RESEARCH ARTICLE

Genetic interaction and mapping studies on the *leaflet development* (*lld*) mutant in *Pisum sativum*

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Abstract

In *Pisum sativum*, the completely penetrant *leaflet development* (*lld*) mutation is known to sporadically abort pinnae suborgans in the unipinnate compound leaf. Here, the frequency and morphology of abortion was studied in each of the leaf suborgans in 36 genotypes and in presence of auxin and gibberellin, and their antagonists. Various *lld* genotypes were constructed by multifariously recombining *lld* with a *coch* homeotic stipule mutation and with *af*, *ins*, *mare*, *mfp*, *tl* and *uni-tac* leaf morphology mutations. It was observed that the suborgans at all levels of pinna subdivisions underwent *lld*-led abortion events at different stages of development. As in leafblades, *lld* aborted the pinnae in leaf-like compound *coch* stipules. The *lld* mutation interacted with *mfp* synergistically and with other leaf mutations additively. The rod-shaped and trumpet-shaped aborted pea leaf suborgans mimicked the phenotype of aborted leaves in HD-ZIP-III-deficient *Arabidopsis thaliana* mutants. Suborganwise aborted morphologies in *lld* gnotypes were in agreement with basipetal differentiation of leaflets and acropetal differentiation in tendrils. Altogether, the observations suggested that *LLD* was the master regulator of pinna development. On the basis of molecular markers found linked to *lld*, its locus was positioned on the linkage group III of the *P. sativum* genetic map.

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Introduction

Leaves differ in their size and architecture within individual plants (heteroblasty) and between species. They comprise simple determinate or compound semideterminate leafblade, attached to stem node by petiole. Leafblades of compound leaves have two or more leaflet (pinna) blades joined to the distal end of petiole in palmately compound leaves and/or distributed on rachis, the distal extension of petiole, in pinnately compound leaves (Zomlefer 1994; Champagne and Sinha 2004). In some species, stem node produces lateral stipules on the sides of the leaf attachment site. Leaf morphogenesis occurs by a succession of events associated with the processes of initiation (leaf primordium separation), primary morphogenesis, secondary morphogenesis and expansion (Canales *et al*. 2010; Efroni *et al*. 2010; Floyd and Bowman 2010; Hasson *et al*. 2010; Yamaguchi and Tsukaya 2010; Nicotra *et al*. 2011; Townsley and Sinha 2012).

Leaf primordium is formed in the peripheral zone of shoot apical meristem (SAM) from undifferentiated stem cells, at the site of auxin maximum, which is identified by *PIN-FORMED-1*-mediated auxin efflux activity (Esau 1997; Takada *et al*. 2001; Aida *et al*. 2002; Benkova *et al*. 2003; Reinhardt *et al*. 2003; Vroemen *et al*. 2003; Hibara *et al*. 2006; Barkoulas *et al*. 2008; Berger *et al*. 2009; Veit 2009; Efroni *et al*. 2010; Hasson *et al*. 2010; Floyd and Bowman 2010). The primordium is separated from SAM by the formation of a boundary of cell layers made quiescent for cell division by *CUC* activity. Primordia for simple and compound leaves are separated similarly; however, they are

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patterned differently (Aida *et al*. 1997, 1999, 2002; Benkova *et al*. 2003; Furutani *et al*. 2004; Laufs *et al*. 2004; Mallory *et al*. 2004; Koyama *et al*. 2007; Blein *et al*. 2008; Berger *et al*. 2009; Veit 2009; Efroni *et al*. 2010; Hasson *et al*. 2010).

The simple leafblade patterning involves placement of palisade parenchymatous tissue on the dorsal (adaxial) side (that faces the next upper leaf) and spongy parenchymatous tissue on the ventral (abaxial) side. The two sides are merged in the middle except where intervened by the occurrence of veins, and both sides are bounded on the outside by epidermis. In the genetic regulatory mechanism of simple leaf patterning, a feature shared by the simple leaves is nonexpression of homeodomain transcription factor *KNOX* genes in the leaf primordium (Ori *et al*. 2000; Hake *et al*. 2004). In *Arabidopsis thaliana* the downregulation of the KNOX class genes *KNAT1* and *KNAT2* is achieved by the expression of *ASYMMETRIC LEAVES-1* (*AS1*, an orthologue of MYB domain transcription factor gene *PHANTASTICA* (*PHAN*) of *A. majus*) and *AS2* and *SERRATE* genes (Xu *et al*. 2003; Eckardt 2004; Grigg *et al*. 2005; Yang *et al*. 2006). The meristematic activity of the primordium is maintained and establishment of adaxial identity to the concerned tissue mass is promoted by the interactive effects of class III homeodomain leucine zipper (HD-ZIP III) transcription factor proteins PHABULOSA (PHB), PHAVOLUTA (PHV) and REVOLUTA (REV) and ARGONAUTE (AGO) and PINHEAD (PINH). The MYB transcription family proteins AS1 and AS2 also participate in this process (McConnel *et al*. 2001; Xu *et al*. 2003; Emery *et al*. 2003; Prigge *et al*. 2005; Chitwood *et al*. 2007; Wenkel *et al*. 2007; Braybrook and Kuhlemeier 2010). The abaxial identity to the counterpart tissues is rendered by the actions of KANADI (KAN) class of transcription factors, microRNA (miRNA) 165/166 and class I auxin response factor genes *ETT* (*ARF3*) and *ARF4* (auxin-mediated enhancers of transcription) (Pekker *et al*. 2005; Hunter *et al*. 2006; Fahlgren *et al*. 2006; Chitwood *et al*. 2007, 2009; Kidner 2010). HD-ZIP III RNA accumulation in abaxialized cells is prevented by cleavage of transcripts led by miRNAs 165/166 (Zhou *et al*. 2007). Analogously, ETT/ARF mRNAs are cleaved by ta-siRNAs in adaxialized cells (Williams *et al*. 1990; Nogueira *et al*. 2007; Chitwood *et al*. 2009). YABBY (YAB) type of transcription factors promote lateral growth in the tissues intervening adaxial and abaxial tissues (Seigfried *et al*. 1999; Eshed *et al*. 2004; Hasson *et al*. 2010). The interplay of auxin and MONOPTEROS (MP) and PIN-FORMED (PIN 1) transcription factor proteins is involved in the determination of procambium/mid-vein which develops from the leaf primordium base to leaf primordium apex acropetally as a radiation from vascular bundle of stem (Sieberer and Leyser 2006). Subsequently veins develop in the apex–base direction (Wenzel *et al*. 2007). The ARHB-8 HD-ZIP III and KANADI and other proteins are involved in the control of polarity of mid-vein and higher-order veins (Baima *et al*. 2001; Kerstetter *et al*. 2001; Scarpella *et al*. 2006). The correspondence between the events and their genetic control between simple leaves and simple pinnae of compound leaves is not fully proved.

Two genetic pathways of compound leaf patterning have been identified. In tomato *Solanum lycopersicum* (*Lycopersicon esculentum*) degree of *KNOX* gene (*LeT 6* and *TKN 1*) expression in basipetally developing leaf (primordium) determines level of rachis branching: higher the expression more the complexity of compound leaf (Hareven *et al*. 1996; Janssen *et al*. 1998; Koltai and Bird 2000; Bharathan *et al*. 2002; Shani *et al*. 2009). The optimal KNOX expression requires repression of gibberellic acid (GA) biosynthesis and negative regulation by the *LePHAN* gene (the tomato homologue of *PHAN* gene of *A. majus*) (Kim *et al.* 2003a, b). The dynamic expression of *LePHAN* is required for proper compound leafblade growth and pinna formation. *KNOX* gene expression has been observed to be generally related to compound leaf development in many plant species other than tomato (Kim *et al*. 2003a; Hay and Tsiantis 2006). In pea, *Pisum sativum*, the synergism between the actions of *UNIFOLIATA* (*UNI*), an orthologue of the *FLORICAULA* (*FLO*) gene of *A. majus* and *LEAFY* (*LFY*) gene of *A. thaliana*, and *STAMINA PISTILLOIDA* (*STP*), an orthologue of *FIMBRIATA* (*FIM*) gene of *A. majus* and *UNUSUAL FLORAL ORGANS* (*UFO*) gene, is the determinant of pinnate leaf compounding (Hofer *et al*. 1997; Taylor *et al*. 2001). The *KNOX*-independent *FLO*/*LFY* pathway has been observed to also function in species other than pea (Champagne and Sinha 2004; Floyd and Bowman 2006; Wang *et al*. 2008).

P. sativum plants demonstrate heteroblasty in compounding of their imparipinnate leaves, such that the leafblades of nonembryonic leaves starting from the cotyledonary end to the first flowering node on the main stem are increasingly complex and decrease in complexity to maturity (Taylor *et al*. 2001; Yaxley *et al*. 2001; Mishra *et al*. 2009; Kumar *et al*. 2009). The wildtype most complex leaf bears 15 simple pinnae on leafblade, three pairs of leaflets in the domain proximal to petiole, four pairs of tendrils in the domain distal to petiole, and single tendril in the apical / terminal domain (Prajapati and Kumar 2001). The normal leafblade pattern is differentially altered by mutant alleles of the major genes *AFILA* (*AF*), *TENDRIL-LESS* (*TL*), *MULTIFOLIATE PINNA* (*MFP*) and *INSECATUS* (*INS*), besides the mutations in *UNI* and *STP* genes. The leafblade structure is simpler than wildtype in *uni* and *stp* mutants (Hofer *et al*. 1997; DeMason and Schmidt 2001; Taylor *et al*. 2001; S. Kumar, unpublished observations). The *uni* homozygotes for the alleles such as *uni-224* produce simple leafblades and those for *uni-tendrilled acacia* (*uni-tac*)-1 or *uni-tac-2* form leafblades with normal proximal domain, fewer tendril pairs in distal domain, and terminal leaflet (Gourlay *et al*. 2000; Prajapati and Kumar 2002). The *stp* mutants mimic the *uni-tac* leafblade except that the apical tendril is not replaced by leaflet in their leaves (Taylor *et al*. 2001). In *tl* mutant, simple leaflets are produced in all the leafblade domains (Marx 1989). The pinnae of the

proximal leafblade domain of *af* mutant comprise branched rachides that terminate in tendrils (Marx 1989). In the distal domain, the *multifoliate-pinna* (*mfp*) mutants bear multifoliate pinna pairs in place of tendril pairs (Kumar *et al*. 2004). The pinnae of all the domains occur in the form of variously branched rachides whose termini bear small-sized leaflets, tendrilled leaflets and structures of intermediate nature in *af tl* and *af mfp*, and *af tl mfp* double and triple mutants, respectively (Kumar *et al*. 2004, 2010; Mishra *et al*. 2009). The *tl uni-tac* double mutant has fewer leaflet pinnae than *tl* mutant and has no tendrils like the latter (Marx 1987; Gourlay *et al*. 2000; Prajapati and Kumar 2002; Mishra *et al*. 2009). Presence of *uni-tac* mutation reduces the pinna compounding of the *af, af tl, af mfp* and *af tl mfp* mutants (Mishra *et al*. 2009). In the *af uni-tac* and *af mfp uni-tac* mutants the branched rachides terminate in leaflets apically and respectively bear tendrils or tendrilled leaflets at the lower termini. All rachide ends are occupied by leaflets in *af tl uni-tac* and *af tl mfp uni-tac* triple and quadruple mutants. The leafblade morphologies of *af, af tl, af mfp, af uni-tac, af tl uni-tac, af tl mfp* and *af tl mfp uni-tac* mutants show that the compound pinnae at the petiole end of rachis are the most complex while the pinna at the apex is the least complex and there is gradual decrease in complexity of pinna structures in the proximal to distal direction (Mishra *et al*. 2009). In *ins*, *ins tl*, *ins mfp* and *ins tl mfp* mutants, the proximalmost pinnae are incised and in the cleft an adventitious/ectopic blade is formed from the mid vein. Pinnae in the adventitious blade have the structure of distal domain, genotypewise (Kumar *et al*. 2010). The above *ins*, *mfp* and wildtype leafblade phenotypes suggest domainwise regulation of pinna structural complexity varying from simple to compound tendril and simple leaflet to pinnablades of leaflets of different shapes and sizes, at different positions on rachis, including formation of adventitious blades on proximalmost leafleted pinnae.

In *P. sativum* the *COCHLEATA* (*COCH*) and *STIPULE REDUCED* (*ST*) genes, the latter epistatic to the former, are known to regulate the size and structure of the wildtype peltate-shaped pea stipules (Gourlay *et al*. 2000; Yaxley *et al*. 2001; Kumar *et al*. 2009). *UNI, AF, TL*, *INS* and *MFP* genes get expressed in stipules in the absence of COCH function (Gourlay *et al*. 2000; Yaxley *et al*. 2001; Kumar *et al*. 2009, 2010). There is evidence that *COCH* downregulates *UNI* in the differentiating leaf primordium, and whether *ST* is expressed in leaf is not known (Sharma *et al*. 2012). An infertile *cist* mutant has been reported in which stipules were fused perfoliately and formed a circular disc around the stem nodes and the nodes were leafless (Kumar and Sharma 1975). Thus both stipuleless and leafless variants are known in *P. sativum*.

In pea, the *LEAF-LET DEVELOPEMNT* (*LLD*) gene is known to control pinna morphogenesis (Prajapati and Kumar 2001). In *lld lld* plants, due to low expressivity of the *lld* allele, the loss of LLD function is manifested only in occasional pinnae and in the affected pinnae LLD function is lost at all different stages of pinna development. The phenotypes of aborted pinnae in *lld, af lld, tl lld, uni-tac lld, af tl lld, af uni-tac lld, tl uni-tac lld* and *af tl uni-tac lld* single, double, triple and quadruple mutants suggest that development of simple pinnae is autonomous and in compound pinnae development of each rachis branch is also independent (Prajapati and Kumar 2001). Whether or not the gene network for leaf development is shared by the adventitious blades formed on *ins* mutant is not known. The interaction of LLD function with MFP, INS and COCH functions remains to be elucidated. The leaflets formed at the termini of branched pinna rachides of *af tl, af mfp* and *af tl mfp* mutants are much smaller in size and varied in shape between them compared to leaflets formed on the wildtype and *uni-tac* and *tl* mutants and proximally on *mfp* mutant (Mishra *et al*. 2009). As wax deposition has been observed on both upper and lower surfaces of the miniature leaflets of *af tl wlo* triple mutant (*wachslos* (*wlo*) mutation suppresses wax deposition on adaxial surfaces of pinnae of pea leafblade), a morphogenetic pathway for *af tl* leaflets different from that for normal leaflets is suspected (Gourlay *et al*. 2000). In this regard, the observations on aborted leaflets in leafblades of *af tl lld*, *af mfp lld* and *af tl mfp lld* genotypes and on adventitious blades of *ins* genotypes are considered informative (Prajapati and Kumar 2001; Kumar *et al*. 2010).

The present study is an extension of the previous study on *lld* (Prajapati and Kumar 2001) and gives frequencies and description of the phenotypes of aborted pinnae in the stipule blades and leafblades of genotypes wherein *coch, af, ins, tl, mare*, *mfp, uni-tac* and *lld* mutations have been variously combined. Genetic mapping of *lld*, the as yet unmapped leaf-mutant of pea, is also described. The observations suggest that LLD function plays the role of master control element in pinna development.

Materials and methods

Plant material

The origin of *lld* as a spontaneous mutant and construction of *tl lld* homozygous line SKP-201 are described in Prajapati and Kumar (2001). Derivation of *mfp* as an ethylmethanesulphonate-induced mutant in the form of homozygous line SKP-100 is described in Kumar *et al*. (2004). The lines SKP-100 and SKP-201 were crossed and in the F_2 generation a *mfp mfp lld lld* (*mfp lld*) plant was isolated on the basis of the known phenotypes of *mfp* and *lld* mutations and developed as an inbred line called SKP-301. A *coch* mutant line of the Blixt (1972) collection and SKP301 were crossed and in the F_2 generation a *coch mfp lld* plant was isolated on the basis of expected phenotype and developed into an inbred line. The already available *af tl uni-tac* line (Prajapati and Kumar 2002) was crossed with the *coch mfp lld* line to derive the various permutated combinations of the six mutations in F_2 to F_4 generations on the basis of expected phenotypes. The phenotypes of *af*, *tl*, *mfp*

and *uni-tac* mutant combinatorials, both in the absence and presence of the *coch* mutation have been described earlier (Mishra *et al*. 2009; Kumar *et al*. 2009). Their *lld* counterparts were identified by observing many leafblades for aborted pinnae or their components in each of the segregants. The *ins* line was also from the Blixt (1972) collection. The *ins lld* and *ins tl lld* plants were isolated from the F_2 generation of the cross SKP-201×*ins* on the basis of expected phenotypes and developed into inbred lines. The above genotypes were developed progressively in the period 1983 to 2009 while working at the Indian Agricultural Research Institute, New Delhi, Central Institute of Medicinal and Aromatic Plants, Lucknow, and at NIPGR, New Delhi, India. The following material was developed at the NIPGR from 2004 onwards. The *lld* homozygous line SKP351b was crossed with the line JI833 homozygous for the *mare* (*maximo-reducticus*; Naidenova 2001) mutation to construct the *lld mare* double mutant line. The cross SKP351b×RMP1a (stipule reduced, green pod, pigmented, LLD; isolated from SKP-100 by JI15) gave a F_2 mapping population. JI15 and JI833 lines were obtained from Mike Ambrose, John Innes Centre, Norwich, UK. Ninety-six F_2 plants of the cross SKP351a×RMP1a were also advanced to develop recombinant inbred lines (RILs). The $F_{2:5}$ RIL population was used for the bulk segregant linkage analysis of *lld* locus in relation to a variety of DNA markers. The *LLD* and *lld* bulks comprised of five and seven RILs, respectively. To prepare a bulk, equal quantities of leaves from the component RILs were pooled/bulked.

Growth conditions and recording of observations about the field grown plants

Pea crops for the study of *lld* phenotype in various genotypes and for mapping of the *lld* locus were raised at the experimental farm of the institute at New Delhi in the winter/rabi (October–March) seasons of 2004–2011. For characterizing the leaves of various genotypes, 10 seeds were sown per genotype in 1-m long row in a completely randomized design replicated twice. In these experiments row-to-row distance was kept as 75 cm. In all the experiments, seed rate, row size and row-to-row distance were kept as above. The field plots were prepared for seeding by the following sequence of operations: solarization, ploughing, irrigation, application of N, P and K fertilizers at the respective rates of 60, 50 and 50 kg/ha, harrowing and leveling. After the onset of flowering, crops were applied 0.1% chlorpyrifos and dithane M-45 to prevent insect infestation and fungal infection. Five plants per genotype per replication were labelled at the onset of flowering. Observations on leaves were recorded twice, first at the onset of flowering and next two weeks later. Morphologies of the leaves borne on the first flowering node and two nodes immediately below it and two nodes immediately above it were recorded. All the individual F_2 plants and five plants in each of the $F_{2:5}$ RILs were screened for the LLD/lld phenotype as above. The F_2 and $F_{2:5}$ plants were sampled for leaves (for DNA extraction) three weeks after the onset of flowering. The leaves of F_2 single plants and $F_{2:5}$ lines were stored at −80°C and samples were drawn for DNA extraction as and when required.

Growth conditions and recording of observations about in vitro grown shoots

Shoots of the *tl lld* double mutant were grown *in vitro* by inoculating 10 to 15 single node explants on 70 mL medium in 375 mL wide-mouth jars (bottles, Allied Scientific Sales, New Delhi, India). The medium consisted of Murashige and Skoog salts and Gamborg's vitamins, 3% sucrose, 0.8% agar and 11 μM 6-benzylaminopurine (all from HiMedia, Mumbai, India). The elicitors, indoleacetic acid (IAA), 1-, N-naphthylphthalamic acid (Npa), GA and paclobutrazol (PBZ) (Sigma-Aldrich, St Louis, USA) were added individually to autoclaved medium cooled at 75°C to obtain their desired concentrations. A seed of *tl lld* was raised into a plantlet *in vitro*. The single nodes extracted from this plant served as starting material to multiply *tl lld* explants. The inoculated cultures were incubated at 25°C, 65% humidity and 16 h : 8 h light : dark cycle in which fluorescent tubes were the source of 3000 lux white light. Cultures were examined periodically while they incubated for six weeks. At the end, each shoot of the cultures was photographed. Observations on lld expression were recorded on two to four nodes per shoot, on at least 75 nodes per treatment.

Photographing of leaves and shoots

Leaves were scanned using Hewlett Packard PSC 750 scanner. Shoots were photographed using the Nikon Coolpix L24 digital 14 MP camera and/or AZ-100 Nikon multi-objective stereozoom microscope.

Protocols for the linkage mapping of lld by bulk segregant analysis

The approach used for the genetic mapping consisted of the following steps. DNAs of LLD and lld bulks prepared from $F_{2:5}$ RILs and parents of the cross from which mapping populations were derived were primed with a large number of primers / primer pairs to identify markers that distinguished the parents and/or bulks. The individual $F_{2:5}$ lines that formed the bulks were screened for the presence or absence of the identified DNA markers. F_2 population was studied for the segregation of morphological markers and DNA markers that distinguished the parents and/or bulks. Linkage analysis was performed on the segregational patterns of morphological and DNA markers in F_2 population, using the Mapmaker/Exp v3.0.

In all, 260 RAPD (Operon Technologies, Alameda, USA), 44 ISSR (Bioneer, Daejeon, South Korea) primers, 51 *Medicago truncatula* EST-SSR primer pairs (Eujayl *et al*. 2004) and 97 pea microsatellite and CAPS primer pairs (Gupta

et al. 2007; Agrogene, Moissy Cramayel, France) were used to map the *lld* mutation. Pea microsatellite markers were chosen from among those used earlier workers in pea genetic mapping (Burstin *et al*. 2001; Loridon *et al*. 2005). DNA was extracted using the method of Doyle and Doyle (1990). For RAPD profiling, (PCR) was set up in 25 μL volume containing 1 U of *Taq* polymerase (Invitrogen, Carlsbad, USA), 25 ng of genomic DNA, 0.8 mM of primer, 0.1 mM of each of four dNTPs (Amersham Biosciences, Uppsala, Sweden), 2.5 μL of 10× PCR reaction buffer (500 mM KCl, 200 mM Tris-HCl (pH 8.4)), and 3 mM $MgCl_2$. DNA amplifications were carried out in a iCycler (Bio-Rad, Herclues, USA). The steps used to generate RAPD markers were: 1 cycle consisting of 60 s at 94°C, 30 s at 36°C and 60 s at 72°C followed by 45 cycles of 5 s at 94°C, 15 s at 36°C, and 60 s at 72°C, and a final cycle of 7 min at 72°C. The ISSR amplification were carried out with a preliminary cycle of 120 s at 94°C, followed by 35 cycles of 20 s at 94°C, 50 s at 50°C, and 90 s at 72°C, and a final cycle of 7 min at 72°C. The amplification products were resolved on 1.2% (for RAPD) and 1.5% (for ISSR) agarose gels (Sigma-Aldrich, USA). The EST-derived SSR primers-based amplification reactions were carried out in a 20 μL volume containing 1 U of *Taq* polymerase (Invitrogen, USA), 25 ng of genomic DNA, 0.80 μM of each primer (forward and reverse), 0.2 mM of each dNTPs, 2.0 μL of 10× PCR reaction buffer and 3 mM $MgCl_2$. DNA amplifications were carried out in a iCycler (Bio-Rad, USA). The amplifications parameter were as follows: 3 min at 94°C, followed by 45 cycles of 60 s at 94°C, 60 s at 50°C, and 120 s at 72°C, and a final step of 10 min at 72°C. The amplification products were resolved on Metaphor agarose gels in TBE (45 mM Tris borate and 1 mM EDTA) (Cambryx Bioscience, Rockland, USA). The pea microsatellite-based amplification reactions were carried out in a 20-μL volume containing 1 U of *Taq* polymerase (Invitrogen, USA), 25 ng of genomic DNA, 0.80 μM of each primer (forward and reverse), 0.2 mM of each dNTPs, 2.0 μL of 10× PCR reaction buffer and 3 mM $MgCl_2$. DNA amplifications were carried out in a iCycler (Bio-Rad, USA). The amplifications parameter were as follows: 3 min at 94°C, followed by 45 cycles of 60 s at 94°C, 60 s at 50°C, and 120 s at 72°C, and a final step of 10 min at 72°C. The amplification products were resolved on Metaphor agarose gel and Page to score polymorphic bands. The bands were photographed using gel documentation system (Alpha Imager, Santa Clara, USA).

For linkage analysis, the markers were tested for their goodness-of-fit to the expected 3 : 1 ratio for a dominant locus in F_2 population by chi-square test ($P < 0.01$). Using the group assign order and ripple command of Mapmaker/Exp v3.0 (Lander *et al*. 1987; Lincoln *et al*. 1992). The linkage groups were built from the segregational data. The marker order on the linkage group(s) was established by using the LOD score of 3.0 and maximum distance of 30 cM. The order of loci on the specific linkage groups was refined by the ripple command. The Kosambi mapping function was used to calculate genetic distances in centimorgans (cM) (Kosambi 1944).

Results

Interaction between lld and af, mfp, tl and uni-tac leaf morphology mutations

The leaflets and tendrils, that make the wildtype pea imparipinnate compound leaf, are individualized pinna organs. Since leaflets have their own blade and stalk (petiolule), they are comparable to simple leaf such as that of *A. thaliana*. Tendrils on the other hand are radial organs, with curled up tip. The *lld* muatation causes mosaicism (Marcotrigiano 2001) in the compound leafblade structure, by aborting the development process in occasional pinna(e). Since the LLD functional loss is temporally variant in different pinnae, development in the affected pinnae is arrested at its different stages (figure 1). Thus, the *lld* mutation on account of its low expressivity permits a dynamic analysis of initiation and development of each pinna in the background of wildtype and mutant alleles of other genes that affect pinna structure and overall leafblade architecture (figures 2 and 3).

The wildtype unipinnate leafblade consists of 15 simple pinnae, three pairs of leaflets in the proximal domain, four pairs of tendrils in the distal domain, and a terminal apical tendril. Thus there are eight nodes on the laefblade rachis. The last node bears apical tendril which is an extension of the primary rachis. The pinnae pairs at the other nodes of primary rachis are secondary organs, extensions of the secondary branches of rachis in the form of petiolules. All pinnae are simple in *tl* and *uni-tac* mutants. Pinnae of distal and apical domains are compound blades in the *mfp* mutant. The *af* mutant produces compound pinnae in the proximal domain. In the double, triple and quadruple mutants involving *af*, *mfp*, *tl* and *uni-tac* mutants, leaf blades are unipinnate or bi-, tri- or more-pinnately compound in proximal, distal and/or apical domains. Therefore, it was desired to study how the *lld* mutation affected development of suborgans in variously compound pinnae. The *af*, *mfp*, *tl* and *uni-tac* mutations that are known to affect overall leafblade morphogenesis were combinatorially recombined with *lld*. The observed frequencies of abortion events are presented rachis-branch-level-wise for each node position on primary rachis in table 1. The structures of the aborted suborgans of all the 16 genotypes are shown in figures 2 and 3. It can be seen from table 2 that *lld* expressed in all the 16 genotypes and the pinnae present at all the positions in their leafblades, irrespective of whether simple or compound structure underwent developmental abortion. In the compound pinnae, the ramifications were aborted at the secondary, tertiary and all the higher levels of rachide divisions. In the genotypes wherein

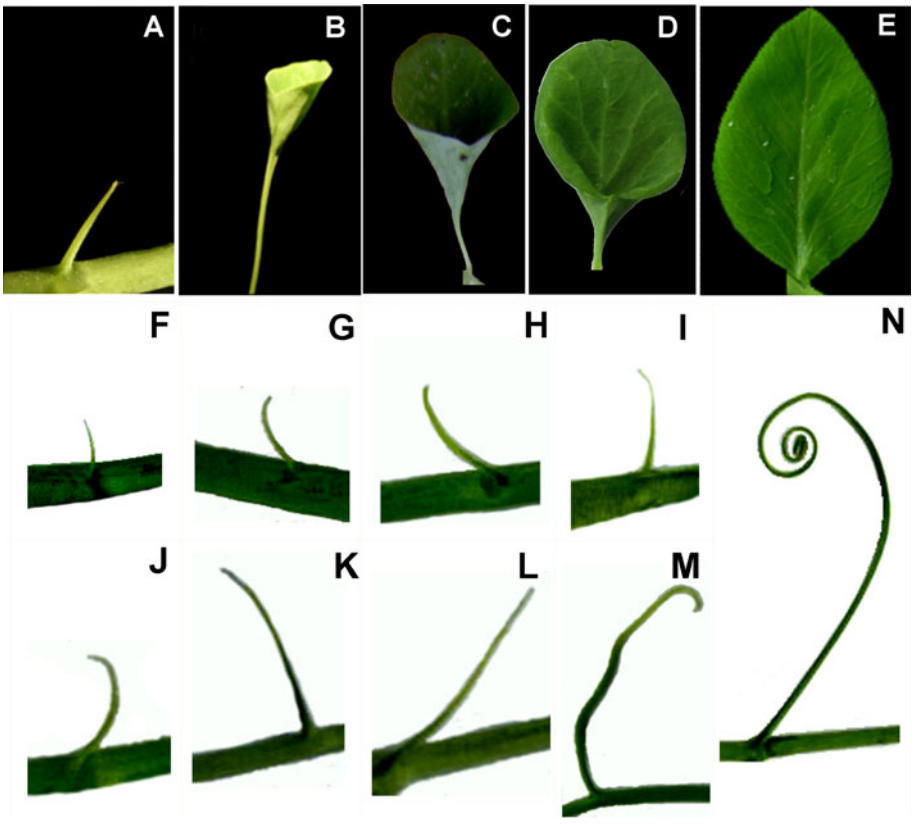

Figure 1. Expression of the *leaflet development* (*lld*) mutation led abortion in pinnae of the unipinnately compound leaf of *Pisum sativum*. A–D, Leaflets aborted at different stages of their development. The aborted leaflets have rod (needle) and cup/bell/trumpet/lotus like morphologies because of the abortion of leaflet differentiation at its different stages; E, normal leaflet; F–M, tendrils aborted at different stages of their differentiation/development; N, normal tendril.

pinnae in the leafblades were simple (*lld*; *tl*/*uni-tac*/*mfp lld*; *tl uni-tac*/*mfp lld*; *uni-tac map lld* and *tl mfp uni-tac lld*), pinna abortification was low at the first rachis node as compared to that at the node numbers two to six. On the other hand, the proximal most compound pinnae, in the genotypes of ramified leafblades (*af lld*; *af tl*/*uni-tac*/*mfp lld*; *af tl uni-tac*/ *mfp lld*; *af uni-tac mfp lld* and *af tl uni-tac mfp lld*), manifested the abortion events at higher frequencies than the compound or simple pinnae at 2 to 6 node of primary rachis. The pinna abortions in the *lld af tl* genotype in some leafblades led to drastic curtailment in the latters complexity (figure 2, L&M). Both rod and trumpet shaped aborted leaflets were visualized in *af tl lld* leaves. The pinnae of the *lld af mfp* and *lld mfp tl* and *lld af mfp uni-tac* triple and quadruple mutant genotypes manifested more than 3-fold, more abortion events than the average of pinna abortion events in the leafblades of all genotypes (figure 3, D,F&H). It can be seen from the figure 3, B,D,F&G that when *lld* and *mfp* mutations were present together, such as in the *lld mfp*, *lld mfp af* and *lld mfp af uni-tac* genotypes, the abortion events occurred at a very high frequency in the domain(s) occupied by tendrilled leaflets.

Interaction between lld and ins

The *ins* mutation allows ectopic growth of midrib of the proximalmost pinnae into leafblades of the morphology as per the genetic background. In the *ins tl* and *ins tl lld* and *ins tl lld* leaves, the proximalmost pinnae (leaflets) were apically cleaved and each bore an adventitious/ectopic leafblade. Such ectopic blades were often trifoliate (figure 3, I–L). Whereas the leaflets of adventitious leafblades were of normal morphology in the *ins tl*, those in the *ins tl lld* were often differentially aborted (figure 3, J–L). The *lld* caused abortion of pinnae in the adventitious blades of *ins tl lld* genotype was observed at a frequency of about 35% (table 2). Both rod and trumpet shaped aborted leaflets were seen.

Interaction between lld and mare

The *mare* mutant leafblades inherently bore highly reduced leaflets in their proximal domain and normal-looking tendrils in the distal and terminal domains. The *lld* mutation was expressed in all the pinnae (tendrils) of leafblade distal

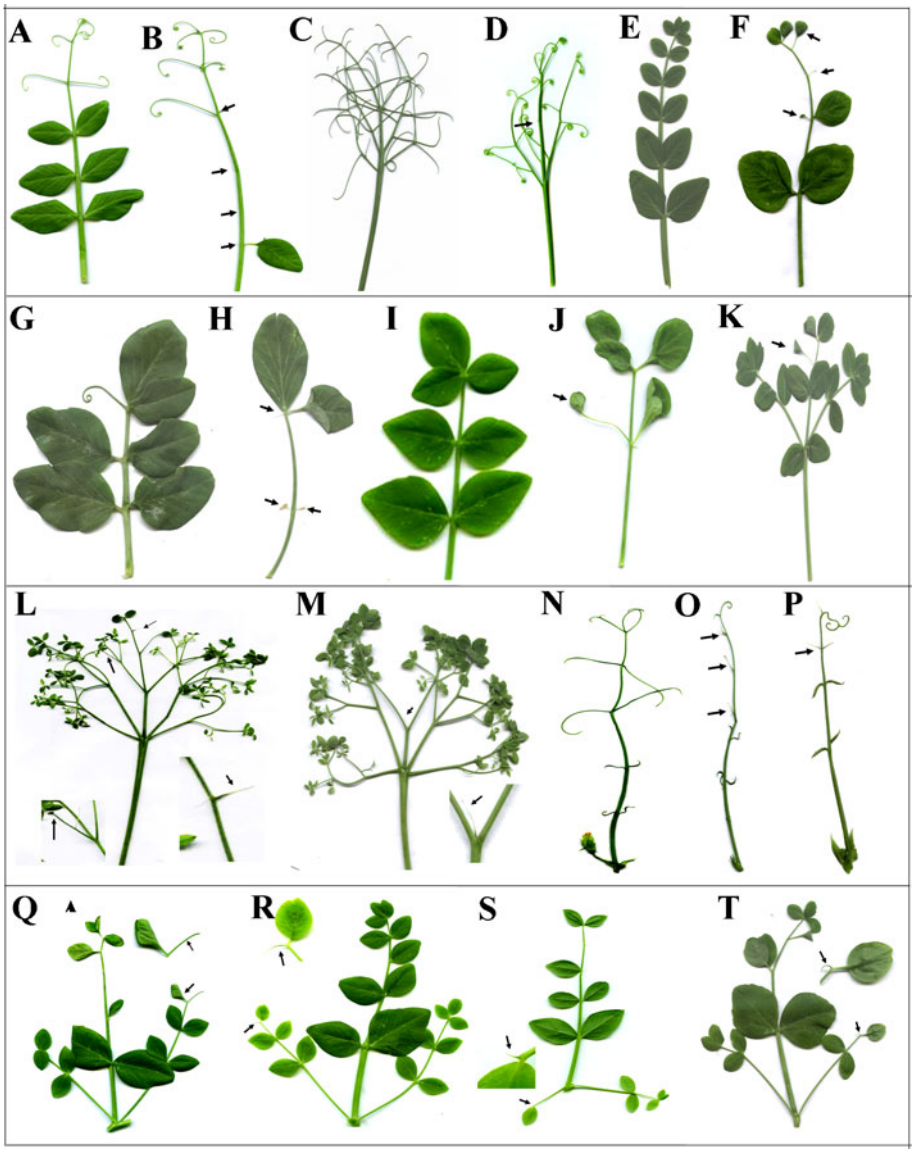

Figure 2. The *lld* (*leaflet development*) caused abortion of one or more tendrils and or leaflets in the leafblades of different leaf morphology mutants of *Pisum sativum*. A, wildtype leaf; B–P, mutant leaves; Q–T, mutant leaves and stipules. Leaf mutations, *af*, *afila*; *tl*; *tendril-less*; *uni-tac*; *unifoliata tendrilled-acacia*; *mare, maximo-reducticus*. Stipule mutation, *coch*, *cochleata*. B, *lld* leaf in which several leaflets and a tendril are aborted; C, *af* leaf; D, *af lld* double mutant leaf in which several tendrils are aborted; E, *tl* leaf; F, *tl lld* leaf in which all the leaflets appear to be aborted at different stages of development; G, *uni-tac* leaf; H, *uni-tac* leaf in which all pinnae except the apical leaflet are aborted and the aborted pinnae are needle or trumpet shaped; I, *tl uni-tac* leaf; J, *tl uni-tac lld* leaf in which all the leaflets are aborted, K, *af tl uni-tac* leaf in which a trumpet shaped aborted leaflet is seen; L, *af tl lld* leaf in which a needle shaped aborted leaflet borne on primary rachis and a trumpet shaped aborted leaflet borne on secondary rachis are visualized; M, *af tl lld* leaf in which primary rachis is seen aborted into a needle in the distal domain of leafblade; N, *mare* leaf; O and P, *mare lld* leaves in which tendrils are seen aborted; Q–T, *coch tl lld* stipulated leaves in which leaflets in leafblades and in leaf-like stipule blades are aborted into trumpet shaped (Q) and needle shaped (R–T) structures.

domain of the *lld mare* double mutant (figure 2, N–P). The proximal leaflets appeared to be unaffected.

Interaction of lld and coch allowed the expression of lld phenotype in stipule blades

It is known that *UNI* (*UNI-TAC*) *coch* stipules are compound, like leafblades, depending on the allelic status of *AF, TL* and *MFP* genes and that *uni-tac coch* stipules are simple, irrespective of the allelic status of *AF, TL* and *MFP* genes (Kumar *et al*. 2009). Table 3 is presented with the stipule phenotypes of 15 genotypes in which *coch* and *lld* mutations are variously combined with *af, tl*, *mfp* and *uni-tac* mutations. It can be seen that lld phenotype is expressed in the compound stipules (stipuleblades) of *coch lld, coch lld af, coch lld tl, coch lld mfp, coch lld af tl*, *coch lld af mfp* and

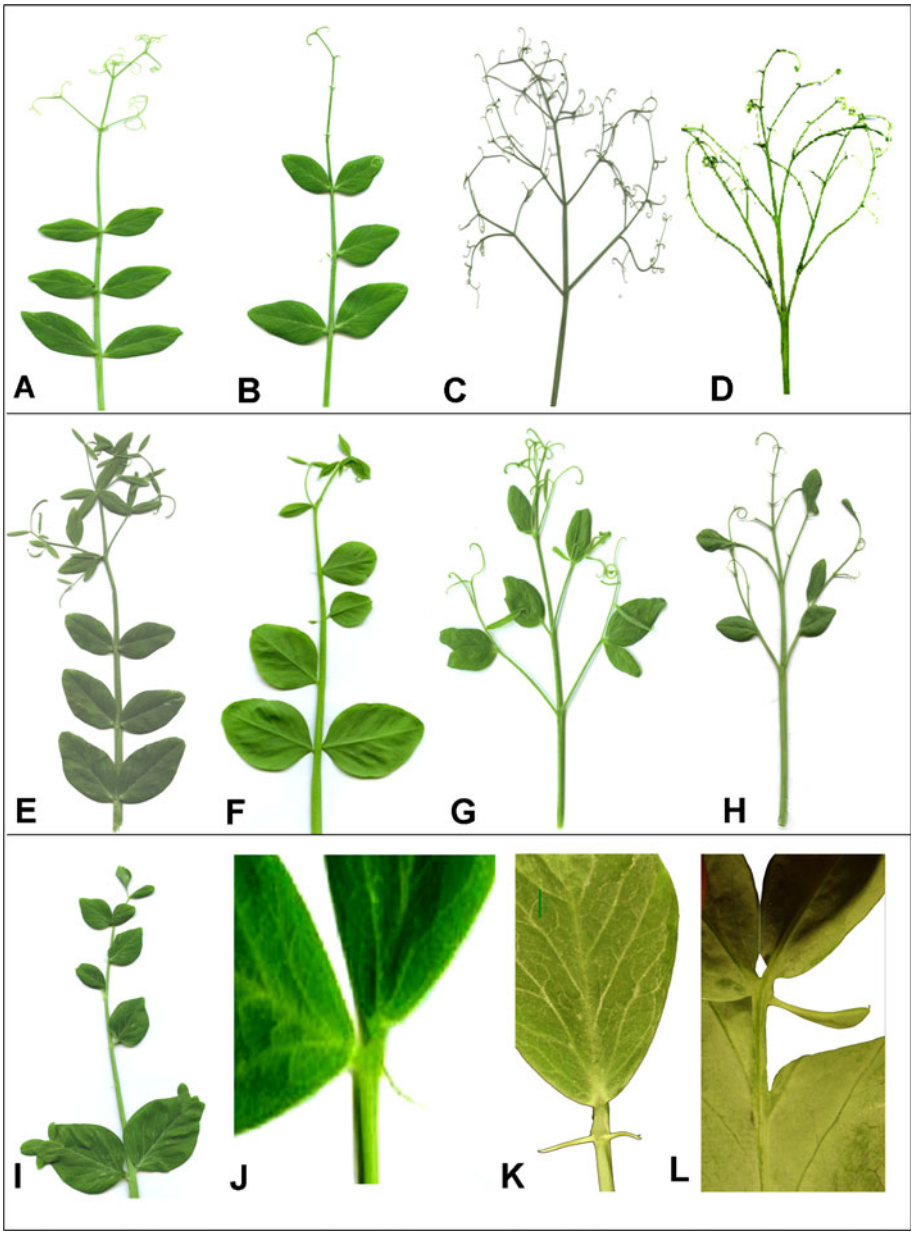

Figure 3. The abortive effect of *lld* on the development of leafblade pinnae in the background of leaf morphology mutant *multifoliate-pinna* (*mfp*) and *insecatus* (*ins*) in *Pisum sativum*. A, *mfp* leaf; B, *mfp lld* leaf in which a leaflet and several tendrils are aborted; C, *af mfp* leaf; D, *af mfp lld* leaf in which abortifying effect of *lld* has led to loss of several pinnae and in pinnae loss of many tendrilled leaflets; E, *tl mfp* leaf; F, *tl mfp lld* leaf in which several leaflets, and tendrilled leaflets in the distal domain blades have aborted; G, *af uni-tac mfp* leaf; H, *af uni-tac mfp lld* leaf in which many tendrilled leaflets have aborted; I, *ins tl lld* leaf in which several leaflets are seen to have aborted. J–L, enlargements of the ectopic blades formed on proximal most leaflets showing abortion of leaflets giving rise to needles and a trumpet shaped structure. Leaf mutants, *mfp*, *multifoliate pinna*; *af*; *afila*; *tl*; *tendril-less*; *uni-tac*; *unifoliata tendrilled-acacia*; *ins*; *insecatus*.

coch lld af tl mfp genotypes, such that occasional pinna in the stipule blades was aborted like in leafblades. However, *lld* caused organ abortion was not observed in the simple stipules formed in the above genotypes, where some of the nodes bore one or two simple stipules, and on the *coch lld uni-tac, coch lld uni-tac af, coch lld uni-tac tl, coch lld uni-tac af mfp* and *coch lld uni-tac af tl* genotypes where all the stipules formed were simple in morphology. These results demonstrated that *lld* mutation indeed expressed at the level of pinna initiation and development in both leafblades and stipule blades of pea, when leafblades and stipules had compound architecture.

The average pinna abortion frequency in the stipule blades of a *coch lld tl* line was observed to be 20% as compared to 26% for pinnae in leafblades (figure 2, Q–T). It will be seen from the observations given in table 4 that the

Table 1. Interaction between *lld* and *af, tl, uni-tac* and *mfp* mutations: the rachis-node-wise distributions of *lld* mutation caused abortion events, at various levels of pinnae ramification, in the leaf blades of 16 genotypes in which *af*, *mfp*, *tl* and *uni-tac* mutations had been permutedly recombined in *Pisum sativum*.

Genotype[a]					Level of pinna	Average[g] number of abortion events at the rachis node pair position from the petiole end								Total of abortion events at all levels of ramification in a leaf blade[i]
Allelic status at the gene														
LLD	*AF*	*TL*	*UNI-TAC*	*MFP*	ramification	1	2	3	4	5	6	7	T	
−	+	+	+	+	P[b]	[h]							0.1	
					S[c]	0.1	0.3	0.4	0.3	0.2	0.4			1.8
−	−	+	+	+	S	0	0.1	0.3	0.2	0.2				4.2
					T[d]	1.9	0.4	0.1						
					Q[e]	1.0								
−	+	−	+	+	P								0.1	4.1
					S	0.1	0.8	0.9	0.8	0.6	0.4	0.4		
−	+	+	−	+	P								0.1	1.1
					S	0.3	0.3	0.4						
−	+	+	+	−	S	0.1	0.3	1.6	1.6	2.0	2.0	1.8		9.4
−	−	−	+	+	S	0.1	0.1	0.3	0.2	0.3	0.5			3.5
					T	0.4	0.2	0.1	0.1	0.1				
					Q	0.4	0.2	0.1	0.1					
					H[f]	0.2	0.1							
−	−	+	−	+	S	0	0.3	0.4	0.3	0.3				5.7
					T	1.3	0.4							
					Q	2.7								
−	−	+	+	−	S	0	0.5	0.5	1.0	1.0	1.0			20.9
					T	6.2	2.5	3.0						
					Q	0	4.8							
					H	0.4								
−	+	−	−	+	S	0	0.5	0.6	0.5	0.7				2.3
−	+	−	+	−	S	0	0.5	0.5	1.0	0.5	0.5			3.0
−	+	+	−	−	S	0	0.5	0.3	0	0	0			0.8
−	−	−	−	+	S	0	0	0.2	0.2	0.3				1.8
					T	1.0	0.1							
−	−	−	+	−	S	0	0	0.3	0.4	0.4	0.2	0.3		22.1
					T	2.0	1.6	0.8	0.2	0.1	0.2			
					Q	3.1	2.5	2.0						
					H	3.9	3.1	1.0						
−	−	+	−	−	S	0	1.0	1.0	1.0	1.0	1.3			19.8
					T	8.0	0.6	0.5	1.0	0.8				
					Q	3.6								
−	+	−	−	−	S	0	0	0.2	0.5	0.6	0.3			1.6
−	−	−	−	−	S	0	0	0	0.1	0.2	0.1			1.5
					T	0.5	0.1	0.1	0	0.1	0.1			
					Q	0.2								

[a]All the genotypes were homozygous for the concerned genes/alleles; [b]primary level or pinna at top; [c]secondary level; [d]tertiary level; [e]quaternary level; [f]higher than quaternary level; [g]average of leafblades taken from the flowering node and ones immediately above and below it on 10 plants; [h]empty space in these columns means nonapplicability. [i]The morphologies of the aborted suborgans of leaf-blade are shown in the figures 1–3. Genes: *LLD, LEAFLET DEVELOPMENT*; *AF*, *AFILA*; *TL*, *TENDRIL-LESS*; *UNI-TAC, UNIFOLIATA TENDRILLED-ACACIA*; *MFP, MULTIFOLIATE-PINNA*. The observations were recorded in 2008–2009 winter season.

pattern of pinnae abortion in stipule blades of the *coch tl lld* line mimicked that of leafblades, about 15% in proximal leaflets and much higher at ≥80% in the distal and apical leaflets.

Effect of elicitors on lld expression

To examine the role(s) of auxin (IAA) and GA in the expression of lld phenotype, the *tl lld* explants were grown into shoots *in vitro* in the individual presence of IAA and auxin transport inhibitor NPA and GA and its antagonist PBZ. The observations are summarized in table 5 and figure 4, A–J. The frequency of aborted leaflets in the *in vitro* grown *tl lld* control shoots was very high (~84%) as compared to field-grown plants (~40%). Exogenous supplementation with IAA did not change the frequency and pattern of occurrence of *lld* led abortion of leaflets *in vitro* grown shoots. Frequency of leaflet abortion on proximal and distal pinnae

Table 2. Interaction between *lld* and *ins*: expression of *lld* mutation in the *ins* directed adventitious blades borne on proximal leaflets of *ins tl lld* in *Pisum sativum*.

Homozygosity for the genes				Number of proximal leaflet pairs	
INS	*LLD*	*TL*	Examined	Having normal pinnae in adventitious/ectopic blades	Having lld pinnae in ectopic blades
−	+	−	20	20	0
−	−	−	20	13	7

These observations were recorded in the 2010–2011 winter season. Genes: *INS, INSECATUS*; the names of other genes are given in table 1.

Table 3. Interaction of *lld* (*leaflet* development) and *coch* (*cochleata*): expression of pinaa abortion in the *coch* and *lld* mutation carrying combinatorials of *afila* (*af*), *tendril-less* (*tl*), *multifoliate pinna* (*mfp*) and *unifoliata-tendrilled acacia* (*uni-tac*) gene mutations in the leafblades and stipules in *Pisum sativum*[a].

Genotype							
Allelic status at the gene						Structural pattern	Whether *lld* phenotype
COCH	*LLD*	*AF*	*TL*	*MFP*	*UNI-TAC*	of stipule[b]	was seen in stipule pinnae
−	−	+	+	+	+	Compound[c]	Yes
−	−	−	+	+	+	Compound	Yes
−	−	+	−	+	+	Compound	Yes
−	−	+	+	−	+	Compound	Yes
−	−	+	+	+	−	Simple[d]	No
−	−	−	−	+	+	Compound	Yes
−	−	−	+	−	+	Compound	Yes
−	−	−	+	+	−	Simple	No
−	−	+	−	−	+	Compound	Yes
−	−	+	+	−	−	Simple	No
−	−	+	−	+	−	Simple	No
−	−	−	−	−	+	Compound	Yes
−	−	−	+	−	−	Simple	No
−	−	−	−	+	−	Simple	No
−	−	+	−	−	−	Simple	No
−	−	−	−	−	−	Simple	No

[a]The phenotype was also observed in the *lld mare* homozygote; [b]TL leafblades had compound structure in all the genotypes; [c]in all genotypes the compound stipule blades mimicked the structure of leafblades; [d]simple stipules were usually small spoon shaped organs. These observations were recorded in 2009–2010 winter season.

Table 4. Expression of *lld* mutation in the stipules of *coch tl lld* genotype in *Pisum sativum*.

Genotype in terms of nature of the alleles in the genes[a]			Number of		
COCH	*TL*	*LLD*	Compound stipules examined	Normal stipules (number of leaflets borne on them)	Stipules carrying aborted leaflets (number of normal leaflets + aborted leaflets borne on them)
−	−	+	20	20 (105)	0
−	−	−	20	6 (28)	14 (53 + 20)[b]

[a]The two genotypes were homozygous for the concerned genes/alleles. [b]Among the aborted pinnae, about 85% were in the distal + apical domains of the compound stipules. These observations were recorded in 2009–2010 winter season. Names of the genes are given in the tables 1 and 3.

Table 5. Effects of gibberellic acid and indole acetic acid and their antagonists on the expression of *lld* phenotype shoots grown *in vitro* in the leaves of *tl lld Pisum sativum*.

Treatment (concentration)	Average number of pinnae in leaves born on explants	Per cent lld phenotype		
		In the proximal pinnae	In the distal and apical pinnae	In pinnae at all position
Control	4.9 ± 0.3	74.0 ± 2.7^{c}	86.5 ± 3.1^{c}	83.39 ± 3.0
Gibberellic acid (50 μm)	4.1 ± 0.1	20.8 ± 1.2^{a1,e}	66.1 ± 2.7^{f}	51.0 ± 2.2
Paclobutrazol (2 μm)	5.4 ± 0.3	41.5 ± 8^{a2,g}	73.12 ± 2.7^{b1,h}	66.8 ± 3.8
Indole-3-acetic acid (2 μm)	5.2 ± 0.2	78.4 ± 1.6^{d}	86.3 ± 0.7^{d}	84.3 ± 0.9
1-N-naphthylpthalamic acid (50 μm)	3.2 ± 0.1	19.2 ± 0.9^{a3,i}	53.4 ± 2.6^{b2,j}	42.3 ± 2.0

$^{a1-a3}$Treatment effects at proximal pinnae position found significantly different from the control at 5% probability using *t*-test (t_6, a1 = 7.8; a2 = 11.9; and a3 = 22.06); ^{b1,b2}treatment effects at distal and apical pinnae position found significantly different from control at 5% probability (t_6, b1 = 5.1; and b2 = 8.5); $^{c-j}$*lld* expression between proximal pinnae versus distal and apical pinnae was significantly different where the superscript letters are different (t_6 at 5% probability, for e and f = 2.62; for g and h = 3.21; and i and j = 8.48). Gene names are given in table 1.

was similar in control and IAA treated shoots (figure 4, A&D). However, shoots grown in the presence of GA, PBZ or NPA demonstrated lower levels of leaflet abortion (figure 4, B,C&E). These results suggested that auxin had stimulatory effect on the *lld* led leaflet abortion events.

Mapping of lld gene

The *lld* trait is inherited as a Mendelian character (Prajapati and Kumar 2001). The inheritance of the *lld* allele was also studied in the present experiment. The identification of lld phenotype could be scored in any of the postjuvenile leaves borne in F_2 plants. The expression of lld phenotype was most pronounced in the postflowering stage of plant approaching maturity as described in the previous section. The inheritance and mapping of *lld* allele are described below.

Inheritance pattern of lld

The F_2 generation obtained from the cross between SKP351b possessing *lld/lld* and RMP1a segregated into 22 *lld/lld* and 74 *LLD/LLD* + *LLD/lld* plants whose pattern gave a good fit

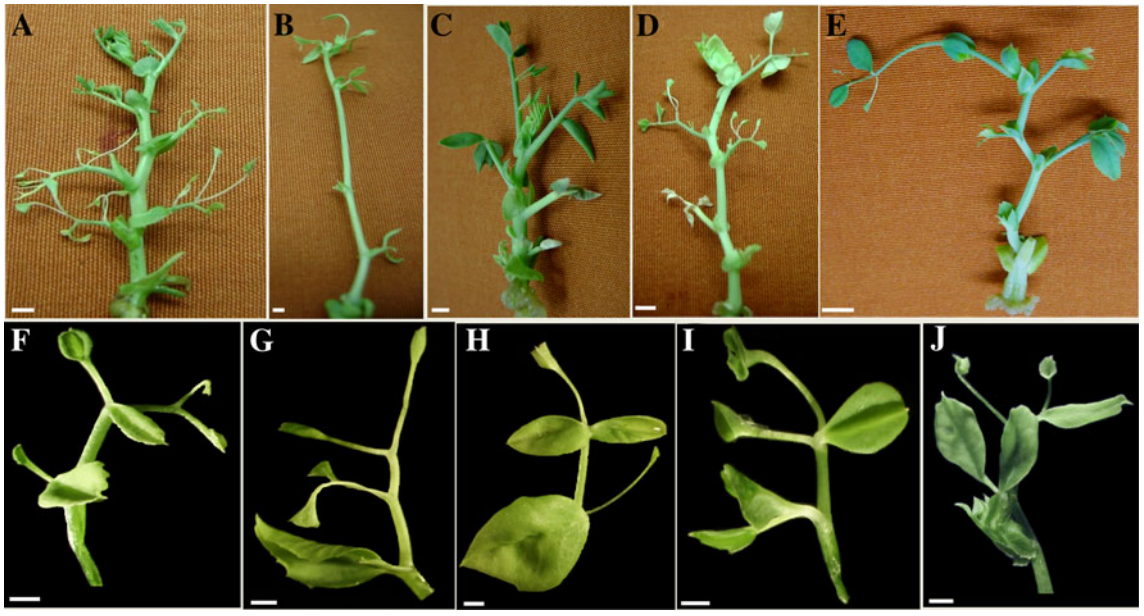

Figure 4. Expression of lld phenotype in the *in vitro* grown shoots of *tl lld* in the absence and presence of elicitors. A, control; B, gibberellic acid; C, paclobutrazol; D, indole acetic acid; E, 1, N-naphthylpthalmic acid; F–J, enlargements of *in vitro* grown shoot segments in which the aborted pinnae can be seen for their varied structures.

to 1 : 3 ratio expected of single gene inheritance ($\chi^2 = 0.21$). The observations in F_3 generation showed that the number of *lld/lld, LLD/LLD* and *LLD/lld* lines was 22, 25 and 49, respectively. The Mendelian inheritance of *lld* locus was confirmed.

Bulk segregant analysis (BSA)

For the BSA, *LLD/LLD* bulk of leaves was made by pooling equal quantities of leaves taken from five *LLD/LLD* $F_{2:5}$ lines. Likewise, leaves from plants of seven *lld/lld* $F_{2:5}$ lines were used to comprise *lld/lld* bulks. DNAs isolated from parental lines, *LLD* and *lld* bulks were primed with 452 primers/primer pairs of different types (table 6) to identify those which distinguished the two parents and bulks. Four DNAs were amplified with 260 RAPD and 44 ISSR primers and 148 SSR and CAPS primer pairs. This led to the short listing of 15 RAPD, 1 ISSR and 7 SSR primers/primer pairs that distinguished the parents and bulks. Their further use in amplification of DNAs of 12 single lines whose leaves went into making of bulks revealed that one SSR primer pair and one RAPD primer gave a band each with the *lld* parent and bulk, but not with *LLD* parent and bulk. The SSR primers related to the DNA band or marker linked to the *lld* were/was identified as PSPO4SG (figure 5), and the RAPD primer related DNA band or marker found linked to *lld* was named as OPH-9 (table 7).

Linkage mapping of lld

A total of 220 DNA markers (161 RAPD, 24 ISSR and 35 SSR) distinguished the parents of the F_2 mapping population, namely SKP351b and RMP1a (table 6). In addition there were four morphological markers that segregated in the F_2 population comprising of 96 plants. The entire F_2 population was studied for the lld and other morphological characters and for amplification of 220 DNA markers by the use of 140 DNA primers/primer pairs individually. The linkage analyses on the phenotypic and DNA marker segregational data by use of Mapmaker/Exp v3.0 produced a linkage group on which 14 molecular markers (tables 6 and 7) along with two morphological markers (including *lld*) were mapped (figure 6). The *lld* linkage group had a length 144.9 cM. The average distance of markers on this linkage group was 9.05 cM.

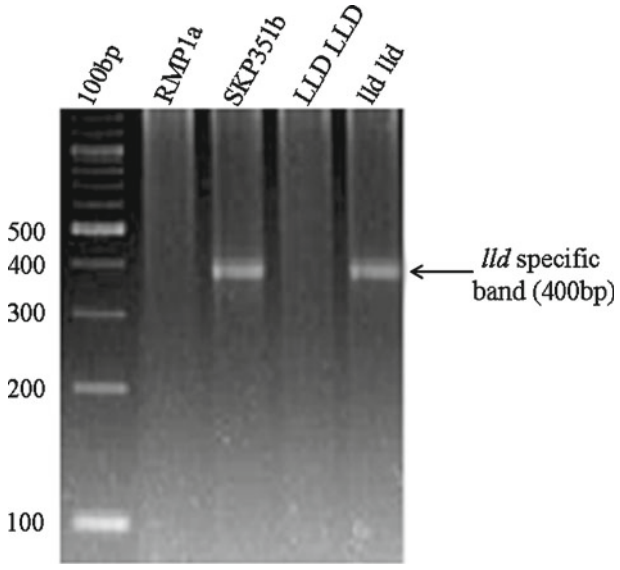

Figure 5. Gel images for the parents and pools (bulks) for the amplified DNA marker that distinguished *lld* genotypes from *LLD*. The genomic DNAs of the parents and bulks had been amplified with the primer pair called PSPO4SG. The size of the band specific to *lld* is also mentioned. RMP1a was the *LLD* parent; SKP 351b was the *lld* parent; *LLD LLD* lane represents the LLD pool; and *lld lld* lane the lld pool.

Alignment of linkage group with published map

To determine the location of *lld* gene in relation to the published and consensus linkage maps of pea, linkage groups of two reference maps, on which some of the markers were common with the *lld* carrying linkage groups were aligned with the latter. The *lld* linkage group developed in this study has two microsatellite markers, namely AA175 and AD73, that are placed in the linkage group III of the map generated by Loridon *et al*. (2005). One microsatellite marker, namely PSPO4SG of the linkage group III of the map of Prioul *et al*. (2004) is present on the *lld* linkage group constructed in this study. One morphological marker *st* also present on *lld* linkage group had been earlier mapped on linkage group III by Ellis and Poser (2002). These results suggest that *lld* is located on the linkage group III of the conventional genetic map of pea (figure 6).

Table 6. Molecular markers used in the bulk segregant analysis for the identification of DNA markers linked to the *lld* marker.

Marker type	Primers/primer pairs screened	Number of primers/primer that distinguished the parents	Number of markers generated	Markers that showed linkage with *lld* marker	Number of markers that got mapped
RAPD	260	104	161	15	11
ISSR	44	9	24	1	0
Mt_ESSR + pea microsatellite, CAPS	148	27	35	7	3
Total	452	140	220	23	14

Table 7. The DNA markers, their sizes and primer sequences that could be placed relative to *lld* locus on the linkage group III of the established genetic map of *Pisum sativum.*

Primer name/ morphological trait	Sequence (5'–3')/ (F 5'–3', R 5'–3')	Band size of the marker	Distance (cM) between marker and *lld* on LG III
OPC2	GTGAGGCGTC	2000	18.2
PSPO4SG	CAACCAGCCATTATACACAAACA GGCAATAAAGCAAAAGCAGA	400	0
lld[a]	–	–	0
OPH9	TGTAGCTGGG	1000	9.7
AA175	TTGAAGGAACACAATCAGCGAC TGCGCACCAAACTACCATAATC	260	12.2
OPG13	CTCTCCGCCA	1000	20.8
OPA9	GGGTAACGCC	1600	40.2
OPC7	GTCCCGACGA	800	40.2
OPD11	AGCGCCATTG	600	56
St[a]	–	–	61.7
OPD13	GGGGTGACGA	2200	64.5
OPA3	AGTCAGCCAC	1700	79.7
AA278	TGATGTAAGTTCCGCCGTGTAT TGCTTGTGTCAAGTGATCAGTG	300	79.7
OPB10	CTGCTGGGAC	1200	92.9
OPK4	CCGCCCAAAC	1300	99.3
AD73	CAGCTGGATTCAATCATTGGTG ATGAGTAATCCGACGATGCCTT	230	126.7

[a]Mutant marker names = *lld*, *leaflet development*; and *st*, *stipule reduced*.

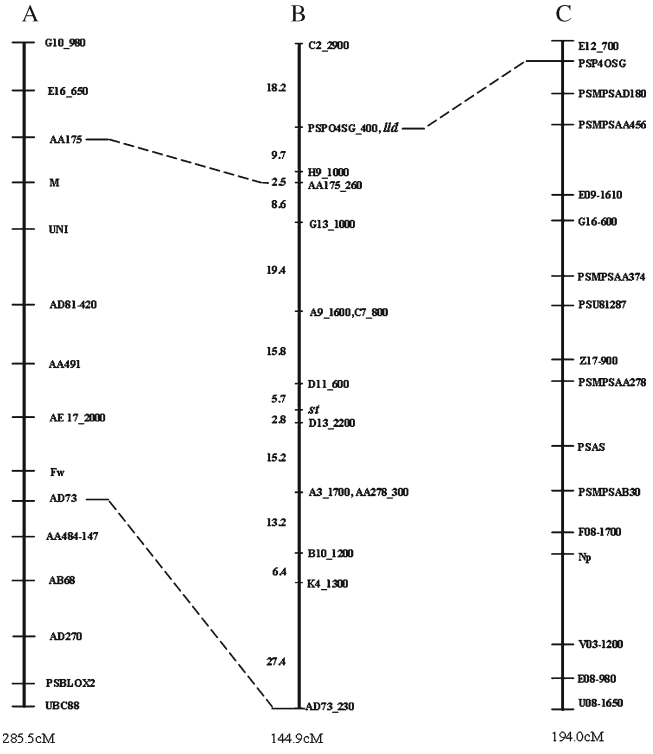

Figure 6. Map of the chromosome / linkage group III region of pea *Pisum sativum* showing the position of the *lld* trait with reference to DNA markers. The map constructed in this study (B) is shown in relation to the maps developed by Loridon *et al*. (2005) (A) and Prioul *et al*. (2004) (C). The sizes of bands amplified by primers common between A and B were AA175 (260 bp), AD73 (230 bp), respectively and the size of band common between B and C and amplified by the primer PSP4OSG was 400 bp (see table 7).

Discussion

The results described above have shown that in *P. sativum* the (i) LLD function is involved in the maintenance of primordium competence for the development of pinna in all the domains of leafblade and compound stipuleblade, (ii) expressivity of *lld* mutation is increased by the mfp function, and (iii) leaflets grow and develop basipetally while tendrils and tendrilled leaflets are formed acropetally. These aspects and the map locations of *lld* and *mfp* mutations vis-à-vis other mutations that affect leafblade and stipule development are discussed below.

LLD function is essential for pinna morphogenesis in leafblades

The leafblades of *P. sativum* lines, in which the mutations in *AF, TL, MFP* and *UNI* genes are permutationally combined, demonstrate variously ramified architectures; among the 16 genotypes, several genotypes bear leafblades with simple pinnae in one or more domains and others produce compound pinnae in all the domains (Mishra *et al*. 2009). When the *lld* mutation was recombined into these genotypes, the expression of *lld* led to abortion of development in one or more pinnae in some of the leafblades, in all the 16 *lld* genotypes studied. There was no leaf architectural difference between the corresponding *LLD* and *lld* genotypes. Among the *lld* genotypes, individual pinna aborted at temporally different stages of morphogenesis. Abortion events in pinnae of the same leafblade were spatially independent. Pinnae at all positions in all domains of leafblades of each of

the genotypes were found to have aborted, at different stages of morphogenesis. These observations are consistent with autonomous morphogenesis of each pinna in all the domains of leafblade, as per the genetic programme specified by the genotypes.

The *P. sativum* wildtype leafblades produce leaflets in the proximal domain and tendrils in the distal domains. The *lld* mutation does not affect the phyllotaxy as well as the organ composition of the leafblade domains. However, *lld* mutation has abortifying effect on both leaflets and tendrils (figures 1–3). Clearly, the *lld* defect is primarily in pinna organ development and not on pinna organ initiation.

At each of its nodes, the meristem of the growing rachis seperates primorda for two pinna primordia. In the compound pinnae, such as those formed in *af* leafblades, secondary rachis seperates primordia for the suborgans. In the primordia for the distal pinnae in unipinnate leafblades and primordia for suborgans, in the bi-, tri- or higher order-pinnately divided pinnae, the population of stem cells is expected to be smaller. The observed high frequency of abortion in distal pinnae of unipinnate leafblades and distal suborgans of compound pinnae may be related to low supply of stem cells in their primordia.

The leaflet or tendril morphogenesis must involve harmonious interplay of several growth and developmental pathways. Apparently, the leaflet morphogenesis entails growth in apical–basal and lateral directions for lamina formation, establishment of dorsiventrality in lamina, assembly of venation network and control over cell division, differentiation and expansion processes. The spectrum of aborted leaflets of *lld* and *lld tl* genotypes shown in figure 1 indicates that the interacting pathways of morphogenetic development in leaflets cease their progression from the time of the expression of *lld* mutation or loss of LLD function. The LLD function possibly couples the various developmental pathways of pinna morphogenesis.

LLD function may be a master regulator of pinna morphogenesis

The aborted parts of pinnae (suborgans), in various *lld* genotypes, are radial structures in which the dorsiventrality has not been established and the outer tissues have abaxial identity. An extreme form of this characteristic is seen in the early or severaly aborted pinnae or pinnules, where a pin-like radial structure is formed in place of leaflet, tendril or tendrilled leaflet, as a result of *lld* expression, irrespective of whether leafblade is genetically programmed to produce simple or compound pinnae. In the late aborted *lld* pinnae, while the distal cup-/saucer-like (lotus-like) structures have dorsiventrally developed pinna segment, the proximal part is radial abaxialized shaft like structure in which dorsiventral development did not occur due to premature abortment. The aborted pinnae and pinnules of *P. sativum lld* mutant are reminiscent of similar structures seen in certain mutant types of heterologous model plant species (Eckardt 2004).

Aborted simple leaves of radially symmetrical shape is a common feature of leaves formed by eight mutants of genes whose products are known to be involved in the determination of dorsiventrality and / or formation of proximo–distal axis in the form of mid-vein. These include mutants in the *PHAN* genes of *A. majus* and its orthologues *AS1* in *A. thaliana* and *LePHAN* in *L. esculentum*, *PHB, PHV, REV, KAN, PNH* (*PIN HEAD*) and *AGO* (*ARGONAUTE*) genes in *A. thaliana* (Waites *et al*. 1998; Byrne *et al*. 2000; McConnel *et al*. 2001; Kim *et al*. 2003a,b; Kidner and Martienssen 2004; McHale and Koning 2004; Scarpella *et al*. 2006; Wenzel *et al*. 2007) and *LBL1* (*LEAF BLADE LESS-1*) in *Z. mays* for the establishment of dorsiventrality (Timmermans *et al*. 1998) and *PIN, MP* and *AUX* (*AUXIN*) in *A. thaliana* for mid-vein formation (Wenzel *et al*. 2007). The mid-vein or primary vein, which brings in the nutrition also provides the shaft, serves as the epicentre for growth and development. The *KAN* and *YAB* genes are redundantly required to establish the abaxial identity to the tissue growing around the mid-vein, since abaxial domain is the default state of lamina development (Eshed *et al*. 2004). The *AS1*, *PHB*, *PHV, REV, PNH* and *AGO* genes are all involved in the establishment of adaxial domain in the lamina. Overexpression of *LITTLE ZIPPER PROTEINS* (*ZPR*s), which are small interacting peptides (siPEPs) that diminish activities of PHB, PHV and REV, results in abaxialized rod-shaped and trumpet-shaped leaves in *A. thaliana* (Wenkel *et al*. 2007; Kim *et al*. 2008). All the above genes involved in lamina development are known to control pathways of proximo–distal extension of mid-vein, lateral lamina growth in distal–proximal direction and origin of adaxial domain, in various combinations (Kidner and Timmermans 2007). Almost complete phenotypic correspondence between the attenuated leaves of triple HD-ZIP III gene mutants heterozygous for *phb* in *A. thaliana* and *lld* leaves of *P. sativum* implies that adaxialization process of leaflet development in *P. sativum* is similar to that of leaf in *A. thaliana* (Prigge *et al*. 2005). Comparative investigations on homologous function in plants have revealed that generally orthologous genes perform similar functions in heterologous plants. It is possible that the orthologues of the above genes of *A. thaliana* involved in simple leaf development control the development of simple pinnae in *P. sativum*. Indeed, the individual pinnae of *crispa* (*cri*) mutant (CRI = pea *PHAN* analogoue) are abaxialized, as expected (Tattersall *et al*. 2005). Since the phenotypes of aborted pinnae of *lld P. sativum* show that growth and development of mid-vein in proximo–distal directon, lateral growth in distal–proximal direction and establishment of adaxial domain altogether cease with the loss of LLD function, it can be inferred that LLD function is a master regulator of pinna morphogenesis. Or *LLD* controls the expression of other genes involved in the determination of pinna differentiation (coregulators) and downstream it functions in concert with coregulators.

Leaflet formation in wildtype and that in af tl leafblades and ins adventitious leafblades are similar

Gourlay *et al*. (2000) raised the issue whether the tiny leaflets produced on highly ramified leafblades of *af tl* genotype are formed like simple leaves of *A. thaliana* or simple leaflets of the proximal domain of wildtype leafblades of *P. sativum*. A related question had arisen whether leaflets formed on adventitious blades of proximal leaflets of *ins tl* leafblades developed like the normal proximal leaflets of *P. sativum*. In this regard, rod-shaped and trumpet-shaped aborted leaflets of the kind seen on *lld* leaflets were visualized among aborted leaflets in *af tl lld* and *af tl uni-tac lld* leaves (figure 2, L,M&K, respectively). The same kind of aborted leaflets were seen in the adventitious blades of *ins tl* leaves (figure 3, J–L). Thus, it is possible to suggest that there is only one kind of process of attaining bifaciality in the leaflets of large size of wildtype and tiny leaflets of *af tl* leaves and those of adventitious blades formed on *tl ins* leaflets.

LLD function is required for the maintenance of meristematic activity in leafblade primordium

The primordium of acropetally growing compound leafblade has dual function, it reiteratively partitions subprimordia for the distichously placed pinnae at each rachis node as well as it must replenish its own meristematic activity for the subsequent rachis growth. In the *lld af tl* triple mutant whose leafblades bear supercompound pinnae, some of the leafblades had well developed proximal pinnae but the rachis failed to form the distal domains. This observation means that LLD function is required for the maintenance of meristematic activity in the primary leafblade primordium.

Synergism between lld and mfp mutations for pinna attenuation

Loss of MFP function leads to replacement of tendrils by blades of tendrilled leaflets in the distal domain of *P. sativum* leaves (Kumar *et al*. 2004; Mishra *et al*. 2009). In *lld mfp* double mutants the abortion/attenuation of pinnae occurred in the different domains of leaf, especially in the distal domain, at a very high frequencies. Loss of MFP function increased the frequency of loss of LLD function. Earlier, it had been hypothesized (Prajapati and Kumar 2001) that LLD gene is linked to or is a part of DNA insertion element (transposon) present in *P. sativum* genome. There are two copies of it in the diploid sporophytic pea plant. *LLD* is expressed from a upstream promoter afforded by the insertion element. The recessive *lld* mutation has rendered the insertion element unstable. *lld* phenotype appears when both the copies of insertion element have been lost. The variation in *lld* phenotype is due to asynchrony in the loss of insertion element present in two copies. It appears that *MFP* is some how involved in the stability of the insertion element whose promoter is deployed in the expression of *LLD* in *P. sativum*. Loss of MFP destabilized the insertion elements by increasing the frequency of loss of function from both copies of *LLD* leading to appearance of lld phenotype in the affected pinnae early and among pinnae of various leaf domains in high frequencies. Interestingly, the DNA marker PSPO4SG which has been found in this study to be very closely linked to *lld* lies in a organ specific gene called P4. This gene has been reported to contain two copies of a repeat 26 amino acids sequence towards the terminus of the gene and 32 ATT repeats in the promoter sequence. There is another gene S2 which has four copies of the amino acid repeat sequence and four ATT repeats. Whether or not these repeat sequences are involved in the instability of *lld* locus remains to be known.

The lld mutation is expressed in compound stipule blades

The wildtype stipule is a simple peltate structure. A stipule is formed on the either side of the site of attachment of leafblade petiole to stem node. In mutant, one or both stipules formed at the preflowering nodes are compound in structure such that the stipuleblade mimics the morphology of leafblade (Sharma 1981; Marx 1987; Gourlay *et al*. 2000; Yaxley *et al*. 2001). The other nodes may have no stipule, one or both simple petiolated spoon-shaped stipules or one simple-petiolated stipule and one compound stipule (Gourlay *et al*. 2000; Yaxley *et al*. 2001; Kumar *et al*. 2009). The compound stipule blades of *coch af, coch tl, coch mfp, coch af tl*, *coch af mfp, coch tl mfp* and *coch af tl mfp* genotypes have the same architecture as that of the corresponding leafblades (Kumar *et al*. 2009). In the *coch uni-tac, coch af uni-tac, coch tl uni-tac, coch af tl uni-tac* and *coch af mfp uni-tac* genotypes despite the presence of *coch* mutation, the stipules formed have simple petiolated structure (Kumar *et al*. 2009). When *lld* mutation is present together with *coch*, in the former group of genotypes, occasional pinnae in the compound stipuleblades show the same kind of abortion as in the corresponding leafblades. Simple stipules do not show any development defect in the genotypes where in *lld* is present together with *coch* in the second group of genotypes (those carrying *uni-tac* mutation along with *af, tl, mfp* mutations in different combinations). In *P. sativum* the *LLD* function is involved in pinna morphogenesis, in both leafblades and stipuleblades.

Leaflets grow basipetally and tendrils acropetally

The morphologies of the leaflets and tendrils aborted at different stages of their development are suggestive of the directions of growth and development in two kinds of pinnae. Attenuated leaflets carry lamina at the apex, usually in the form of a cup of varying size attached to rachis via radial structure much like petiolule/rachis/stem in morphology. The sizes of cup and its basal stem were negatively correlated; larger the cup, smaller the size of its stem (figure 1). This suggested that lamination of leaflet occurred backwards from apex to base or basipetally.

Tendrils are much like rachis, morphologically and anatomically; apical tendril is an extension of rachis (Kumar *et al*. 2010; Sharma *et al*. 2012; V. Sharma, A. Kumar and S. Kumar, unpublished observations). A characteristic feature of tendrils is their curled apex (ringlet). The attenuated tendrils, irrespective of their size, had the tendency of apical curl intact. In the attenuated/aborted tendrils, the part nearer to the rachis was more developed than the part distal to rachis or apical part (figure 1). These features of lld tendrils were suggestive of acropetal growth in tendrils.

Distributed map locations of leafblade and stipule morphogenesis genes

The linkage map positions of the leafblade architectural mutations *af, mfp, stp, tl* and *uni* and stipule morphology mutations *coch* and *st* are known; they map on the linkage group I, IV, VII, V, III, V and III, respectively (Ellis and Poser 2002). In the present study the *lld* mutations has been mapped on the linkage groups III. Among the listed genes, *UNI, AF, TL* and *MFP* determine leafblade ramification, *ST* and *COCH* control stipule growth and development and *AF, TL, MFP* and *LLD* interact in pinna development, yet no two members of these three groups map on the same linkage groups. In *P. sativum*, the genes whose functions are interrelated are not closely clustered but widely dispersed on the genome.

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