of polymorphism (although much more limited in commercial varieties), but there are also another 100 or so full gene sequences on the NCBI database that make useful comparisons. Nearly all the sequences I generated are new (JI 261 had been sequenced before), so although many of the varietal sequences are identical, we have an excellent sample of the variation in the genus.

## **The Accessions Analyzed**

Table 2 presents the set of accessions and varieties analyzed. The accessions included a sampling of fresh market and freezer cultivars, dry pea varieties, and a few representatives of other commercial types. Most of these were analyzed for all 22 genes. In addition, a wide sampling of landraces, including at least one accession of *P. sativum* ssp *abyssinicum*, was included in order to sample as much of the genetic diversity as possible within the domesticated pea germplasm. Again, most of these were analyzed for all 21 genes. Accessions of what were believed to be wild *Pisum* germplasm (here defined as *P. sativum* ssp. *elatius* and *P. fulvum*) had been received by the PI from germplasm storage facilities and collaborators over the last 40 years. Except for a few accessions such as JI 1794 and *P. fulvum*-19, sequences from these were obtained for only about half of the genes. The reasons for the more limited survey of the wild germplasm were that the more extensive germplasm survey represented an add-on to the original focus of the project and resources became limited near the end of the project.

Over 40 different alleles were identified within the freezer and fresh market lines (not including the PI's or wild accessions), permitting us to easily distinguish all the commercial varieties examined. Similarly, nearly all dry pea varieties were able to be individually identified by a combination of sequence variability and a small set of monogenic morphological traits such as green vs. yellow cotyledon color, normal vs. semi-leafless type, colored vs. white flowers, etc.

The broader analysis of *P. fulvum* and the 'wild' P. sativum accessions revealed that P. fulvum represents a distinct but highly variable taxon, as has been determined by numerous previous studies. Not all the accessions designated as *P. sativum* ssp. *elatius* (or *P. elatius*) possessed dehiscent pods. The result of the sequencing analysis confirmed that many of these 'wild' accessions were either generated by hybridization between domesticated and wild lineages or had been misidentified and were clearly *P. sativum* ssp *sativum* (note that neither *P. fulvum* nor *P. sativum* ssp. *abyssinicum* accessions displayed such ambiguity). The sequences obtained from the broader germplasm analysis will be used to better define this germplasm taxonomically.

Seed samples obtained by selfing each of the lines/accessions examined will be submitted to WRPIS as voucher specimens.

#### Table 2. Lines or accessions investigated

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Admiral	Dakota	
Agassiz	Darien	
Alaska	Dark Skin Perfection	
Almota	Delta	
Alsweet	Early Columbia	
Amarillo	Early Freezer	
Ambassador	Encore	
Amigold	Frimousse	
Aragorn	Frisson	

Mozart Navarro Neptune Novella II Old Muffin Oregon Sugar Pod Orka Perfect Freezer Primo

Arcadia Arvika Atlas	Gain Green Arrow Golden	Progress #9 Ranger St. Mauren
Badger	Gunner	Salamanca
Bohatyr	Hyline	Scout
Bolero	K2	Shamrock
Bonneville	K90-2131	Spider
Blue-podded Shelling	Lifter	Stirling
Bridger	Little Marvel	Striker
British Wonder	Lincoln	Sutton's Harbinger
Carneval	Majoret	Telephone
Champion of England	Mars	
Cooper	Mexique	
Cruiser	Miragreen	
LISDA Plant Introduction accossi	ons and their affinity group as desc	ribod in Kwon ot al. (2012)
PI 109866	Venezuela	Group b of Kwon et al. (2012)
PI 206838	USA	
	USA	Group d of Kwon et al. (2012)
PI 210583		Group b of Kwon et al. (2012)
PI 220174	Afghanistan	Group a-2 of Kwon et al. (2012)
PI 250440	Czech Republic	Group b of Kwon et al. (2012)
PI 261624	Spain	Group c of Kwon et al. (2012)
PI 269804	United Kingdom	Group c of Kwon et al. (2012)
PI 285730	Poland	Group c of Kwon et al. (2012)
PI 343338	USA	Group d of Kwon et al. (2012)
PI 411142	New Zealand	Group d of Kwon et al. (2012)
Pisum 'elatius' accessions		
JI 261	Southern Turkey	Group a-1 of Kwon et al. (2012)
JI 1794	Golan Heights, Israel	Group a-1 of Kwon et al. (2012)
Pisum fulvum accessions		
VIR 6070	Israel	
VIR 6070 VIR 6071	Israel	
	101 0 01	Abbo
P. fulvum numbered accessions	Israel	UUUA

#### References

Kwon S.J., A. Brown, J. Hu, R. McGee, C. Watt, T. Kisha, G. Timmerman-Vaughan, M. Grusak, K. McPhee, C. Coyne. 2012. Genetic diversity, population structure and genome-wide marker-trait association analysis emphasizing seed nutrients of the USDA pea (Pisum sativum L.) core collection. Genes and Genomics 34:305-320.