# Isolation of *Pseudo Response Regulator* genes and evaluation as candidate genes for photoperiod response loci

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**Introduction**

The genetic control of flowering in pea has been studied for more than five decades, and several loci affecting photoperiod response are known (1). Mutations at the *Sn, Dne, Ppd* or *Hr* loci result in early-flowering under short-day conditions (2, 3, 4, 5) whereas loss-of-function mutations in the *PhyA* or *Late1* genes cause late flowering under long-day conditions (6, 7). In arabidopsis, many genes that affect photoperiodic flowering have a primary role in regulation of the circadian clock, and we recently showed that *Late1* is the pea ortholog of the clock-related arabidopsis gene *GIGANTEA (GI)* (6). This study also showed that *Late1* interacts genetically with *Sn,* and that *sn* mutant impairs the diurnal expression rhythm of *Late1* (6). This provides the first direct evidence that *Sn* might be involved in the clock mechanism in some way.

one potential route to the identification of the *Sn* gene could be to assess homologs of arabidopsis circadian clock-related genes as candidates. We previously isolated several clock-related genes in pea (8), but found that none of them mapped close to known photoperiod response loci. However, the list of potential candidate genes for these loci has been extended with recent identification of additional clock-related genes in arabidopsis. Isolation of the corresponding pea genes has been greatly assisted by recent progress in sequencing of the medicago genome and advances in comparative genome analysis between medicago and pea (9, 10, 11).

Several recent studies have examined the contribution of the *PSEUDO RESPONSE REGULATOR (PRR)* gene family to the circadian clock mechanism (12, 13, 14, 15). This family includes the core clock gene *TIMING OF CAB EXPRESSION 1 (TOC1)* and four other members; *PRR9, PRR7, PRR5,* and *PRR3.* All five genes show diurnal and circadian regulation, with distinct peaks of expression that occur sequentially every 2 hours after dawn (12). This finding has suggested that like TOC1, other members of the *"PRR* quintet" might also form part of the central oscillator. We recently observed that the effect of the *sn* mutant on the expression of *LATE1* is similar to the effect of the *prr5 prr7 prr9* triple mutant on *GI* in arabidopsis (16), raising the possibility that in some respects *Sn* might act similarly to *PRR* genes. We therefore set out to isolate *PRR* genes in pea, in order to examine their potential role within the pea circadian clock, and also their potential identity as candidate genes for photoperiod response loci.

**Materials and Methods**

Sequences of *PRR* homologs from *Medicago truncatula* and other species were obtained using tBLASTn searches of the Genbank database (http://www.ncbi.nlm.gov) and the medicago EST database at http://compbio.dfci.harvard.edu. To isolate members of the *PRR* gene family in pea, degenerate primers were designed within conserved domains using the CODEHOP strategy (http://blocks.fhcrc.org/codehop.html) (17). The full length *PsPRR37* and partial *PsPRR59* cDNA were obtained by 5' and 3' RACE-PCR using the BD-SMART RACE cDNA amplification kit

(CLONTECH). Protein alignments of various PRRs were performed with ClustalX (18) and adjusted using GENEDOC (Nicholas et al. 1997; http://www.psc.edu/biomed/genedoc). Relationships among PRR amino acid sequences were determined using phylogenetic analyses in PAUP* 4.0b10 (http://paup.csit.fsu.edu).

The origin of the WT line NGB5839 (cv. Torsdag *le-3)* and the *sn-2 and sn-4* mutants have been described previously (19, 6). The *sn-3* mutant is an additional recessive mutant isolated in the same screen as *sn-4* (6). All plants were grown in the Hobart phytotron, using previously-described growth media, light sources and phytotron conditions (6).

Information and approximate map positions of pea genes in the bottom half of LGVII was obtained from several published maps (20, 21, 22). To identify medicago homologs of pea genes in this region, tBLASTx searches were performed against the medicago genomic database at the J. Craig Venter Institute (http://www.jcvi.org/cgi-bin/medicago/index.cgi). The map positions of relevant genes were obtained by using Medicago Genome Browser (http://gbrowse.jcvi.org/cgi-bin/gbrowse/medicago_imgag/).

**Results and Discussion**

*Identification of PRR homologs in Medicago truncatula*

PRR proteins are characterized by two conserved domains; the Pseudo-regulator (PR) domain and the CONSTANS, CONSTANS-LIKE, and TOC1 (CCT) domain (12). Several reports have identified PRR genes in a number of species including rice (23), Lemna *gibba, L. paucicostata* (24) and *Populus trichocarpa* (25). Phylogenetic analysis shows that these genes fall into three major groups, which can be designated as *PRR1* , *PRR59* and *PRR37,* on the basis of the arabidopsis sequences they include (Figure 1). Two accessions from Lemna and one from rice *(OsPRR59)* show affinity to the *PRR59* clade at the sequence level, but fall outside this group in the phylogenetic analysis, most probably because they do not contain complete sequences for both conserved domains.

BLAST searches of the *Medicago truncatula* genomic (NCBI) and EST databases identified seven

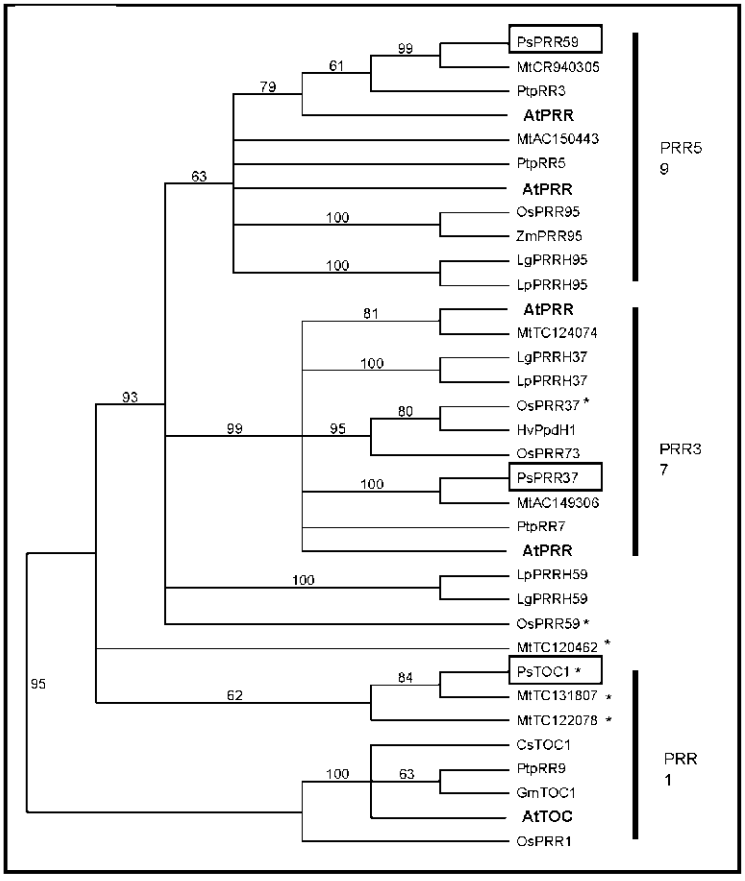


***Figure 1. Phylogenic tree of PRRs. Amino acid sequences of PRR and CCT domains were aligned and used to produce the neighbour-joining tree as described in materials and methods. The bootstrap values are indicated as a percentage above each branch. Ps, Pisum sativum; Mt, Medicago truncatula; At, ArabidopsisArabidopsis thaliana; Lg, Lemna gibba; Lp, Lemna paucicostata; Os, Oryza sativa; Hv, Hordeum vulgare; Pt, Populus trichocarpa; Cs, Castanea sativa; Zm, Zea mays; Gm, Glycine max. *C: sequence contains C-terminal; *N: sequence contains N-terminal.***

distinct *PRR* sequences, including three genomic and four ESTs. Additional EST contigs corresponding to three of the genomic sequences were also identified. Four of these sequences were predicted to encode full-length PRR proteins, including two in the *PRR59* clade and two in the *PRR37* clade. The remaining three EST sequences were only partial. Two distinct ESTs from the 5' region grouped with the partial pea *TOC1* sequence described previously, while the other from the 3' region did not show a clear relationship to any other PRR.

The three medicago BAC contigs containing the genomic *PRR* sequences have all been assigned map positions (www.medicago.org), on chromosome 3 (CR940305), 7 (AC150443) and 4 (AC149306). These positions predict positions for the corresponding pea sequences in the middle of LGIII, near the top of LGV, and in the bottom half of LGVII, respectively. We noted in particular that AC149306 was located in a region of chromosome 4 corresponding to the region of pea LGVII known to contain the *Sn* locus. *Sn* was previously reported to show close linkage with the amylase locus *Amy1* (26) and was mapped between isozyme loci *Aldo* and *Gal2* (27) on the lower section of pea linkage group VII. The similar positions of *MtPRR59* (CR940305) and the *Dne* locus also suggested a possible candidate gene relationship.

*Isolation of PRR homologs from pea*

Using degenerate primers targeting the PR and CCT domains two distinct fragments from the PR domain were amplified by PCR. These sequences were extended to both 5' and 3' ends using RACE PCR to obtain one partial (70%) and one full-length coding sequence. Phylogenetic analysis showed that these sequences belonged the *PRR59* and *PRR37* clades, respectively, and they were designated as *PsPRR59* and *PsPRR37*. Figure 1 shows that these sequences are apparent orthologs of the medicago *PRR* genes on CR940305 and AC149306.

*Mapping of PRR genes and evaluation of PsPRR37 as a candidate for Sn*

Sequencing of *PRR59* and *PRR37* from mapping parents JI1794 and "Slow" identified polymorphisms that were used to map both genes in the RIL population derived from these parents (22). The results confirmed positions for *PRR59* in LGIII near *Dne* and for *PRR37* in LGVII, consistent with the positions predicted by the location of the orthologous medicago genes. The relationship between *PRR59* and *Dne* was not explored further, as *Dne* has been identified as the pea ortholog of the arabidopsis *ELF4* gene (28). However, for *PRR37*, no recombination was detected with the Amy locus on LGVII, and as *Amy* was previously noted to be tightly linked to *Sn* (26), this indicated that *PRR37* is in the region of *Sn*.

In order to carry out fine mapping of *Sn*, we also generated a new mapping population derived from a cross between the *sn-4* mutant in the NGB5839 background and cv, Terese. Unfortunately, we found that the coding region of *PRR37* was identical in NGB5839 and Terese, precluding the straight¬ forward mapping of *PRR37* relative to *Sn* in this cross. Instead, the entire coding sequence of *PRR* was determined from the three known induced *sn* mutants and from their isogenic wild-type lines Borek (sn-2) and NGB5839 *(sn-3* and *sn-4).* In all three cases the *PRR37* coding sequence was identical in mutant and wild-type, indicating that the *sn* phenotype does not result from a mutation that affects *PRR37* protein structure. It will obviously be of interest in future to isolate flanking sequence of *PRR37* and identify an appropriate polymorphism that will allow the cosegregation of *PRR37* and *Sn* to be directly tested.

*A comparative map of the* Sn *region*

As an aid to future mapping studies in the region of *Sn*, we generated a comparative map using markers anchored in published pea linkage maps and the medicago physical map (Figure 2). This

identified a broad region likely to contain *Sn*, bounded approximately by the *Aldo* and *Sod9* genes. In medicago, the physical map of this region is estimated to span approximately 5Mb, although it still contains several gaps where adjacent BAc contigs are not yet joined. We are now using this information as a basis for the design of additional markers for the mapping of *Sn* and for the identification of other potential candidate genes. The first step will be the mapping of *Sn* relative to other markers shown.

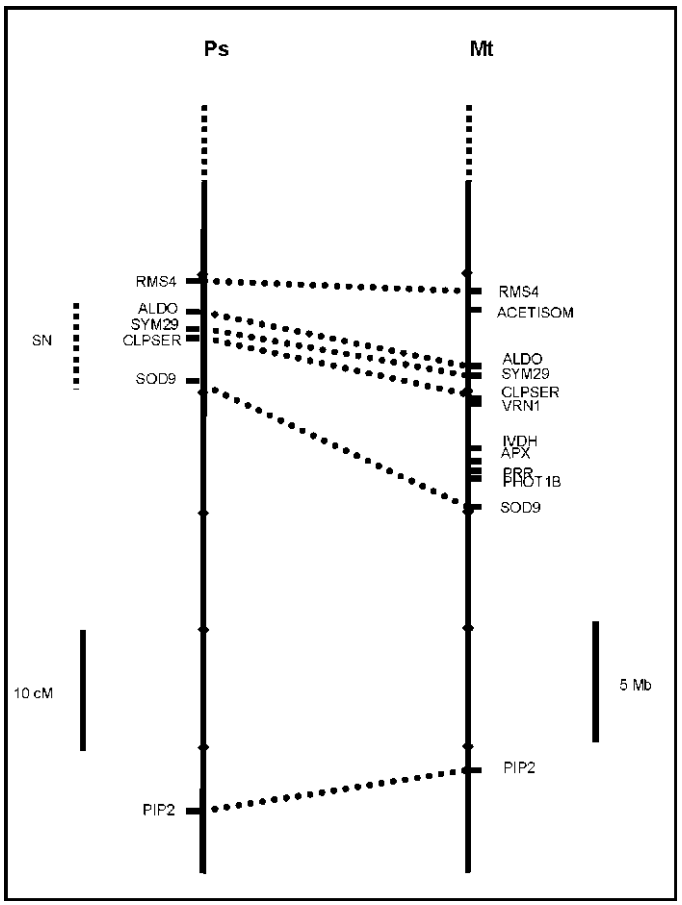


*Figure 2. Comparative map between pea(Ps) and medicago(Mt). Orthologous genes in the two species are joined by a dash line. The scale on the left give approximate recombination distances(cM) for pea linkage group VII whereas the scale on the right give approximate physical distance (Mb) for medicago chromosome 4. Actual map postions of medicago genes are as follows: RMS4 (AC174294; 24.68), ACETISOM (AC135231; 24.59), ALDO (AC174316; 20.81), SYM29(AC149637; 20.36), CLPSER (AC225505;19.82), VRN1 (AC137825; 19.44), IVDH (AC152176,16.68), APX (AC145164,16.35), PRR (AC149306; 16.24), PHOT1B (AC148218; 16.13), SOD9 (AC126007; 15.06), PIP2 (AC153003; 4.50)*

*Medicago information accessed on 5th Nov 2009.*

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