Nodule-Expressed *Cyp15a* Cysteine Protease Genes Map to Syntenic Genome Regions in *Pisum* and *Medicago* spp.

Jason L. Vincent,¹ Maggie R. Knox,¹ T. H. Noel Ellis,¹ Péter Kaló,² György B. Kiss,² and Nicholas J. Brewin¹

¹Department of Genetics, John Innes Centre, Norwich Research Park, Colney Lane, Norwich, NR4 7UH, U.K.; ²Institute of Genetics, P.O. Box 521, Szeged H-6701, Hungary

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PsCyp15a is a gene that encodes a vacuolar cysteine protease expressed in wilt-induced shoots of *Pisum sativum* (pea) and in root nodules. To further the understanding of nodular *PsCyp15a* expression, a region 5' to the coding sequence of the gene was cloned. Varying lengths of 5' untranslated sequence were fused with the *uidA* coding region and introduced from *Agrobacterium rhizogenes* into "hairy roots" of *Vicia hirsuta*. In this transgenic root nodulation assay, a promoter sequence of 900 bp was sufficient to give an expression pattern indistinguishable from that obtained in pea nodules by in situ hybridization. An orthologue of *PsCyp15a* was cloned from nodule mRNA of *Medicago sativa* and a corresponding gene identified in *M. truncatula* was also shown to express strongly in nodules. With molecular mapping techniques, it was demonstrated that these genes map to a syntenic genome location in pea and *Medicago* spp., but the map positions of the *Cyp15a* genes cannot be correlated with existing nodulation mutants.

Additional keyword: transformation.

In the pea root nodule, *PsCyp15a* has been identified as a plant cysteine protease transcript (Kardailsky and Brewin 1996). The gene product is localized to lytic compartments of uninfected cells and, additionally, to the symbiosome compartment in host cells harboring *Rhizobium* spp. bacteroids as endosymbionts (Vincent and Brewin 2000). Initially identified as a stress-induced mRNA in pea shoots subjected to water-limitation (Guerrero et al. 1990; Jones and Mullet 1995), the gene product showed, in subsequent studies of root nodules, strong expression at the nodule apex—a zone associated with meristematic activity and rhizobial invasion—and lower expression throughout the central infected tissue where bacteroidal nitrogen fixation takes place

(Kardailsky and Brewin 1996). Immunolocalization of *PsCYP15A* in the nodule apex shows antigen associated with numerous cytoplasmic vesicles similar to those observed in wilt-induced shoot tissue (Vincent and Brewin 2000).

To extend the molecular genetic analysis of *PsCyp15a* in legume nodules, promoter::*uidA* fusions were constructed to allow delimitation of the region of *PsCyp15a* necessary to give tissue-specific expression following transformation of *Vicia hirsuta*. Furthermore, on the assumption that *Cyp15a* may have an essential function in nodule development, we attempted to find orthologous genes expressed in the nodules of other legume species. However, it has not yet proved possible to identify a symbiotically defective mutant with a lesion in *Cyp15a*. We demonstrate that an orthologous gene is expressed in nodules of *Medicago* spp. and maps to a position that shows synteny with *PsCyp15a* on the pea restriction fragment length polymorphism (RFLP) map. Further studies of *PsCyp15a* and its orthologues can now be conducted in pea, *Vicia* spp., or *Medicago* spp., as appropriate.

RESULTS

Amplification of *PsCyp15a* promoter.

Several adapter-ligated libraries of pea genomic DNA were generated to amplify the sequence 5' to the coding region of the *PsCyp15a* gene. Following nested touchdown polymerase chain reaction (PCR), two libraries yielded single products of approximately 350 bp (*SspI*) and approximately 950 bp (*HpaI*), as indicated in Figure 1, which shows the sequence obtained from the *HpaI* amplification product. For purposes of clarity, the first nucleotide of the initiation codon has been designated as +1, as the transcriptional start of the gene has yet to be determined. Putative TATA and CAAT boxes are evident at –123 and –194 bp, respectively. The sequence AAAGAT, which is present at position –137 bp, may correspond to one half of the bipartite nodule-specific "organ specific element" (OSE) found in the promoters of late nodulin genes such as leghemoglobin, at positions approximately –140 bp from the transcriptional start (Ramlov et al. 1993). A number of tandem and inverted repeats are also evident in the sequence, which may have functional roles in the regulation of this promoter.

Corresponding author: N. J. Brewin; Fax: +44 1603 450045; E-mail: nick.brewin@bbsrc.ac.uk

Current address of Jason L Vincent: Zeneca Agrochemicals, Jealott's Hill Research Centre, Bracknell, Berkshire, RG42 6ET, U.K.

Nucleotide and/or amino acid sequence data have been deposited in the EMBL data base as accession numbers AJ250432 (*pPsCyp15a*) and AJ245868 (*MsCyp15a*).

Generation of transgenic nodules.

To construct promoter::uidA fusions, two segments of the p*PsCyp15a* promoter sequence were produced, P1 and P2, corresponding to 251 and 900 bp, respectively. Figure 2 shows examples of β -glucuronidase (GUS)-stained root and nodule tissue following transformation by *Agrobacterium rhizogenes*. Plants transformed with the P1 construct (251 bp) exhibited GUS expression that was apparently specific to stele and vascular tissue, and did not extend into the distal areas of the nodule. Weak GUS expression was visible in developing nodule primordia. Plants transformed with the P2 construct (900 bp) showed GUS expression associated with a layer of cells immediately adjacent to the stele of the root and strong expression in primary root tips and emerging laterals. GUS expression driven by this construct was also observed in nodules as early as 5 days post inoculation (dpi) with rhizobia. In ma-

ture nodules (14 dpi) expression of GUS was detected at the highest levels in the apex, corresponding with the persistent meristem, and in a layer of cells associated with the outer cortex. Lower levels of expression were apparent throughout the infected zone. These observations for the mature nodule match those for in situ hybridization patterns for *PsCyp15a*, as previously reported for pea nodules (Kardailsky and Brewin 1996). The GUS levels for P2 nodules at 24 dpi were unchanged from those in the mature stage and spatial expression did not vary.

Analysis of roots/nodules expressing the cauliflower mosaic virus p35s::gusA-int control construct (Quandt et al. 1993) showed very strong histochemical GUS staining throughout all tissues (data not shown). Similarly, roots induced by the transforming strain devoid of expression construct (ARQua1; Quandt et al. 1993) did not exhibit GUS activity (data not shown).

Fig. 1. DNA sequence for the promoter region of *PsCyp15a* derived from the amplification product of a *HpaI* prepared, adapter-ligated, genomic library. Putative TATA and CAAT elements are in bold. An AAAGAT sequence that may correspond to one half of the nodulin "organ specific element" (OSE) is bold and underlined (Stougaard et al. 1987; Ramlov et al. 1993). Certain inverted and tandem repeats are underlined. The start codon is italicized. The last 44 bp are aligned with the first 44 bp of *PsCyp15a* cDNA sequence (bottom line) and show discrepancy at the extreme end of the cDNA (Guerrero et al. 1990). Nested, gene-specific primers used for amplification of this product are positioned approximately 60 bp downstream of the start of the cDNA sequence, in the coding region of the gene. Adapter sequence is not shown. Primers used for amplification of specific segments of this promoter for fusion to reporter genes are indicated as arrows above sequence. This sequence has been deposited in the EMBL data base as accession AJ250432.

Fig. 2. Sections of roots and nodules showing histochemical β -glucuronidase (GUS) staining (blue) of transgenic *Vicia hirsuta* expressing pPsCyp15a::uidA fusion constructs. **A**, Longitudinal section of a mature (14 days post inoculation [dpi]) nodule expressing the P1 construct (251 bp). Infected host cells exhibit a brown coloration. **B**, Developing nodule primordium (5 dpi) expressing the P2 construct (900 bp). **C**, Mature P2 nodule (14 dpi) showing strong GUS expression in the apex and outer cortex. **D**, Magnification of central infected tissue in **C**, showing low levels of GUS expression. **E**, Magnification of apex from **C**, showing strong GUS expression associated with meristem. **F**, P2 nodule (24 dpi) showing GUS expression similar to the 14 dpi nodule in **C**. **G**, Lateral root tip expressing P2 construct showing strong GUS activity in meristem. **H**, Longitudinal section through a root expressing P2 construct showing region of GUS activity adjacent to stele. Bar = (A–C,F) 500 and (G,H) 100 μ m.

Identification and expression of *Cyp15a* homologues in *Medicago* spp.

In Southern blots of digested *M. truncatula* genomic DNA probed with a radiolabeled PsCyp15a probe, a single band could be determined in each lane, indicating the presence of a single-copy *Cyp15a* homologue in this species (data not shown). To allow comparative analysis of the *PsCyp15a* gene with homologues in *Medicago* spp., rapid amplification of 3' cDNA ends (3' RACE) was employed to amplify an approximately 900-bp product from total RNA of mature nodules of *M. sativa*. Comparison with PsCyp15a indicated that homology of 92% (identity) at the amino acid level was conserved. This partial cDNA was named MsCyp15a (*M. sativa*), and its sequence is shown in Figure 3.

Quantitative reverse transcription (RT)-PCR with MsCyp15a-specific primers (see Figure 3) gave an indication

of expression levels of *MtCyp15a* in different parts of the *M. truncatula* plant (Fig. 4). Expression is seen to be the highest in leaves, followed by nodules and inflorescences, with shoots, roots, and buds exhibiting lower levels.

Molecular mapping in pea and *Medicago* spp.

An RFLP-based approach was used to place *PsCyp15a* on the linkage map of pea (Hall et al. 1997; Laucou et al. 1998). An RFLP was identified between the parental lines JI15 and JI399 when genomic DNA was digested with *EcoRV* and probed with a PsCyp15a radioprobe (Fig. 5A). Consequent screening of 82 individuals derived from the F₁₁ generation of a JI15 × JI399 recombinant inbred (RI) population showed linkage of this marker with the previously mapped markers cDNA 56/1, 1B8, and pAT-T4/4, placing *PsCyp15a* at an end of linkage group II. This position does

Fig. 3. Sequence data of the partial MsCyp15a cDNA. Amino acid translation is depicted below nucleotide sequence and shows conserved active site residues cysteine and histidine (bold and underlined), common to this class of protease. Arrows above sequence indicate primers used for quantitative polymerase chain reaction of MtCyp15a. This sequence has been deposited in the EMBL data base as accession AJ245868.

not correspond to any known locus for a (symbiotically) defective allele in pea.

Similarly, single-strand conformational polymorphism (SSCP)- and RFLP-based approaches were applied to place *MsCyp15a* on the *M. sativa* linkage map (Kaló et al. 2000). *MsCyp15a*-amplified fragments from the individuals of the mapping population were subjected to SSCP analysis, resulting in the designation of *MsCyp15a* as a co-dominant marker. An RFLP marker was also detected between the F₂ individuals of the segregating population with amplified, radiolabeled *MsCyp15a* fragments used as a probe (Fig. 5B). These genetic analyses determined the map position of the *Cyp15a* locus at an end of linkage group 1, co-segregating with the previously mapped markers, AAT2 and DFR. This position does not correspond with a known, mapped mutant allele for *M. sativa* but does, however, indicate synteny between *Pisum* and *Medicago* spp. for the *Cyp15a* locus (Fig. 5C).

Fig. 4. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *MtCyp15a* expression. **A**, Upper panel, equivalent loadings of *MtCyp15a* RT-PCRs after 20 cycles, run on an agarose gel; lower panel, equivalent loadings of ubiquitin RT-PCRs after 30 cycles for the same RNA samples. M = Marker (numbers to the side in bp); S = Shoot; L = Leaf; R = Root; N = Nodule; I = Inflorescence; B = Bud. **B**, Graphic comparison of densitometry data from **A** after standardization to ubiquitin controls. Scale shown is an arbitrary measure of *MtCyp15a* expression and is, therefore, dimensionless.

DISCUSSION

For many decades, pea has been an excellent model system for the study of the biochemistry, physiology, and genetics of legumes. Increasingly, however, the advantages of model legume systems, e.g., *M. truncatula* (Cook et al. 1997; Cook 1999) are becoming apparent for the future development of physical and functional genomics. In this study, several *Cyp15a*-related sequences have been investigated from different legumes, illustrating the ability to study a particular gene (or orthologue of such a gene) in a variety of genetic backgrounds.

In *V. hirsuta*, transgenic investigations of *pPsCyp15a* activity tested the efficacy of short (P1) and long (P2) promoter segments as fusions with the *uidA* coding sequence. Although GUS expression was evident with both constructs, only the P2 construct (900 bp) demonstrated the expected expression patterns for *PsCyp15a* in mature nodules of transgenic *V. hirsuta*. Furthermore, continual GUS expression driven by this construct indicated a consistent involvement of *PsCyp15a* during nodule organogenesis and maturation. Interestingly, strong GUS expression in the nodule apex driven by the P2 construct was similar to that observed in the meristematic zones of developing roots and lateral roots. Thus, a strong correlation of *PsCyp15a* expression with meristematic zones of the root and nodule is apparent, confirming previous results from *in situ* hybridization experiments (Kardailsky and Brewin 1996).

With a proteomics approach, another possible orthologue of *PsCyp15a* (SwissProt accession number P25804) has recently been identified as a component of isolated symbiosomal membranes from soybean nodules (Panter et al. 2000): this provides further evidence that this class of protease is widespread in legume nodules and that it is associated with the symbiosome compartment. Southern blotting has identified a single-copy homologue of *PsCyp15a* in the model legume *M. truncatula*. PCR cloning of a partial cDNA of a *PsCyp15a* orthologue from *M. sativa* root nodules demonstrated 92% conservation of identity at the amino acid level between the two sequences. Another close homologue of *MsCyp15a* is a *Cyp15a* cDNA from *Cicer arietinum* that exhibits 91% identity at the amino acid level (accession no. AJ009878). Expression analysis of *MtCyp15a* in various tissues of *M. truncatula* shows a pattern similar to that of *PsCyp15a* in pea with respect to roots and nodules, but different in terms of a higher expression in leaves and lower expression in buds. Given the association of *PsCyp15a* expression with meristematic zones it is somewhat unexpected to find a relatively low level of *MtCyp15a* transcript in the buds.

Mapping of *PsCyp15a* and *MsCyp15a* to the respective genetic maps shows synteny between the two species for the *Cyp15a* locus, yet does not identify any association with mutant loci in either species. Future work with the model legume *M. truncatula* will enable more precise studies of the physiological and genetic control of *Cyp15a* gene expression and will, eventually, enable gene knock-outs to be obtained, either by mutagenesis or by the development of gene silencing in transgenic lines. It is hoped that these investigations will provide more insights into the functionality of *Cyp15a* protease in the developing root nodule.

In conclusion, we have, in the first instance, investigated gene function in a crop plant, the pea (Vincent and Brewin

2000). This legume has advantages for fundamental biochemical and physiological studies because the plants are easy to handle and the nodules are large and readily harvested. In the second instance, we have turned our attention to the smaller model legume *M. truncatula* to allow further analysis at the level of molecular genetics. The fact that synteny is apparent between the pea and *Medicago* spp. linkage maps (this study; Cook 1999) vindicates the use of *M. truncatula* as a model legume that, like pea, forms nodules with indeterminate meristems. Thus, the ability to cross biochemical and genetic boundaries by translating between traditional and model legume systems promises to advance the study of root nodule symbioses in the same way that comparative research of *Arabidopsis thaliana* has revolutionized the field of general plant molecular genetics.

MATERIALS AND METHODS

Plant material.

Seeds of *V. hirsuta* (obtained from S. M. Walters, Botanical Gardens, University of Cambridge, UK) were surface sterilized for 30 min with concentrated sulfuric acid and washed copiously in sterile water, followed by a further sterilization in 10% sodium hypochlorite for 10 min. After a final wash in sterile water, seeds were placed onto the surface of 1% (wt/vol) distilled water agar solidified in petri dishes, and vernalized by incubation at 4°C for 7 days before germination in the dark at room temperature over a further 3 days.

M. truncatula R108-(c3) (a kind gift of A. Kondorosi, CNRS, Gif-sur-Yvette, France) and *M. sativa* cv. Aragon

Fig. 5. Genetic mapping and synteny. **A**, Autoradiograph of a Southern blot of *EcoRV*-digested genomic DNA from pea parental lines JI15 and JI399, probed with radiolabeled *PsCyp15a*. Restriction fragment length polymorphism (RFLP) marker used to score segregation in recombinant inbred (RI) progeny from a JI15 × JI399 cross indicated by arrowheads. Number to the side, size in kb. **B**, Autoradiograph of a Southern blot of *EcoRI/EcoRV*-digested genomic DNA from *Medicago sativa* parental lines Mcw2 and Mqk93, probed with radiolabeled *MsCyp15a*. RFLP marker used to score segregation in the F₂ RI progeny from an Mcw2 × Mqk93 cross indicated by arrowheads. Numbers to the side, size in kb. **C**, Linkage group diagrams showing position of the *Cyp15a* loci in pea (Ps LG II) and *M. sativa* (Ms LG I). Lines between diagrams indicate conserved markers.

(John Innes Centre Seed Store) were germinated on water-soaked filter paper in covered petri dishes in the dark at room temperature over 5 days. Germinating seedlings were transferred to pots containing a 1:1 mixture of vermiculite and coarse sand/grit, and grown in cabinets at 24°C with a photoperiod of 16 h light and 8 h dark. Plants were fed with a nitrate-free nutrient solution based on Soluplant (N/P/K: 18/6/26; Duclos International, Lunel Viel, France), and inoculated with *Sinorhizobium meliloti* strain 2011 (Rosenberg et al. 1981) at 2 weeks post germination, to induce nodulation.

Bacterial strains.

Agrobacterium rhizogenes strain ARQual, with endogenous streptomycin resistance, was the recipient for the binary transformation plasmids produced in this study and used subsequently for hairy root induction on *V. hirsuta* (Quandt et al. 1993). ARQual2 is a derivative of ARQual carrying the cauliflower mosaic virus p35s::*gusA*-int reporter gene in the binary vector pBIN19 (Vancanneyt et al. 1990). *Rhizobium leguminosarum* bv. *viciae* 3841 (Wang et al. 1982) was made resistant to gentamicin by the introduction of the broad-host-range plasmid pBBR1MCS-5 (Kovach et al. 1995) by triparental mating. The resulting strain (3841 Gm^r) was resistant to gentamicin (10 µg · ml⁻¹), whereas ARQual was sensitive. Both *Agrobacterium* and *Rhizobium* strains were cultured in liquid or on solid tryptone-yeast extract (TY) medium containing the appropriate concentration of antibiotic at 28°C.

Nucleic acid purification.

Genomic DNA was extracted from leaves of mature plants by the CTAB method (Murray and Thompson 1980), and spooled before resuspending in Tris-EDTA (TE) buffer. For total RNA extraction, tissues were harvested under liquid nitrogen, homogenized to a powder with a pestle and mortar, and purified with the RNeasy Plant Mini Kit (Qiagen, Crawley, UK) following the supplier's recommendations.

PCR amplification of genomic and cDNA sequences.

PsCyp15a genomic sequence was amplified by the Genome Walker technique (Clontech, Basingstoke, UK). Pea genomic DNA (JI15) was digested in six separate reactions with six blunt-cutting endonucleases. Digested DNA was ligated to the Genome Walker adapters with T4 DNA ligase to produce six individual "libraries" of adapter-ligated genomic DNA. These libraries were used as templates in PCRs with two forward adapter-specific primers and two reverse *PsCyp15a*-specific primers in a nested approach. Adapter-specific primers were AP1 and AP2 as supplied by Clontech. *PsCyp15a*-specific primers were JLV5 (5' – CGG CGA AGA GGA AGA GAG CG – 3') and JLV6 (5' – AGC GAA GAG GAA ACG GCG – 3'), where JLV5 is used in the first round of nested PCR with AP1, and JLV6 is used in the second and final round of nested PCR with AP2. JLV6 is located upstream of JLV5 on the *PsCyp15a* cDNA sequence, approximately 60 bp downstream of the start of the cDNA sequence, thus allowing a "tag" of known *PsCyp15a* sequence to be amplified with the 5' genomic region to assist in verification of the amplification products as *PsCyp15a* in origin. PCRs were performed under touchdown conditions as described by Hecker and Roux (1996) in a Techne Genius thermocycler (Techne, Cambridge, UK) with Pfu DNA polymerase (Stratagene, Amsterdam).

Amplification of *MsCyp15a* cDNA by the 3' RACE technique of Frohman et al (1988) was with *M. sativa* total nodule RNA as a template. First-strand synthesis was with the oligo d(T)_n primer G1149 (5' – CTC GAG GAT CCG CGG CCG C(T)₂₀ – 3'). Second-strand synthesis was with the degenerate primer G1184 (5' – TGY GGI HSI KGI TGG GCI TT – 3', with I as inosine and other letters corresponding to the GCG code for degeneracy). This primer was originally employed to amplify *PsCyp15a* from pea nodule mRNA (Kardailsky and Brewin 1996). Both G1149 and G1184 were used to amplify cDNA under the same touchdown conditions described above. Two products were generated, approximately 900 bp and approximately 1,000 bp; the latter of which was apparently irrelevant, but the former was shown by sequence analysis to correspond to *Cyp15a*.

PCR products were directly sequenced following agarose gel purification with the ThermoSequenase (Amersham, Amersham, UK) cycle sequencing kit in conjunction with an ABI 377 Prism automated sequencer. Products were sequenced on both strands and sequence data was compiled and analyzed with the GCG package (Genetics Computer Group, University of Wisconsin, Madison).

Quantitative RT-PCR followed a similar procedure to the 3' RACE reaction described above, with 3 µg of total RNA per reaction and primer G1149 to generate first-strand cDNA. To measure the levels of *MtCyp15a* transcript in various *M. truncatula* tissues, forward (JLV24: 5'– GGT CGG GGT GGG CGT TTA G –3') and reverse (JLV25: 5'– CAC CCC AAT TCT GGC CCC –3') *MsCyp15a* gene-specific primers were used. Equal volumes of first-strand reactions were used as the template for quantitative PCR in a Techne Genius thermocycler (Techne, Cambridge, UK) with AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA). PCR cycle parameters were as follows: 94°C for 30 s; 60°C for 30 s; 72°C for 30 s. This cycle was conducted 20 times for *MtCyp15a* amplification and 30 times for ubiquitin amplification. Ubiquitin primers were used in parallel reactions as a control to standardize the levels of *MtCyp15a* products in individual samples relative to the original template (first-strand cDNA) concentrations of the sample set. The forward primer was G3520 (5'– ACC ACC ACG RAG ACG GAG –3'); the reverse primer was G3521 (5'– ATG CAG ATY TTT GTG AAG AC –3'). Equal amounts of RT-PCRs were electrophoresed in agarose gels, ethidium bromide-stained, and UV illuminated. Densitometry of the intensities of the products in these gels was made with the GelDoc 1000 software (BioRad, UK).

Production of promoter::uidA fusion constructs.

To construct *pPsCyp15a* reporter fusions, specific segments of the *PsCyp15a* promoter were amplified from the approximately 950-bp PCR product obtained from the *HpaI* adapter-ligated library. Short (P1) and long (P2) segments were amplified with primer combinations JLV9 and JLV10 or JLV9 and JLV11, respectively. JLV9 is 5'– CAT GGC CCG GGA GCG AAG GTT CG –3', where the underlined bases represent a *SmaI* site; JLV10 is 5'– GAA AGT CGA CAT TTT AAA GGG CCC AGT ATA ATA CTT ATA ATT TCC AC – 3', where the underlined bases represent *SalI* and *ApaI* sites, respectively; JLV11 is 5'– TCA GTC GAC ATT TGG GCC CAT AAC TCT AAA AAA GAT CAT –3', where underlined bases represent *SalI* and *ApaI* sites, respectively. PCR condi-

tions were the same as in the original *pPsCyp15a* amplification. Following agarose gel purification, P1 and P2 products were ligated into the plasmid pIV20 (Ramlov et al. 1993) at the *SalI* and *SmaI* sites. The resulting plasmids contained either P1 or P2 fused transcriptionally 5' to the coding sequence of the *uidA* (GUS) gene flanked at the 3' end by the *NOS*-terminator sequence. The *uidA* sequence used in this study was not the *gusA*-int intronic version that prevents endogenous agrobacterial GUS expression (Vancanneyt et al. 1990). The *pPsCyp15a::uidA::NOS*-term fusions were subsequently excised by cutting once with *ApaI* at the 5' end followed by a T4 DNA polymerase fill-in to blunt the terminus, and cutting once at the 3' end of the *NOS*-term by *EcoRI*. The liberated fragments were purified on agarose gels before subcloning into the binary transformation vector pE6NOSBAR, which was linearized by cutting the unique *EcoRI* and *ClaI* sites inside the T-DNA region. The *ClaI* site was filled in by T4 DNA polymerase to provide a compatible terminus for the filled-in *ApaI* termini of the fusion cassettes. The pE6NOSBAR vector consists of pRK4-*BglIII* (Bevan 1984) containing a 815-bp fragment made-up of *lacZ'* and the pBlueScript polylinker flanked by synthetic left and right T-DNA borders based on pTiC58 (P. Mullineaux, *personal communication*).

Nodulation of hairy roots of *V. hirsuta*.

Germinated seedlings on distilled water agar plates, having epicotyls 1.0 to 2.0 cm in length, were infected with transforming strains of *A. rhizogenes*. Epicotyls were stabbed with a 26-gauge hypodermic needle coated with a log-phase culture of the desired strain, as described by Quandt et al. (1993). Wounded seedlings were transferred to sterile tissue culture tubes (C5916; Sigma, Poole, UK) containing 1% (wt/vol) agar slants with Fahraeus medium (Fahraeus 1957) supplemented with 10 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin to inhibit growth of *Agrobacterium* spp. except at the wound sites. Inoculated seedlings were maintained in growth room conditions of 16 h light at 20°C, and 8 h dark at 15°C, until emerged hairy roots were greater than 1 cm long (approximately 2 weeks). At this point, the primary root was excised just below the cotyledon and the hairy roots were inoculated with a diluted culture of freshly grown *R. leguminosarum* bv. *viciae* 3841 Gm^r. Plants thus treated were maintained in the same growth conditions until 5, 14, or 24 dpi, when a sample (greater than 40 plants) was removed for histochemical GUS analysis of each construct. The effect of adding gentamicin to the growth medium of *V. hirsuta* was assessed in a separate trial, and it was found that no deleterious effects were evident at concentrations below 100 $\mu\text{g} \cdot \text{ml}^{-1}$. Addition of gentamicin (10 $\mu\text{g} \cdot \text{ml}^{-1}$) to the plant growth medium allowed the exclusion of agrobacteria with endogenous GUS expression from all areas of the plant, save at the original site of inoculation, without impairing nodulation by 3841 Gm^r.

Histochemical GUS analysis.

GUS expression in transgenic tissue was assayed by incubation in a solution containing X-gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) at 37°C for 16 h (Jefferson et al. 1987). GUS-stained tissue was sectioned (80 to 100 μm) with a vibratome (TAAB series 1000; TAAB, Aldermaston, UK) and observed with a Zeiss Axiophot microscope with bright field illumination. Digital image capturing was achieved with

a personal computer coupled to the Zeiss microscope via a CCD digital camera and operating ImageProPlus software (Media Cybernetics, Leiden, The Netherlands).

RFLP and SSCP mapping.

Basic techniques for RFLP mapping have been previously described for pea (Ellis et al. 1992) and *M. sativa* (Kiss et al. 1993). Southern blots of genomic DNA (digested with *EcoRV*) from pea parental lines JI15 and JI399 and the F₁₁ RI progeny resulting from crossing the two lines were probed with a random-primed, ³²P-labeled PsCyp15a probe generated with the Boehringer Hexanucleotide 10x Mix (Boehringer-Ingelheim, Mannheim, Germany) per the supplier's instructions. Hybridization was performed overnight at 65°C in a solution containing 10% (wt/vol) dextran sulfate (in 4x SET; 10x Denhardt's; 0.1% sodium dodecyl sulfate [SDS]) and the blot was washed twice in 0.1x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate)/0.3% SDS at 60°C before being exposed to a phosphoimaging plate (Fuji, London). Plates were scanned in a Fuji Phosphoimager after 12 h at room temperature, and autoradiographic digital images were generated with MacBas1000 software (Fuji). Linkage relationships were calculated by processing the segregation information with MapMaker version 3.0 software (Whitehead Inst., Cambridge, MA; Lander et al. 1987).

SSCP analysis of segregation of PCR-generated *MsCyp15a* markers, from individuals of the F₂ RI progeny of a cross between diploid *M. sativa* parental lines (subsp. *quasifalcata* k93 [Mqk93] and subsp. *coerulea* w2 [Mcw2]; Kiss et al. 1993) was performed following the theory of Orita et al. (1989). *MsCyp15a*-specific primers were MscypU1 (5'-GGT GGG CGT TTA GTA CAA CT -3') and MscypD1 (3'-AGA GGC CGA AAT GTA TGT GG -5'), and PCR conditions were as described by Kaló et al. (*in press*). Samples (5 μl) were taken from each 25- μl PCR and digested with *EcoRI*. The digests were denatured in stop buffer (95% formamide, 20 mM EDTA, 0.02% xylene cyanol, 0.02% bromophenol blue) at 95°C, and fractionated by electrophoresis in a 10% polyacrylamide gel (acrylamide:bisacrylamide = 49:1) in 0.5x Tris-borate buffer at a constant 8°C, 400 V for 16 h. In addition, RFLP analysis of *MsCyp15a* was performed. Polymorphic markers were defined on Southern blots of the F₂ RI genomic DNA (described above), following digestion with *EcoRI* and *EcoRV*, and probing with ³²P-labeled *MsCyp15a* fragments amplified with the primers MscypU1 and MscypD1 from *M. sativa* (Feinberg and Vogelstein 1983). Probe hybridization was performed at 63°C as described by Kiss et al. (1993). Recombination fractions were calculated with the "special" maximum-likelihood equations of Lorieux et al. (1995) by the method described by Kaló et al. (*in press*). Recombinant fractions were converted to map distances according to the Haldane function. Map position was also confirmed by Colormapping (Kiss et al. 1998).

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