Pheophorbide a monooxygenase (*Pao*) is located on LG VII near *Amy* in pea and lentil

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Introduction

The gene coding the enzyme pheophorbide a monooxygenase (PAO) was first identified by a light dependent lesion mimic in *Zea mays*, called the lethal leaf spot mutation (*lls1*) (6). The leaves of this mutant stay green during dark incubation and accumulate pheide *a*, causing a light-dependent lesion mimic phenotype. The protein contains a Rieske iron–sulfur cluster and conserved C-terminal motif in both plant and cyanobacteria (6). PAO catalyzes the third step in chlorophyll degradation (Fig. 1).

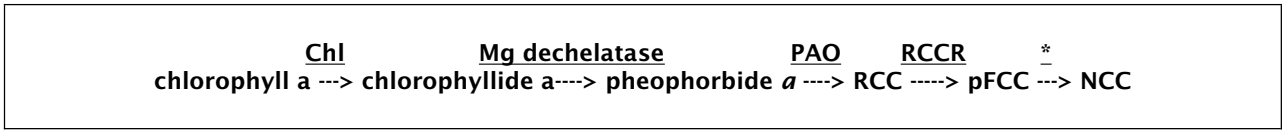

Fig. 1. Basic pathway of chlorophyll degradation.
Substrates: abbreviations – RCC, red chlorophyll catabolite; pFCC, primary fluorescent chlorophyll catabolite; NCC, non-fluorescent chlorophyll catabolite)
*Enzymes: abbreviations – Chl, chlorophyllase; PAO, pheophorbide a oxygenase; RCCR, red chlorophyll catabolite reductase; *low pH and multiple enzymes)*

PAO has also been shown to be a member of a small non-heme oxygenase family that includes chlorophyll *a* oxygenase (CAO), choline monooxygenase (CMO), a 55 kD inner chloroplast membrane translocation protein (Tic55), and a 52 kD translocation protein for protochlorophyllide oxidoreductase A in chloroplasts (Ptc52) (7). Homologs of *Lls1* (*Pao1*) have been subsequently identified in *Lycopersicon esculentum, Oryza sativa, Arabidopsis thaliana, Medicago truncatula, Pinus tuneada, Solanum tuberosum*, and *Vigna unguiculata* (8, 21). The *A. thaliana* homolog, *accelerated cell death* (*Acd1*, At3g44880, gi: 42565541), displayed less than 80% sequence identity with the *Zea mays* gene and was not universally accepted as the *Pao1* ortholog until the latter gene was used to complement an *Acd1* null mutant in *Arabidopsis* (21). Additional articles have been published confirming and restating the *Lls1* product is PAO (8, 9, 12).

The 'stay-green' phenotype of the *lls1* mutant lead some researchers to propose *Pao* as a candidate for Mendel's *i* gene in pea, particularly after it was demonstrated that PAO activity was greatly reduced in green cotyledons (15). This study has led to the acceptance by many that the genetic basis of the *i* mutant is known (4, 15, 16). Here we present evidence that the *Pao* gene in pea is located on LG VII, not on LG I and, therefore, cannot be synonymous with the genetic defect generating the green/yellow cotyledon polymorphism.

Materials and Methods

Amplification of PsPao: Homologous mRNA sequences were obtained using a tomato EST (AF321984 [gi: 16973464]) for BLAST at NCBI; identifying *Arabidopsis thaliana* (At3g43880 [gi: 42565541]), maize (U77346 [gi: 1935910]), *Medicago truncatula* (TC100809) and *Vigna unguliata* (gi: 30088963) for comparison of highly conserved regions and positions of introns. The gene structure (Fig. 2) and expected fragment sizes of the primer sequences in *Pisum, Lens, and Phaseolus* (Table 1) is presented below. The primer sequences used for amplification were Pao-F2 (5'GGAGAGATCATTGGTAYCCAGTTTC3') and Pao-R3 (5'GTGAAMGTGAGSRTTGTGTACTG3'). In order to prevent mis-priming at lower annealing temperatures a touchdown protocol was used with each primer

pair for sequencing. Cleaved Amplified Polymorphic Sequences (CAPS) analysis (11) was performed with a standard PCR protocol (all primer combinations worked with a 58° C annealing temperature and a 2 minute extension) using *Taq* polymerase (Promega).

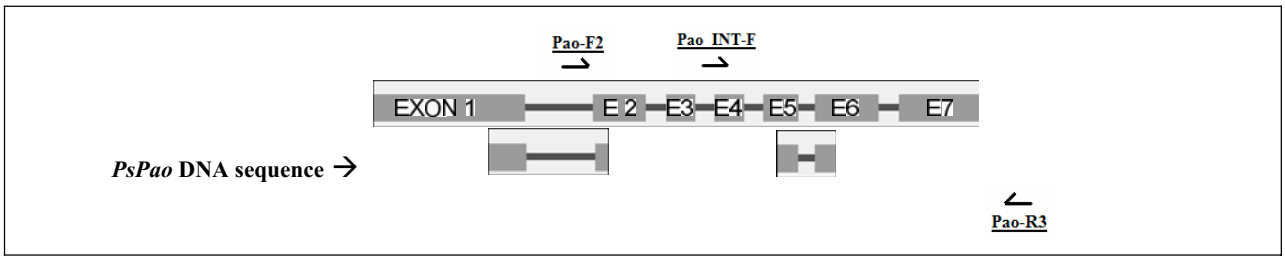

Fig. 2. PsPao *gene model based on the gene splicing of AtPao.*

The two *PsPao* fragments were cloned using a pCR2.1 kit (Invitrogen) according to the manufacturer's recommended procedures. The two clones PsPao-WL808 and PsPao-MN313 were partially sequenced from both ends. Internal primers were designed to permit sequencing of the remaining genomic sequence (final sequence not available).

Amplification of other gene fragments: PCR procedures used for isovaleryl dehydrogenase (IVDH), β-amylase, Sym29 and RAPDs were all done with Promega *Taq* polymerase. IVDH, β-amylase, and Sym29 were amplified using a PCR protocol with a 60° C annealing temperature, a 2 minute extension and 40 cycles. The RAPDs were done with a 37° C annealing temperature, 2 minute extension and 35 cycles.

Sequencing: Sequencing was carried out by the CORE facility at WSU-Pullman. Purified sequence and primers were sent to the sequencing lab for dye incorporation and sequencing.

Populations used for mapping PsPao: The sequence generated by Pao-F2/-R3 was initially mapped in the JI1794 x Slow RIL population that has been used as the basis of the *Pisum sativum* consensus map (3, 20). The map position was confirmed in two other pea RIL populations. The first of these is a [*P. sativum* ssp. *sativum* x *P. s.* ssp. *abyssincum*] x *P. s.* ssp. *sativum* BC_1F_5 population consisting of 62 lines. The second is an F_2-derived F_5 population from the cross PI 220174 x A1078-239. PI 220174 was collected in Afghanistan and possesses a phenotype (tall stem, round and yellow cotyledons, brown-mottled testa, small seeds) typical of many landraces in that region. A1078-239 is a multiple-marker line developed by and obtained from G.A. Marx. A final confirmation of the position of *Pao* was performed in the F_8 RIL population derived from the cross *Lens culinaris* 7 x *L. ervoides* 32 (13, 18, 19).

Detection of polymorphism: Polymorphism in DNA fragments was detected based on original fragment size, when different, or CAPS analysis. Fragments were separated on 2% agarose gels. The β-amylase polymorphism was also confirmed in the pea populations by isozyme analysis on a tris-citrate/lithium borate system (1) using young leaf extracts.

Estimation of recombination frequencies: Recombination frequencies between pairs of loci in F_2-derived RILs were obtained by the equation r = R/(2-2R) (5). For small recombination frequencies (r<0.1) this equation also provides a good approximation for r in the backcross RIL population.

Results

The fragment generated by the Pao-F2/R3 primer pair in pea was approximately the same size as predicted from the arabidopsis gene model (Table 1). Sequencing of the fragments amplified from MN313 and WL808 gave the end sequences presented in Fig 3. Approximately 95% homology was observed between the two lines with five possible SNPs, as presented in Fig. 3. Two additional primers were designed to the verified nucleotide sequence

and were used in mapping Pao. Sequences for the additional primers are Pao_INT-F: 5'-GCAGGAGGGAAGGATAGATGAG and Pao_INT-R: 5'-CTATCNCTTCTTCCTGTGACC.

Table 1. Primer Combinations – Expected vs. Actual Fragment Size in 2% SB agarose gels

	Exp. Pea Size	Act. Pea Size	Lens Size	Phaseolus Size
Pao_INT-F/INT-R	700bp	>700bp	900bp	Faint band
Pao-F2/INT-R*	1100bp	~1100bp*	1300bp*	Faint band
Pao_INT-F/R3*	1300bp	~1300bp/1200bp*	~1050bp	~1150bp
Pao-F2/R3+	1616bp	~1600/1700bp+	Faint band	Faint band

***Primers used to map Pao.**
+Primer pairs used in sequencing Pao.
SB – Sodium-borate buffer.

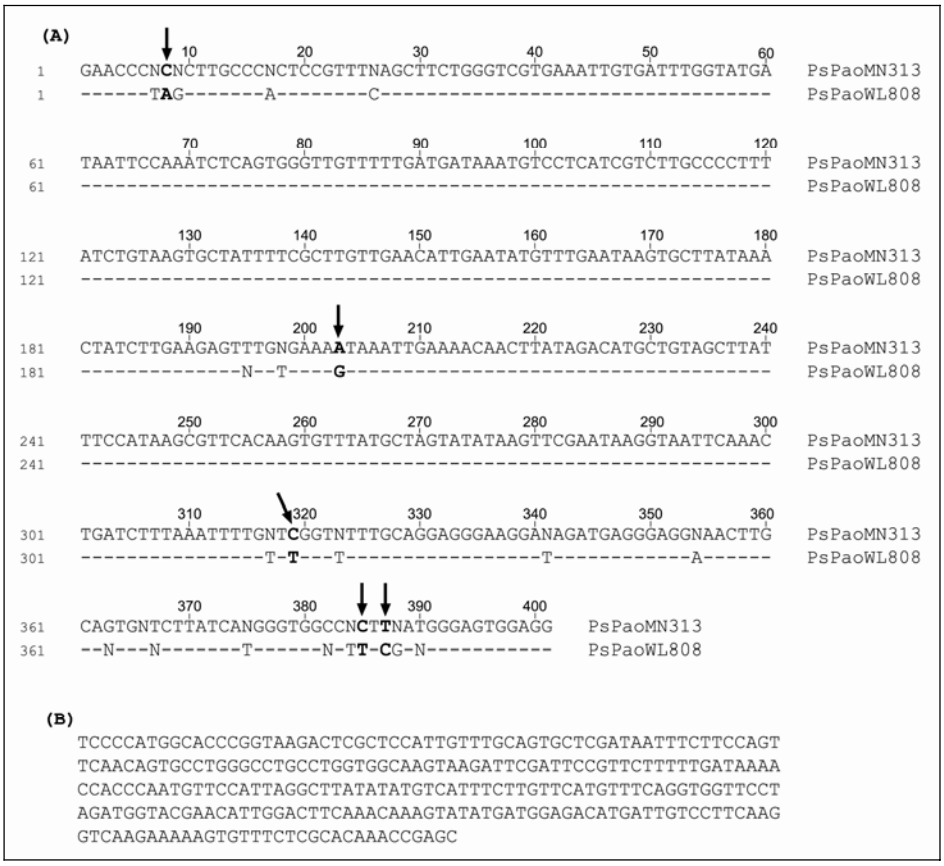

Fig. 3. End sequences of PsPao. (A) Comparison of MN313 (top) and WL808 (only changes indicated) sequences at the primer Pao-F2 end of the cloned fragments. Apparent SNPs are indicated by arrows. (B) Sequence (reverse complement) of the Pao-R3 end of PsPao-WL808 clone.

The size of the *Pao* fragment amplified by Pao_INT-F/R3 or F2/R3 differed between JI 1794 and Slow, allowing the segregation pattern to be observed directly on the 2% SB agarose gel without further restriction (Fig 4A). Similarly, a size difference was observed in the direct products amplified in the two *Lens* parents when the same primer pairs were used. In the other two populations CAPS was used to reveal a polymorphism, with Pao-F2/INT_R being used in the Abyssincium backcross RIL with *Cfo*I (Fig 4B) and Pao_INT-F/R3 in the Afghanistan RIL cut with *Hinf*I. In both pea and lentil a size polymorphism could be viewed when the second half or two-thirds of the gene was amplified, when the first half of the gene was amplified no size polymorphism was observed. Thus in both pea and lentil two alleles, both differing in the second half of the gene by ~100 bp, were observed.

Segregation of the various markers investigated in the analysis did not differ significantly from the expected 1:1 or 3:1 ratios expected in the F_2-derived or BC-derived RILs, respectively. Joint segregation analysis between *Amy* and *Pao* in each of the pea populations indicted a recombination value between 3 and 7 cM (Table 2). A comparable recombination value was obtained between the two loci in *Lens*.

Table 2. Joint segregation analysis between the loci *Amy* and *Pao* in four RIL populations Number of lines with designated genotype

Population	P_1P_1	P_1H	P_1P_2	HP_1	HH	HP_2	P_2P_1	P_2H	P_2P_2	N	r
JI1794 x Slow	19	0	3	0	0	0	0	0	27	49	3.3
BC1F5	11	0	3	0	0	0	2	0	46	62	4.4
Afghanistan	8	0	0	1	0	1	2	0	12	24	7.1
L.c. 7 x L.e. 32	22	0	2	0	0	0	0	0	22	46	2.3

Pao could be further fine-mapped in the JI1794 x Slow population using the dense marker map available for this set of RILs. The segregation data unambiguously placed *Pao* on LG VII between *Amy* and *Sym29* (Fig. 5).

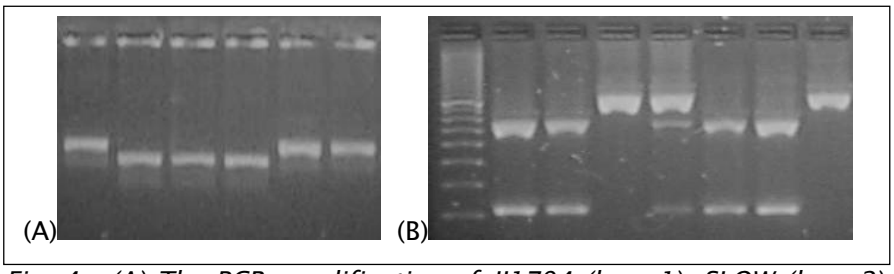

Fig. 4. (A) The PCR amplification of JI1794 (lane 1), SLOW (lane 2), JI1794 x Slow progeny (lanes 3-6) showing 2-JI1794 and 2-Slow genotypes. (B) Abyss. x Sparkle w/CfoI, 2-Abyss., 4-Spark., and 1 heterozygous genotype.

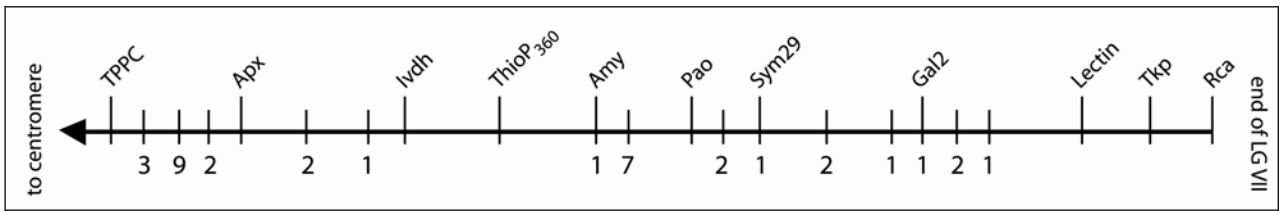

Fig 5. Linkage map for the distal 34 cM of the lower arm of LG VII in the JI1794 x Slow RIL population. Isozyme or STS loci are given above the horizontal bar, and the numbers below the bar indicate the position and number of RAPDs in the region.

Discussion

A mutation at the *Pao* locus could provide an explanation for the green cotyledon phenotype of pea because of the enzyme's participation in the short pathway of chlorophyll degradation. PAO converts green chlorophyllide a to light green pheophorbide a, and a mutation at *Pao* could cause the cotyledons to remain green as a result of the lack of chlorophylide a breakdown. However, our mapping data here demonstrate that *Pao* is located on LG VII of pea, not at the *I* locus on LG I, thereby eliminating *Pao* as a candidate gene for *I*. The finding by Thomas et al. (15) that PAO activity is significantly reduced in pea lines with green cotyledons suggests that PAO may be directly involved in producing the green cotyledon phenotype in seeds but that the genetic mechanism is specific to the cotyledon tissue (chlorophyll degradation in leaf or pod tissue does not appear to be affected by the *i* mutation). We are forced to conclude that *I* is most likely either a factor controlling transcription of the *Pao* gene in cotyledons or influencing the transport/processing of the initial translation product in the same tissue. A less likely possibility is that *I* codes for a factor influencing another enzyme in the degradation pathway in cotyledons, possibly an upstream enzyme such as chlorophyllase.

It has been suggested that PAO is expressed only in senescent tissue (17), making the coding sequence a more plausible candidate gene for a 'stay-green' phenotype. However northern blot analysis of Pao expression in tomato (M.D. Moffet and D.R. Bergey, unpublished) and detection of PAO in etiolated pea seedlings using antibodies (21) establish that Pao is generally constitutively expressed in many tissues and may be involved in other functions besides senescence-related chlorophyll breakdown. PAO may not be an appropriate candidate gene for many 'stay-green' mutants. For instance, 'stay-green' in bean is only associated with the retention of thylakoid proteins, indicating regulation of PAO activity does not cause the stay-green phenotype in *Phaseolus* (2). Similarly, *Pao* is not responsible for the 'stay-green' mutation in arabidopsis (14).

Linkage analysis of *Pao* in *Lens* indicated conservation of synteny, with lentil *Pao* also displaying linkage with *Amy* and other markers on pea LG VII. Much of the region also appears to be conserved in *Medicago truncatula*. For example, the *Medicago* BAC mth2-11o4 contains a sequence that, except for apparent introns, is identical to the *Pao* mRNA from *M. truncatula*. This BAC has been placed on linkage group 4 (http://medicago.org), the linkage group identified as partially homologous to pea LG VII (10). A *Sym29* sequence is located on this same BAC, paralleling the tight linkage between *Pao* and *Sym29* observed in pea. Furthermore, ascorbate peroxidase and isovaleryl dehydrogenase sequences are found on linked BACs (mth2-27j21 and mth2-65c4, respectively). One interesting aspect of the comparison of the different *Pao* genes is that the introns in *MtPao* are much larger than those in any of the other genes investigated (pea, lentil, *Phaseolus* and arabidopsis).

Finally, the introns of the *Pao* gene appear to be relatively polymorphic, even within species. The primers used generally amplified a single major product in each of the genera examined, suggesting that they will reliably amplify orthologous loci in different legume species. Thus, *Pao* should be very useful in PCR-based map generation and marker assisted selection (MAS) in breeding projects.

Acknowledgment: Thanks to Dr. Dan Bergey formerly of MSU-Bozeman, now at Black Hills State-SD, for starting the senior author on this project as an undergraduate.

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