RESEARCH ARTICLE

Genetic control of leaf-blade morphogenesis by the *INSECATUS* gene in *Pisum sativum*

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Abstract

To understand the role of *INSECATUS* (*INS*) gene in pea, the leaf blades of wild-type, *ins* mutant and seven other genotypes, constructed by recombining *ins* with *uni-tac, af, tl* and *mfp* gene mutations, were quantitatively compared. The *ins* was inherited as a recessive mutant allele and expressed its phenotype in proximal leaflets of full size leaf blades. In *ins* leaflets, the midvein development was arrested in distal domain and a cleft was formed in lamina above this point. There was change in the identity of *ins* leaflets such that the intercalary interrupted midvein bore a leaf blade. Such adventitious blades in *ins, ins tl* and *ins tl mfp* were like the distal segment of respective main leaf blade. The *ins* phenotype was not seen in *ins af* and *ins af uni-tac* genotypes. There was epistasis of *uni-tac* over *ins*. The *ins, tl* and *mfp* mutations interacted synergistically to produce highly pronounced ins phenotype in the *ins tl mfp* triple mutant. The role(s) of *INS* in leaf-blade organogenesis are: positive regulation of vascular patterning in leaflets, repression of UNI activity in leaflet primordia for ectopic growth and in leaf-blade primordium for indeterminate growth of rachis, delimitation of proximal leaflet domain and together with TL and MFP homeostasis for meristematic activity in leaflet primordia. The variant apically bifid shape of the affected *ins* leaflets demonstrated that the leaflet shape is dependent on the venation pattern.

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Introduction

The papilionoid grain legume crop *Pisum sativum*, variously used as protein rich human food and animal feed, is emerging as an important model plant for understanding the genetic control of leaf (Champagne *et al*. 2007; Mishra *et al*. 2009), inflorescence (Singer *et al*. 1999) and flower (Wang *et al*. 2008) morphogenesis. Despite the large genome size and relatively long annual cycle, the facility with which induced mutants can be isolated, and the abundant variation in landraces and ease in fertility control, make *P. sativum* a competitive model for comparative developmental genetics (Amurrio *et al*. 1992; Dalmais *et al*. 2008; Mishra *et al*. 2009). Novel pathways of compound leaf blade and simple-stipule-blade morphogenesis have been revealed in this system (Yaxley *et al*. 2001; Kumar *et al*. 2009; Mishra *et al*. 2009).

On account of heteroblasty, *P. sativum* bears leaves typical for the genotype at the first flowering node and a few to several nodes below and above it (Yaxley *et al*. 2001, Kumar *et al*. 2009). A node bears two simple foliaceous peltate sessile stipules and between them a compound leaf-blade. The leaf blade has up to three pairs of simple leaflets on the petiole side (proximal domain) and up to four pairs of tendrils (distal domain) plus an apical tendril (terminal domain) furthest to petiole. Mutant alleles at several genes/loci are known to significantly alter the morphology of leaf blade and stipule blade. The gene mutants that have proved to be particularly useful in the dissection of pea leaf-blade morphology are: *unifoliata* (*uni*) and another mutant allele in the same gene *unifoliata-tendrilled acacia* (*uni-tac*), *afila* (*af*), *tendril-less* (*tl*) and *multifoliate-pinna* (*mfp*) (de Vil-

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morin and Bateson 1911; White 1917; Eriksson 1929; Lamprecht 1933; Kujala 1953; Goldenberg 1965; Sharma 1972; Sharma and Kumar 1981; Kumar *et al.* 2004; Hofer *et al.* 2009). In *uni-tac* leaf blades, a leaflet replaces the apical tendril, the distal tendrillar domain is abridged and proximal domain is normal. All the three domains are tendrillar in *af* leaf blades and leafletted in *tl* leaf blades. The distal domain bears compound pinna blades of tendrilled leaflets in *mfp* leaf blades. The interactions among *uni-tac, af, tl* and *mfp* mutations have revealed the functional roles of the four genes in leaf-blade morphogenesis (Marx 1987; Hofer and Ellis 1998; Gourlay *et al.* 2000; DeMason 2005; Mishra *et al.* 2009). UNI is an activator of the proximodistal and mediolateral rachis growth. AF downregulates these UNI activities, activates laminated growth of proximal pinnae as leaflets and establishes boundary between proximal and distal domains. The MFP and TL are suppressors of mediolateral rachis growth and promote tendrillar growth in pinnae, in distal domain, and together with UNI establish boundary between distal and terminal domains. They require UNI for their expression (Mishra *et al.* 2009).

The phenotype of relatively less studied mutation *insecatus* (*ins*) implicates *INS* gene in the leaf-blade morphogenesis in *P. sativum* (Lamprecht 1959) (the *ins* mutation is not to be mistaken for the so called *insecatus2* (*ins2*) mutation (Berdnikov *et al.* 2000) which may define the *INS2* gene distinct from *INS* gene). Due to heteroblasty and/or poor penetrance of *ins*, only some of the leaf blades; especially those produced at the time of onset of flowering, show *ins* phenotype (Lamprecht 1959; Hofer *et al.* 2001; Smirnova 2002). In the affected leaf blades, one or both leaf blades of the pair most proximal to petiole show an apical notch. A tiny blade is seen arising from the incision exposed apical end of the leaflet midvein. This ectopic/adventitious blade, an extension of leaf-blade midvein, is tendrillar. The *tl* mutation has been found to interact with *ins* such that dissected leaflet tip may bear one to three leafleted blade (Hofer *et al.* 2001; Smirnova 2002). The interactive effects of *af, tl, mfp* and *uni* (or *uni-tac*) mutations with *ins* are as-yet-unknown. In the present work *uni-tac ins, af ins, tl ins, mfp ins, uni-tac af ins, tl mfp ins* and *uni-tac af mfp ins* genotypes were constructed and characterized for their leaf-blade morphologies. It is shown that the distal part of leaf blade is adventitiously miniaturized on the notched *ins* leaflets, INS is a repressor of the UNI-led adventitious growth on leaflets and overall rachis size is controlled by *INS, TL* and *MFP*. A scheme of interactions between *UNI, AF, INS, TL* and *MFP*, in the development of pinnae at the rachis nodes in proximal, distal and terminal domains, in pea leaf-blade morphogenetic pathways is diagrammed.

Materials and methods

The origins of wild-type, *uni-tac, af, tl, mfp, uni-tac af, af tl, af mfp, tl mfp, af tl mfp, uni-tac af mfp* and *uni-tac af tl* homozygous genotypes have been described earlier (Sharma and Kumar 1981; Prajapati and Kumar 2001, 2002; Kumar *et al.* 2004; Mishra *et al.* 2009). The *ins* line was from the Blixt collection (Blixt 1972). The homozygotes *ins, tl ins, mfp ins* and *tl mfp ins* were isolated as segregants from the F_2 generation of the cross *tl mfp* into *ins*. In the F_2 generation of the cross *tl tl mfp mfp* × *ins ins*, 44 plants out of a total of 198 demonstrated ins phenotype. Thus the *ins* allele proved to be recessive, as reported earlier (Smirnova 2002). The homozygotes of *af ins, uni-tac ins* and *af uni-tac ins* were isolated as F_2 segregants of a cross between *uni-tac af* and *ins*. In the F_2 population of 106 plants, there were 17 plants of af phenotype, 11 plants of clear ins phenotype and eight plants of af uni-tac phenotype. The *af ins* plants were identified by backcrossing of 10 F_2 plants of af phenotype with the *ins* parent. To identify plants of *af uni-tac ins* genotype, five F_2 plants of af uni-tac phenotype were backcrossed with the *ins* parent. Among the tested *af uni-tac* F_2 plants, two plants produced several leaf blades in which the leaflets borne on branched rachis of pinnae most proximal to petiole had inflected margin. Both these plants proved to be of *af uni-tac ins* genotype. The *af uni-tac mfp ins* genotype was isolated from the F_2 population of the cross *af uni-tac ins* × *uni-tac mfp*. In the resulting F_2 population of 212 plants, two plants had af uni-tac mfp ins phenotype; ins feature was present on the leaflet(s) borne on the branched pinnae most proximal to petiole. The presence of *ins* allele was confirmed by backcrossing with *ins*. The *coch* line was crossed with *tl ins* line to isolate *coch tl ins* from the F_2 population.

The genotypes were grown in a field plot of the experimental farm of the National Institute of Plant Genome Research, New Delhi, India, in the winter season (November–April) of the years 2007–2010. Ten seeds were sown per genotype per replication. There were two replications and the genotypes had been arranged in field in a completely randomized design. The agronomy of pea cultivation has been described earlier (Kumar and Sharma 1986; Prajapati and Kumar 2001, 2002; Kumar *et al.* 2009). Observations were recorded genotype-wise twice, first at the onset of flowering and secondly two weeks later. Morphologies of all the leaves present were recorded on at least five plants per replication. Quantitative observations on *ins* expression were recorded on the leaf blades borne on the first flowering node and two nodes immediately below and above it.

The venation patterns in *INS* and *ins* leaflets were studied by clearing them and by transverse sectioning of their midrib region, near to, below and above the site where from the adventitious blade seemed to originate. Freshly harvested whole leaflets were cleared by 15–30 min incubation at 90°C in phenol : lactic acid : glycerol : water :: 1 : 1 : 1 : 1 solution. The cleared leaflets were retained in 20% glycerol and were examined with and without safranine staining. Segments of leaflets fixed in 70% alcohol overnight, placed vertically between the split rod of radish root for the support, were cut with hand held razor blade. The cleared leaflets were examined using NIKON SMZ 1500 Stereozoom Microscope (Tokyo, Japan) and photographed with Nikon DXM 1200 cc digital camera. The transverse sections retained in 20% glycerol, stained with dilute safranine were examined using Nikon E100 microscope and photographed with Nikon 8400 digital camera.

Results

Insecatus phenotype

The common features of ins phenotype expressed in *ins, tl ins, mfp ins* and *tl mfp ins* genotypes (figure 1, c–k) were the following. Morphologically, the typical *ins* effect was a notch on leaflet, at its petiolule distal or apical side. There was bifurcation of leaflet lamina over 20–40% of leaflet's petiolule-distal length. The point of bifurcation was the junction of midrib/midvein and sixth, seventh or eighth pair of mediolateral secondary veins, which originate from midvein and traverse their part of lamina, distal to the petiolule (figures 2, a–e and 3). The midvein, at the base of notch, past the junction with the secondary vein pair, was usually extended into ectopic/adventitious blade (figure 2). The architecture and complexity of the blade was genotype specific (figure 3). The adventitious blade was tendrillar in the *ins* mutant and leafletted in the *tl ins* double mutant (figures 1–4). It bore leaflets and tendrilled leaflets in the *tl mfp ins* triple mutant (figures 1 and 4). The leaflet borne at the terminal position

Figure 1. *Pisum sativum* leaf blades of *ins* genotypes. (a) *ins af uni-tac*; (b) *ins af uni-tac mfp* (c) *ins*; (d) *ins tl*; (e) *ins mfp*; (f–k) *ins tl mfp*; and (l) *ins tl coch*. Adventitious blade formation on the leaflets of distal domain, as in figure (g). In the *ins* single mutant leaf blade (c), the length of the adventitious tendril borne on the proximal most right leaflet was of the same size as the terminal tendril borne on the main leaf blade. The average size of leaflets borne on adventitious leaf blades of the proximal most leaflet was 16 times smaller than the leaflets bearing them (d), in *ins tl* double mutant. The sizes of the corresponding organs of the adventitious leaf blades borne on proximal most leaflets and on the main leaf blade in distal domain were of about the same size in *ins tl mfp* triple mutant leaf blades shown in (j) and (k). Scale bars (a–l) = 2 cm.

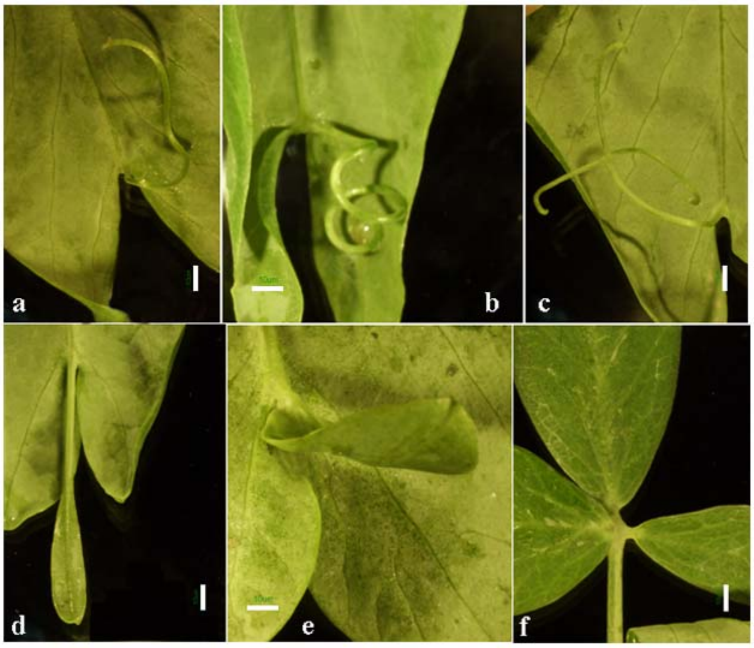

Figure 2. Enlargements of the distal domains of leaflets of certain *ins* genotypes of *P. sativum* showing the ectopic/adventitious blades, emerging from interrupted midvein, in between the cleft formed by bifurcated pinnae. (a–c) *ins*; (d) *ins mfp*; and (e,f) *ins tl*. Scale bars (a–f) = 10 μM.

Figure 3. The proximal most leaflet pairs in *P. sativum* leaf blades of wild type (a), *ins* mutant (b–d) and *ins tl* double mutant (e–g), showing within a leaf blade and between leaf blades variation in the expression of *insecatus* (*ins*) phenotype. Scale bars (a–g) = 2 cm.

of the branched pinna bore tendrilled leaflet as adventitious blade in the *af uni-tac mfp ins* quadruple mutant (figure 1b). The *ins* had poor penetrance and expressivity. The *ins* effect largely occurred on the leaf blades produced around the time of onset of flowering. The site of *ins* effect was the leafletted proximal domain of leaf blade, predominantly the leaflets of the first pinna pair which is most proximal to petiole. The subsequent leaflet(s) of the proximal domain and those of distal domain were affected by *ins* albeit rarely (table 1 and figures 1, e,g&h). Among the proximal most leaflet pairs of affected leaf blades, one or both leaflets bore the *ins* effect (figures 3, b–g). When both leaflets showed *ins* effect, the phenotypes of individual leaflets were often different (figures 3, e–g).

Interaction of ins with uni-tac, af, tl and mfp

The study of leaf blades formed on the wild-type and *ins, ins af, ins af uni-tac, af uni-tac, ins uni-tac, ins tl, ins mfp, ins tl mfp* and *ins af uni-tac mfp* mutant genotypes allowed analysis of interactions between the *ins* mutation on one hand and *af, uni-tac, tl* and *mfp* mutations, singly and in combinations, on the other hand. The *ins* morphology was not detected in *ins af* and *ins af uni-tac* genotypes. Since the leaf blades of *ins af* plants comprised of tendrils and did not have leaflets, the absence of *ins* effect was expected. None of the leaflets borne on the leaf blades of *ins af uni-tac* plants produced the typical ins phenotype. However, the leaflets formed on the proximal-most pinna blades were inflected or bilobed

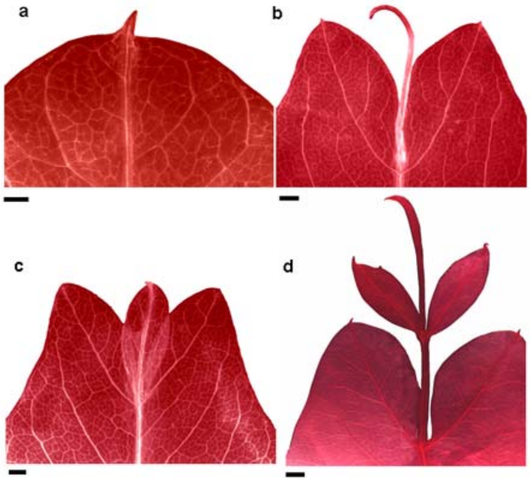

Figure 4. Enlargements of the distal segment of cleared and safranine stained leaflets of wild type (a), *ins* (b), *ins tl* (c), and *ins tl mfp* (d), showing venation patterning in the distal parts of leaflets, borne on the leaf-blade rachis nodes most proximal to petiole, and ectopic blades borne on the ins leaflets. Scale bars (a–d) = 1 mm.

(figure 1a) in the *ins af uni-tac* plants and such lobe was absent on the corresponding leaflets of *af uni-tac* plants. This observation suggested that bilobing of leaflets may be a phenotype of *ins* expression in the *af uni-tac* background.

The frequency with which the ins phenotype was observed in *ins, ins uni-tac, ins tl, ins mfp, ins tl mfp, ins af uni-tac* and *ins af uni-tac mfp* single, double, triple and quadruple mutants are presented in table 1. It will be seen that *ins* expression was similar in *ins, ins tl* and *ins mfp* genotypes (about 1.6/pair of proximal-most leaflets in a leaf blade). The frequency of *ins* effect was marginally higher (about 1.9) in *ins tl mfp* genotype, but much lower in *ins uni-tac* genotype (0.2). Although typical ins phenotype was not seen in *ins af uni-tac* genotype, *ins* expression in the form of adventitious blade was visualized at low frequency in *ins af uni-tac mfp* genotype (0.2).

The organ composition of the *ins* phenotype related adventitious blades formed on *ins, ins uni-tac, ins tl, ins mfp* and *ins tl mfp* leaflets were like that of the distal part of the respective leaf blades. The adventitious leaf blades were composed of simple or compound tendril in the *ins* mutant (figure 2, a–c), leaflet in *ins uni-tac* mutant, leaflet(s) in *ins tl* mutant (figure 2, e–f), tendrilled leaflets in *ins mfp* mutant (figures 1e and 2d) and leaflet(s) and tendrilled leaflet(s) in *ins tl mfp* mutant (figures 1, i–k). In the *ins* mutant, the tendrillar adventitious blades comprised of one or three tendrils. There were up to five leaflets in the leafleted adventitious blades of *ins tl* mutants. Up to seven tendrilled leaflets were noted in *ins mfp* adventitious blades. The largest adventitious blade comprised of 11 leaflets plus tendrilled leaflets in the *ins tl mfp* mutant.

The organs of adventitious blades were generally of the same size as that of the counterpart organs formed in the distal domain of main leaf blades in the *ins* and *ins tl mfp* mutants (figure 1, c, j&k). The size of the leaflets borne on the adventitious leaf blade in *ins tl* mutant was on average basis 16 times smaller than the leaflets bearing them (figure 1d).

Table 1. Frequency of ectopic/adventitious blade formation on the midveins of apically incised leaflets of pinna pairs most proximal to petiole in leaf blades and number and nature of organs formed on the adventitious blades, in various genotypes of *Pisum sativum*[a].

Genotypic homozygosity for[b]					Frequency per leaf blade of the adventitious blade formation[c,d,e]	Characteristics of the adventitious blade formed	
ins	*af*	*tl*	*mfp*	*uni-tac*		Number of organ(s)	Nature of organ(s)
−	+	+	+	+	1.4±0.2	2.3±1.6	Tendril(s)
−	−	+	+	+	0	NA[f]	NA
−	+	−	+	+	1.6±0.3	3.2±1.4	Leaflet(s)
−	+	+	−	+	1.7±0.2	1.7±0.3	Narrow leaflet(s)
−	+	+	+	−	0.2±0.4	0.2±0.4	Leaflet
−	+	−	−	+	1.9±0.1	9.1±6.7	Leaflets and tendrilled leaflets
−	−	+	+	−	0	NA	NA
−	−	+	−	−	0.2±0.4	0.2±0.4	Tendrilled leaflet

[a]The *ins* phenotype, apical incision of petiolated stipule and formation of adventitious blade from the incised stipule was noted in a stipuleblade of *coch tl ins* genotype (figure 1p); the simple COCH stipules were not observed to show ins phenotype in any of many *ins COCH* genotypes examined.
[b]+, wild-type allele and −, mutant allele.
[c]Leaf blades borne on the first flowering node and two nodes above and below it were scored in five plants.
[d]The frequencies with which ins phenotype was visualized in the second and third pairs of proximal domain leaflets in the *ins TL MFP UNI* genotype and distal domain leaflets of *ins tl mfp UNI* genotype were ≤ 1% and 0% and ≤ 1%, respectively, as compared to 70% in the first pair of leaflets.
[e]The leaflets in which cleft was unaccompanied by morphologically perceptible ectopic blade were not treated as *ins* leaflets.
[f]NA, not applicable.

The size of the proximal domain was enlarged to four pairs of leaflets in some of the leaf blades of *ins tl mfp* mutant (figure 1, f, h&k). The rachis was up to 1.5 times bigger in *ins tl mfp* leaf blades as compared to *INS TL MFP, ins TL MFP, INS tl MFP, INS TL mfp, ins tl MFP* and *ins TL mfp* leaf blades.

Vascular tissue reorganization in ins leaflets

The vasculature of the area of cleft formation/origin of adventitious blade in *ins* leaflets was compared with corresponding area of *INS* leaflets, by examination of the cleared whole leaflets and transverse sections. The central vasculature of *ins* leaflets near about the junction of midvein and origin of adventitious blade appeared to be somewhat thicker than at the corresponding position of comparable *INS* leaflets. The tertiary and higher level venation patterns of *INS* and *ins* leaflets around the mid vein and mediolateral secondary veins were similar (figures 4, a–d). The venation patterns in the tendrils, leaflets and tendrilled leaflets of the *ins, ins tl* and *ins tl mfp* adventitious/ectopic blades, respectively, showed no obvious alterations in respect to the corresponding structures formed in the distal domains of *INS, tl* and *tl mfp* leaf blades. The serial transverse sections below, at and above the junction of midvein and ectopic blade in *ins tl mfp* leaflets (figures 5, bB–bH) showed that the single vascular bundle of midvein (5bB=5aA of comparable *INS* leaflet) gave rise to vasculature of the rachis of adventitious blade in two stages. First, the vascular bundle got divided into three bundles (figures 5, bB–bE), upper two laterals for the secondary veins for the two sides of the cleft of mother leaflet and the lower central for the ectopic blade. In the second step, the latter vascular bundle developed into the vasculature of rachis (figures 5, bF–bH), of the kind seen in the distal rachis of *INS* leaf blades (Mishra *et al*. 2009). These observations allow the conclusion that INS represses the conversion of leaflet meristem into a leaf-blade meristem.

Discussion

The present work in *P. sativum* has confirmed that the *ins* allele is inherited as Mendelian recessive and has low penetrance. Further in the *ins* homozygotes, the proximal most leaflets of the affected leaf blades are notched and bear adventitious blades. The adventitious blade is simple or compound. It is tendrillar in *ins* and leafletted in *ins tl* mutants. The new observations were that ins phenotype: is not expressed in *af uni-tac* background, expresses at very low level in *ins uni-tac* double mutant and expression is very high in *ins tl mfp* triple mutant. The adventitious blade is simple in *ins uni-tac* and *ins af uni-tac mfp* and compound in *ins tl, ins mfp* and *ins tl mfp*; the rachis is inordinately enlarged in the *ins tl mfp* genotype. The multiple effects of the loss-of-function *ins* mutation are discussed on one hand with reference to the current understanding of simple leaf morphogenesis in the model plants such as *Nicotiana tabacum, Antirrhinum majus, Arabidopsis thaliana* and *Zea mays* (Dolan 2009; Micol 2009), because of leaflet's analogy with simple leaves, and with regards to compound leaf-blade morphogenesis in *P. sativum*, on the other hand. Distinctness of so called *insecatus2* (*ins2*) mutation from the *ins* mutation is also made evident.

Ins expression (penetrance) is dependent on the critical size of leaflet meristem

Pisum sativum demonstrates leaf-blade heteroblasty such that the fully developed leaf blades of size comprising of 13 to 15 pinna organs are formed at the time of first flowering and early and late formed leaf blades are proportionately smaller in terms of organ number and size. The primordium of the full size leaf blade separates seven pairs of daughter primordia for pinnae on rachis and gets consumed into terminal pinna. The first three pairs of daughter primordia produce leaflets, the next four pairs produce tendrils and the terminal pinna is also tendril. In leaf blades of all sizes the leaflet pairs in the proximal domain and tendrils in the distal domain are progressively smaller from petiole side to the terminus. The *ins* phenotype was expressed in the leaf blades of full size produced at the time of first flowering but was not visualized in early and late leaf blades. In the full leaf blades, *ins* phenotype was seen expressed at high frequency in the proximal most leaflet pair and occasionally in subsequent leaflet pairs. These observations are consistent with the idea that *ins* caused intercalary transformation of leaflet primordium into a leaf-blade primordium requires a meristem of large size, of the kind of size present on proximal primordia pair of full size leaf blades. The other pairs of leaflet primordia have meristems of sub-critical sizes, inadequate for *ins* effect. The absence of *ins* phenotype in leaf blades of smaller than full size may also be the reason of sub-criticality of meristem in their proximal leaflets. Indirect evidence that proximal most primordia have largest meristems comes from the leaf blades of *af, af uni-tac, af mfp, af tl, af mfp tl, af uni-tac mfp* and *af tl uni-tac* and *af mfp tl uni-tac* genotypes (Mishra *et al*. 2009). Each of the proximal compound pinna in these leaf blades is roughly of the same size and complexity as the entire distal blade.

In *P. sativum*, and the dicot model plant species *A. thaliana*, the flowering time is photoperiodically regulated (Putterill *et al*. 2004; Wenden and Remeau 2009), in both species flowering occurs earlier under long-day conditions than under short-day conditions. Transition from vegetative phase to flowering state in *A. thaliana* is initiated by the products of *LEAFY* (*LFY*) and / or *APETALA1* genes which are themselves activated by the products of flowering promoting photoperiod-dependent pathway, including the mobile signal product of *FLOWERING LOCUS T* (*FT*) gene synthesized in phloem tissue of leaf and transported to shoot apical meristem, and hormone gibberellin (Kobayashi and Weigel 2007). Counterparts of *FT* and *LFY* are known in *P. sativum* (Marx 1987; Hofer *et al*. 1997; Hecht *et al*. 2005, 2007). The *LFY* ortholog *UNI* has been shown to be involved in the

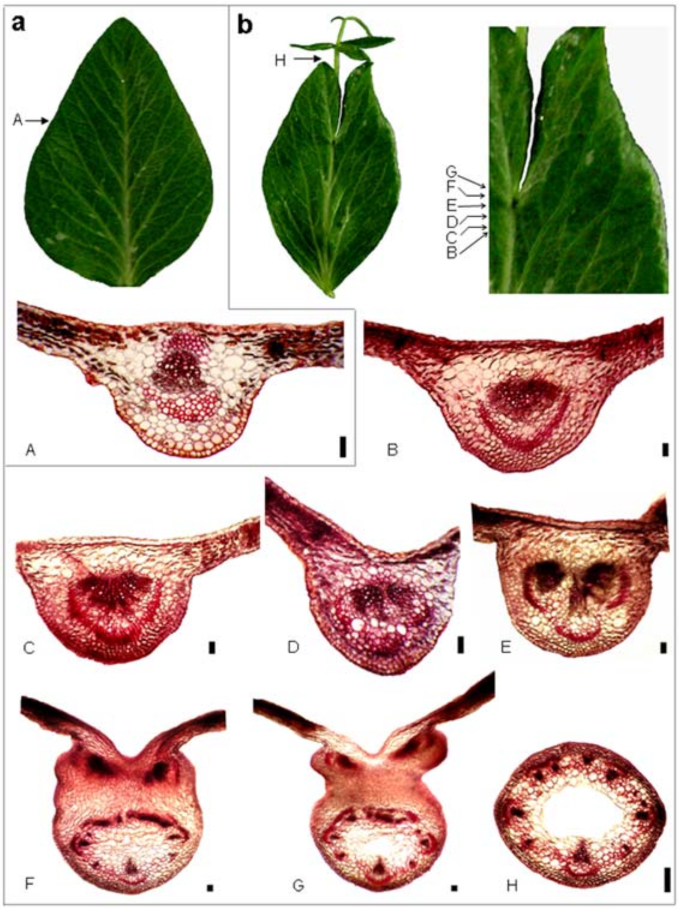

Figure 5. Histological features, as visualized in transverse section, of the midrib region of wild type (a) and *ins tl mfp* (b) leaflets (pinnae). aA, typical features of the midrib region sectioned near to the site of origin of seventh/eighth secondary vein in *INS* leaflet; bB-bH, features of the *ins tl mfp* leaflet sectioned in the midrib region in the area of origin of ectopic blade: bB, midrib of *ins tl mfp* showing features similar to those of *INS* in aA; bC–bE, division of the vascular bundle of midrib into three parts; and bF–bH, development of vasculature for the rachis of ectopic blade. Scale bars (A–D) = 0.1 mm.

development of inflorescence, leaf blade and *cochleata* (*coch*) stipules (Hofer *et al*. 1997; Kumar *et al*. 2009). Formation of cauline leaves in *A. thaliana* and full size leaf blades and leaf-blade-like stipules in *COCH* and *coch* lines, respectively, and expression of ins phenotype in leaflets of *P. sativum* occur coincidently with flowering time. Therefore, it is an attractive possibility that in pea, the system which activates gene(s) for initiation of flowering is also responsible for the formation of full size leaf blades, a requirement for the expression of ins phenotype.

Positive regulation of leaflet vascular patterning by INS

The *P. sativum* leaflet is an elliptic to ovate, flat and entire pinna organ, attached to leaf blade by means of petiolule (Prajapati and Kumar 2001). It is bilaterally symmetrical about the midvein. The vasculature of simple leaf, such as *A. thaliana*, and of simple leaflet in compound leaf blade, like *P. sativum*, is in continuum with that in stem via petiole in the former and petiole, rachis and petiolule in the latter (Carlsbecker and Helariutta 2005). The shoot apical meristem in the course of its forward growth is known to allocate progenitor cells for procambium downwards, for the growth of stem. During simple leaf development, the primordium allocates procambium cells from the petiole and mid-vein, similarly (Burton 2004; Carlsbecker and Helariutta 2005; Barkoulas *et al*. 2007; Heisler and Jonsson 2007). In the compound leaf-blade bearing plants such as pea, the meristematic cells (meristem) of leaf-blade primordium are expected to allocate procambium for vascular tissue of petiole and rachis in leaf blade and for veins in leaflets within latter. Since the secondary veins in the pea leaflets are directed apically, the growth of midvein and origin from it of about 10

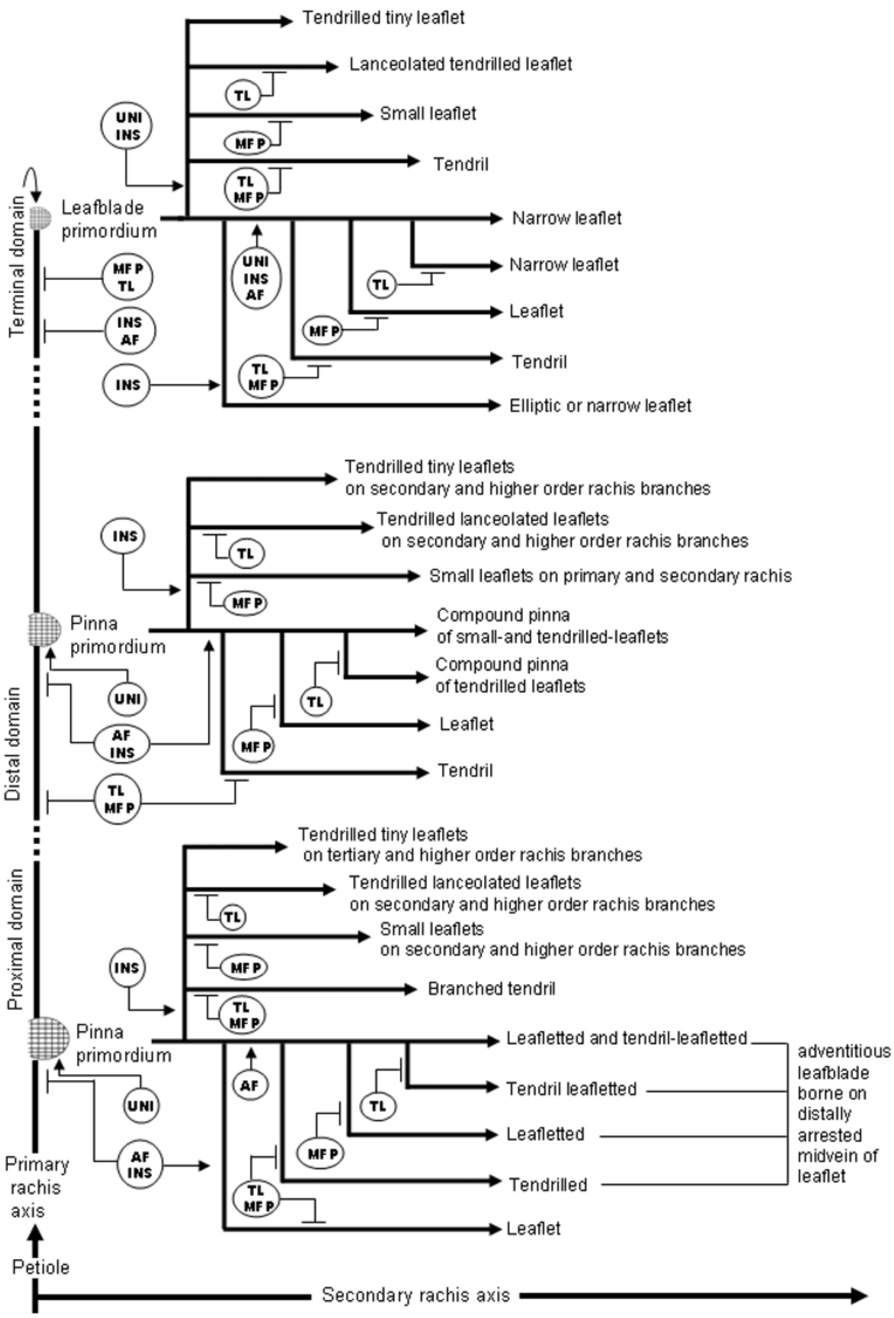

Figure 6. Diagram of the gene regulatory network related to the roles of *INSECATUS* (*INS*) gene in the routes of growth and morphogenesis of *P. sativum* leaf-blade. The interactions of *INS* gene with *AFILA* (*AF*), *TENDRIL-LESS* (*TL*), *MULTIFOLIATE-PINNA* (*MFP*) and *UNIFOLIATA* (*UNI*), in the growth of primary rachis and origin of pinna primordia and their development into pinnae, are depicted. The spectrum of regulatory events is exemplified with respect to first proximal domain pinna, a distal domain pinna and the terminal domain pinna (or leaf-blade primordium). The observations presented in this work and those reported and reviewed in Mishra *et al*. (2009) form the basis of the various pathways depicted for formation of pinna structures at proximal, distal and terminal positions of leaf blade. Each arrow indicates an activation step and a bar a, repressive step.

pairs of secondary veins must be acropetal; or the midvein polarity in leaflets is from petiolule outwards to the tip (Dengler and Tsukaya 2001; Burton 2004; Fujita and Mochizuki 2006). The midvein procambium is known to be patterned very early in the simple leaf ontogeny (Hageman and Gleissberg 1996; Dengler and Tsukaya 2001; Scarpella and Meijer

2004; Floyd and Bowman 2006; White 2006). Analogously, the procambium patterning of the midvein in leaflet must also be early in the leaflet development process. The *ins* mutant is apparently defective in the midvein patterning of leaflet. The midvein development is aborted in the distal side domain of leaflet in the *ins* mutant. The precocious arrest of midvein development must be related to INS deficiency perceived in the meristematic cell population of leaflet primordium (midrib meristem) or, the normal leaflet primordium provided with INS is tailored for the establishment of the pea leaflet specific normal pattern of vascular web. This means that the INS function is essential for the unperturbed ontogenic development of vascular system in the leaflet.

A cleft is formed in the *ins* leaflet above the arrested midvein. Supported by the upper secondary veins already separated from midvein, each of the two lamina sides outgrow the point of midvein abortion and develop their own tip, giving the leaflet top a bifid structure. Venation pattern and leaflet/leaf shape are known to be interdependent (Fujita and Mochizuki 2006). The formation of cleft in the ins leaflets implies that vascular webbing preludes lamina formation or venation patterning is a pre-requisite for leaflet shaping. The latter requires wide dispersal of meristematic cells borne upwards of veins, cell division along all axes and cell differentiation and enlargement. The *ins* caused cleft in the leaflet also implies relationship between variation in simple leaf morphologies and genetic variation in vascular patterning. It can be hypothesized that *INS* family genes are involved in determining leaf shape diversity in plants.

AF is known to be a positive regulator of leaflet formation in the proximal domain of *P. sativum* leaf blade (Marx 1987; Hofer and Ellis 1998; Gourlay *et al.* 2000; DeMason and Chawla 2004; DeMason 2005; Mishra *et al.* 2009). INS may play its role downstream of AF function since *ins* phenotype is realized in the presence of AF function and AF function is essential for the initiation and progression of leaflet formation.

INS mediated negative regulation of UNI dependent ectopic leaflet growth in leaflet

In *ins* leaflets, distal domain undergoes change of organ identity. The midvein interrupted in the distal domain of these leaflets is extended as leaf blade (called here as adventitious leaf blade). The adventitious leaf blade is a copy of the distal part of the main leaf blade. The distal leaflet assumes the identity of distal leaf blade. This paracronic error, which occurs at the midvein arrest point, must involve transformation of small leaflet meristem into a larger leaf-blade meristem. It is known that meristems are niches of pluripotent stem cells and these can deplete or regenerate in response to genetic signals (Brand *et al.* 2000; Schoof *et al.* 2000; Reddy and Meyerowitz 2005; Williams and Fletcher 2005; Muller *et al.* 2006; Wurschum *et al.* 2006; Beveridge *et al.* 2007; Fiers *et al.* 2007; Heisler and Jonsson 2007; Nardmann and Werr 2007; Sablowski 2007). UNI activity has been identified as the positive regulator of growth in the primordium of main leaf blade. The epistatic effect of *uni-tac* mutation over *ins* suggests that the adventitious leaf blade formation is also directed by UNI function. Thus *INS* must repress *UNI* activity in leaflets for the normal *AF* directed leaflet morphogenetic pathway to get accomplished.

In terms of the size of organs formed on adventitious leaf blades, the genotypes could be arranged in the following order: *ins* < *ins tl* and *ins mfp* < *ins tl mfp*. It appears that *TL* and *MFP* are homeostatic for stem cells in the transformed leaf-blade meristems distally located in the *ins* leaflets.

Roles of INS in the delimitation of proximal domain and determinate growth in primary rachis

Each of the leaf-blade primordium, separated node-wise on *P. sativum* plant grows a rachis of certain determinate size, which has on it commensurate number of daughter pinna primordia. The full size leaf blade has a proximal domain of three leaflet pairs. The *ins tl mfp* leaf blades differed from leaf blades of *INS, uni-tac, mfp* and *tl mfp* morphologically in two respects. (i) The proximal domain in *ins tl mfp* leaf blades comprised of four pairs of leaflets, instead of the usual three pairs of leaflets. (ii) The overall size of leaf-blade rachis was larger in *ins tl mfp* as compared to leaf blades of other genotypes. It has been shown earlier that AF determines the boundary between the proximal and distal domains of leaf-blade (Mishra *et al.* 2009). The present results suggest that INS is also involved in this process. It has also been shown earlier that AF determinates UNI-directed growth of primary rachis (Mishra *et al.* 2009). The present results suggest that INS is also involved in the down regulation of UNI activity for rachis enlargement.

Distinctness of ins and ins2 mutations

A leaf-blade morphogenesis mutation has been called *insecatus2* (*ins2*) on account of superficial similarity in the leaf-blade phenotypes of *ins2* and *ins* homozygotes. Some of the differences between the phenotypes of *ins2* and *ins* homozygotes are pointed out below. In the *ins* homozygotes, occasional leaflets, most proximal to petiole, in the leaf blades formed at the time of onset of flowering, are incised and bear ectopic leaf blades of one to three simple tendrils. This phenotype was frequently demonstrated in the early vegetative phase leaf blades of the *ins2* homozygotes. The late vegetative nodes of *ins2* homozygotes bore leaf blades in which two or more leaflets comprising a pinna and several leaflets of different pinnae, of the proximal domain, were incised and bore ectopic leaf blades comprising of simple and compound tendrils. The leaf blades borne on the reproductive nodes of *ins2* homozygotes had afila morphology. The leaf blades of the *ins2* homozygotes had the morphology of *af tl uni-tac* homozygotes. In certain crosses, *ins2* allele was found to be partially dominant over *INS2* allele in *ins2 INS2* heterozygotes. The absence of evidence that *ins* and *ins2* are allelic and grossly different phenotypes of *ins* and *ins2* homozygotes are suggestive of *ins* and *ins2* to be the mutant alleles of different genes.

Concluding remarks

Leaf blade morphologies of *P. sativum* wild type and *ins, uni* and *uni-tac, af, tl, mfp, ins uni-tac, ins af, ins tl, ins mfp, uni-tac af, uni-tac tl, uni-tac mfp, af tl, af mfp, tl mfp, ins uni-tac af, ins af uni-tac mfp, ins tl mfp, uni-tac af tl, uni-tac af mfp, uni-tac tl mfp, af tl mfp* and *uni-tac af tl mfp* mutants are now known (Mishra *et al*. 2009 and present work). Thus, the pathways for the control of leaf-blade morphogenesis stand genetically dissected. Therefore, in *P. sativum* it is possible to model the pathways that regulate the growth of leaf-blade rachis and pinnae, in terms of the concerned genes. It will be seen from figure 6 that the roles of *INS* gene are multilayered and all pervading. *INS* and *AF* appear to play mutually exclusive as well as additive roles, in both leaf-blade rachis growth and leaflet organogenesis.

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