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The *lld* mutation in *Pisum sativum* used as a genetic tool to discern the plant leaflet/leaf developmental process

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Leaves of *P. sativum* the double mutant genotype *tendril-less* (*tl*) *leaflet-development* (*lld*), due to the action of *lld* mutation, produce many leaflets that are aborted at different stages of development. Morphological, vein pattern and histological observations showed that aborted leaflets became cup/bell/trumpet (cup) shaped because of segmental differentiation in the leaflet primordium. Cup's inside lamina surface was adaxial and outer surfaces of cup and its stem were abaxial. The *lld* cups were phenotypically homologous to aborted leaves described in *Arabidopsis thaliana* mutants, *angustifolia* and those which underexpressed the HD-ZIP III proteins. Leaflet primordium was found to grow and establish three dimensional polarities apex-downwards. Primordium produced lateral outgrowth on one side of midvein. Differentiation, in the outgrowth, of secondary veins, whose xylem tissues faced each other, established the adaxial-abaxial polarity. Lateral outgrowth then developed a cavity which got bounded by future adaxial epidermis. Further growth, veinlet formation, differentiation of palisade parenchyma and spongy parenchyma followed. Opening of lateral outgrowth at its outer midline produced a flat leaflet with lateral lamina spans. The structural and functional correspondence between leaflet and simple leaves suggested commonality between leaf and leaflet development mechanisms. A molecular model for the *lld* led leaflet abortion was also provided.

Keywords: Adaxial-abaxial differentiation, Cup shaped leaf, Leaf development mechanism, Leaf vein network, Mediolateral polarity, Proximodistal growth

Eudicot leaves are typically bifacial. Such a leaf consists of several layers of parenchyma, interspersed with a network of veins and bounded on the two sides by epidermis[1]. Leaf differentiation on the whole is adapted for performing photosynthesis. Light capture is facilitated by epidermis and tightly packed chloroplast-rich palisade parenchyma cells on the adaxial (upper) side. Loosely arranged spongy parenchyma cells and stomata rich epidermis on the adaxial (lower side) facilitate gas exchange and temperature control. This anatomical structure of lamina in simple leaves is shared by simple leaflets (sub-organs of compound leaf) in the compound leaves[2,3]. Thus, simple leaves and simple leaflets are functionally and structurally (cell composition-wise) similar organs/sub-organs.

Molecular genetic studies on the model eudicot species *Arabidopsis thaliana* have considerably enhanced knowledge about the gene regulatory network that controls leaf development pathway[4-7]. Leaf is initiated with the separation of its primordium at the periphery of shoot apical meristem (SAM). The site has stem cells enriched with auxin, mediated by *PINFORMED1* (*PIN1*) activity[8-11]. *CUP-SHAPED COTYLEDONS* (*CUC*) induced relative quiescence in cells surrounding the group of stem cells at the site of auxin maxima separates the primordium from SAM[8,11-15]. Class I *KNOX* genes are silenced in the leaf primordial cells by a repressor complex binding to their promoters. This complex is comprised of the MYB-domain transcription factor *ASYMMETRIC LEAVES 1* (*AS1*), LOB domain protein *AS2* and chromatin modeling protein *HIRA*[16-19]. Thereafter, the developments in leaf primordium begin towards its proximodistal, mediolateral and adaxial-abaxial differentiation and growth. Some of the first developmental events in the leaf primordium include acropetal growth of midvein and differentiation of a basal region for petiole formation and an apical region for the formation of leafblade[7,20].

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The development of adaxial-abaxial, mediolateral and proximodistal polarities in the apical region of leaf primordium, destined to become leafblade, are largely interdependent. Interplay between several classes of transcription factors, small RNAs and proteins controls the adaxial-abaxial polarity development (Fig. 1). The *class III HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIP III)* genes *REVOLUTA* (*REV*), *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*) are expressed in cell layers that develop the adaxial domain in the primordium[21-25]. These are also expressed in the xylem of the veins[5,26]. The GARP-domained *KANADI* (*KAN*) and *YABBY* (*YAB*) families of transcription factors and *AUXIN RESPONSE FACTOR 3* (*ARF3*) and *ARF4* specify abaxial fate to cell layers juxtaposed to the adaxializing cell layers in the primordium[5,15, 27-37]. *KAN* genes are also expressed in the phloem of veins[5]. Whereas *KAN* genes are necessary and sufficient for the attainment of abaxial identity, *ARF* and *YAB* genes act downstream of *KAN* in the abaxializtion process. KAN and HD-ZIP III are mutually antagonistic[38]. AS2 and KAN are similarly antagonistic[39,40]. The activity of HD-ZIP III genes is lowered by microRNAs (miR) 165 and 166 and *LITTLE ZIPPER* (*ZPR*) proteins[25,41-45]. The miR 165/166 target the HD-ZIP III transcripts and ZPR proteins form heterodimers with HD-ZIP III proteins and prevent the binding of latter to DNA. AS1-AS2 regulates HD-ZIP III in the other direction[31]. *ARF3* and *ARF4* are negatively targeted by trans-acting small interfering RNAs (ta-si RNA-ARF) derived from *TAS-3* genes[42,44,46-47]. Several large subunit ribosomal proteins (RPL), 28S proteasome, cell division promoting proteins *ANGUSTIFOLIA 3* (*AN3*) and *AS2 ENHANCER 7*) (*AE7*), chromatin modeling and other proteins also participate directly or indirectly in the leaf polarity establishment[7,25,34,48-53]. Over-or under-production of the principal determinants of adaxial-abaxial polarity establishment or one or more genetic alterations that indirectly affect polarity establishment are known to arrest the leaf primordium development. Such premature cessation of polarity development is known to lead to production of rod-, cup-, lotus- or trumpet-shaped illdeveloped leaves in *A. thaliana* (Table 1). The wild type plants of *Ficus krishnae*[54] and *Sarracenia drummondii*[55] are known to bear cone/bell/trumpet shaped leaves. Although much information about the gene regulatory network for leaf differentiation is available, how tissue-wise leaf differentiation occurs in the space of leaf primordium is not known.

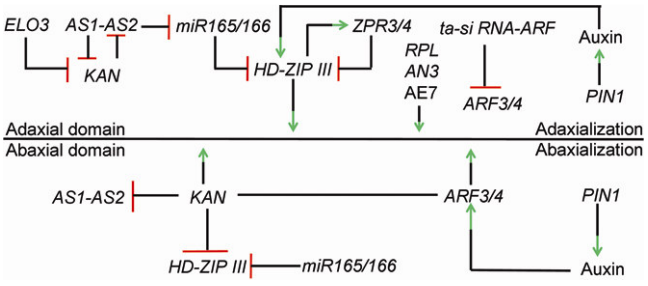


Fig. 1—Diagrammatic representation of the gene regulatory network that renders adaxial or abaxial specification to cells layers juxtaposed in leaf primordium, in its primary morphogenetic stages. More information about the genes mentioned in this figure is provided in the Table 1.

Eudicot compound leaves consist of two or more leaflets attached to a rachis. In such leaves rachis is an extension of the petiole. Gene regulatory network(s) that underlie the compound leaf differentiation are being investigated in several species, including *Cardamine hirsuta*, *Medicago truncatula*, *Pisum sativm* and *Solanum lycopersicon*[4,6,15,56,57]. Leaf primordium in compound leaved species is separated as simple primordium like in simple leaved species[14,58]. However, rachis growth and associated leaflet forming potential in compound leaved species has been shown to be either dependent on *KNOX 1* (*C. hirsuta* and *S. lycopersicon*[59,60]) or on *LEAFY* (*UNIFOLIATA* in *Pisum sativum* and *SINGLE LEAFLET 1* in *M. truncatula*[61,62] orthologs. The leaf polarity determining gene network is largely conserved in compound leaved species. It is thought that separation of leaflet primordium and subsequent morphogenesis of it into mature leaflet occurs largely by the process that leads to simple leaf formation[6].

In the model plant *Pisum sativum*, unipinnately compound leaves consist of upto 15 pinnae, 3 pairs of proximal leaflets and 9 tendrils[63]. All the pinnae are leaflets in the *tendril-less* (*tl*) mutant[64,65]. In another Mendelian recessive mutant called *leaflet development* (*lld*), located on the linkage group III, some of the leaflets do not complete their morphogenetic process[66,67]. In the *tl lld* double mutant about 40% leaflets get aborted in the course of leaflet formation (unpublished observation). The aborted leaflets occur in the shapes of needles, cups, lotuses and trumpets. Their structure mimics the structures of aberrant leaves reported in leaf morphogenesis genetic variants of *A. thaiana*, which are listed among 33 genotypes in the table 1. Unlike many of the *A. thaliana* variants that produce aborted (*lld* aberrant leaflets like) leaves and demonstrate poor growth and sterility, life cycle

Table 1—Genetic modifications, in the gene regulatory network known for the establishment of adaxial-abaxial polarity, mediolateral polarity and vein system in the differentiating leaf primordium, that are abortifacient and lead to formation of rod-, cup-, lotus- and/or trumpet shaped structures in place normal bifacial leaves in *Arabidopsis thaliana*

S. No.	Gene family name	Gene name	Nature of mutation/ genetic modification[a]	Genotype (homozygote unless mentioned)	Nature of leaf abortion		Remark(s)	Reference(s)
					Rod shaped (radial)=Y	Cup-, lotus- or trumpet-shaped=Y		
1	MYB domain transcription factor	*ASYMMETRIC LEAVES 1* (*AS1*)	*as1*	*as1* in Landsberg *ERECTA* (Ler) background	Y	Y	Temperature higher than 22 °C increased the frequency of abaxialized aberrant leaves	76, 77
2	Leucine zipper protein that associates with AS1	*AS2*	*as2*	*as2* Ler background	Y	Y	As above	17, 76, 77
3	Cell cycle progression protein	*AS1 ENHANCER 7* (*AE7*)	*ae7*	*ae7 as2*	Y	b	Cell proliferation is a requirement in polarity establishment	28
4	*Ribosomal large subunit protein (RPL)*	*RPL 28A/5A/5B RPL 28B/*	*rpl5b*	*rbl5b as2*	Y	Y	Adaxialized leaves; RPLs are required for cell proliferation to occur	52
		SHORT VALVE1 (*STV1*)	*stv1*	*stv1 as2*	Y	Y		
		ENHANCER 5 (*AE5*)	*ae5*	*ae5 as2*	Y	Y		
		ENHANCER 6 (*AE6*)	*ae6*	*ae6 as2*	Y	Y		
5	*Growth regulating factor (GRF)-INTERACTING FACTOR1* (*GIF1*)	*ANGUSTIFOLIA 3* (*AN3*)	*an3*	*an3 as2*		Y	Abaxialization genes are over expressed	53
6	Elongator complex component histone acetyltransferase (*ELONGATA=ELO*)	*ENHANCER-OF-ASYMMETRIC LEAVES 2-1* (*EAST1*) OR *ELO*	*east1* (*elo3*)	*east1 as2*	Y	Y	Abaxialization genes are repressed in adaxial domain cells	78
			elo2	*elo2 as2*		Y		
			elo4	*elo4 as2*		Y		
7	Post-transcription gene regulatory network	*RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*)	*rdr6*	*rdr6 as2*	Y	Y	RDR6 and AS2 associated pathways synergistically repress miR165/166 in leaf polarity development process	25
8	Chromatin modeling	*Histone deacetylase* (*HDT*)	*hdt2-RNAi*	*hdt2*	Y	Y	MicroRNA 165/166 levels modified in adaxial and abaxial cell layers	50
9	Post transcriptional control of gene expression	*ARGONAUTE1* (*AGO1*)	*ago1*	*ago1*		Y	MicroRNA 165/166 regulation is disrupted.	73

(*Contd.*)

Table 1—Genetic modifications, in the gene regulatory network known for the establishment of adaxial-abaxial polarity, mediolateral polarity and vein system in the differentiating leaf primordium, that are abortifacient and lead to formation of rod-, cup-, lotus- and/or trumpet shaped structures in place normal bifacial leaves in *Arabidopsis thaliana (Contd.)*

10	micro-RNAs (miR) for posttranscriptional regulation of gene expression	*microRNA 165/166* (*miR165/166*)	Overexpressing promoter *miR165/166*	*miR165/166* overexpressed	Y		miR166 is ovrexpresed leading to adaxialization like in phb-id/+.	25, 41-44, 73
				35S::miR165 as2	Y	Y	Expression of HD-ZIP III genes is prevented by cleavage of their transcripts in the adaxial side	
				35S::miR165 rdr6	Y	Y		
			jabba-1d	*jabba-1d*	Y			
11	*AUXIN RESPONSE FACTORS* (*ARF*)	*ARF3*, *ARF4*	*arf3 arf4*	*arf3 arf4*	Y		Unavailability of ARF3 and ARF leads to poor abaxialization in the abaxial domain.	27, 79
12	*GARP*-domain transcription factors	*KANADI 1*, 2, 3	*kan 1, 2, 3*	*kan 1 kan2 kan3*	Y		Adaxialized radial growth has on it abaxial ectopic outgrowth	29-31
			kan1 kan2 as2			Y		
13	*YABBY* or HMG-like proteins (transcription factors)	*FILAMENTOUS FLOWER* (*FIL*) *YABBY 3* (*YAB 3*)	*fil1* *yab3*	*fil1 yab3*		Y	Poor abaxialization	29-32
				fil1 yab3 as2		Y		
14	Class III homeodomain leucine zipper transcription factors (HD-ZIP III). These have a binding site for miR165/166	*PHABULOSA* (*PHB*) *PHAVOLUTA* (*PHV*) *REVOLUTA* (*REV*)	*phb-1d*	*phb-1d/+*	Y	Y	Adaxial tissue on the stem and abaxial in the bell of the aborted leaflet	21, 22, 25
				phb-1d	Y	Y		
15	As above		*phb* (semidominant)	*phb as2*	Y	Y	Abaxialized in stem and adaxialized in the bell	21-25
			phv (semidominant)	*phv as2*	Y	Y		
			rev (semidominant)	*rev as2*	Y	Y		
				rev	Y	Y		
				phv rev	Y	Y		
				rev phb/+phv		Y		
16	As above	*INCURVATA 9* (*ICU9*)	*icu9*	*icu9*	Y	Y	Adaxialization is promoted due to loss of miR binding site and HD-ZIP III expression throughout the primordia	80, 81
17	As above	*LITTLE ZIPPER* (*ZPR*)	Overexpressed *ZPR 3* and *ZPR4*	*ZPR 3/4* overexpressed		Y	Loss of HD-ZIP III function due to competitive inhibition leading toabaxialization.	45

a = Active/Gain of function/ Dominant mutation is referred by d; all others are loss-of-function mutations; constitutive promoter based expression and RNAi inhibition modifications are indicated; b = Empty spaces means not applicable or information not available

progresses normally in *P. sativum tl lld* plants. The *tl lld P. sativum* genotype therefore offered an opportunity for using the aborted leaflets as tools in understanding how leaflets/leaves undergo development. A histological analysis of aborted *tl lld* leaflets is presented in the present communication. A hypothesis for the *lld* led occurrence of leaflets attenuation at different stages of development is presented. The histological observations have been used to propose a scheme for leaf/leaflet development.

Material and Methods

The *P. sativum* lines *lld* and *tl lld*[66] were grown in field; *tl lld* line was also micropropagated. The normal leaflets borne on the two lines are known to possess similar morphology and anatomy (unpublished observations). The line *tl lld* was chosen for inclusion in the study because its leaves formed more number of leaflets than the *lld* line. Seeds were sown in late November during 2009-2010 and 2010-2011 winter seasons in the experimental field plots of NIPGR. Single nodes from the single seed derived plantlets were used to raise shoots on MS medium containing Gamborg vitamins, 11µM 6-benzylaminopurine (both from Sigma-Aldrich, USA), 3% sucrose and 0.8% agar (both from Hi-Media Laboratories Pvt. Ltd., India). The culture was exposed to 16 : 8 h::light : dark cycle at 25 °C. Single nodes from this culture were then serially propagated on the above medium and incubation condition.

Catharanthus roseus cv 'Self Pollinated' plants which bore some bell-shaped leaves were raised in the NIPGR experimental farm during 2006 summer season[68]. The trumpet-shaped leaves observed on *Amaranthus spinosus* were on its weed plants found growing in the farm in 2008. *Bacopa monnieri* shoots maintained as microcultures sporadically produced cup-shaped leaves. *B. monnieri* microcultures were raised by the method described above for *P. sativum tl lld* microcultures. The field cultivation procedures used for *P. sativum* and *C. roseus* have been described earlier[69,70].

Leaf samples were taken from the microcultures and field grown plants and morphologically and anatomically examined. For morphological study the samples were scanned using a Hewlett Packcard PSC scanner. Lateral organs were fixed in formalin : glacial acetic acid : water : alcohol : : 1 : 1 : 5 : 13. For histological studies, on the one hand, organs were cleared by incubation at 90 °C for 15 min in water : glycerol : phenol : lactic acid : : 1 : 1 : 1 : 1 mixture, stained with dilute safranine and observed microscopically. On the other hand the leaflets were placed in between two slices of radish and sectioned transversally with hand held razor and thin sections stained with dilute safranine were observed microscopically at different magnifications. Nikon E100 microscope was used for the examination of sections and cleared organs at 40X, 100X and 400X magnifications. Microphotographs were taken by attaching Nikon 8400 digital camera to the microscope.

Results

Morphology and venation pattern in the lld *aborted leaflets*—Needle-, cup-, bell-, lotus- and trumpet-shaped leaflets and leaflets of normal morphology were seen on the leaves formed on both the field grown plants (Fig. 2) and microcultured shoots (Fig. 3) of *tl lld* line of *P. sativum*. Clearing followed

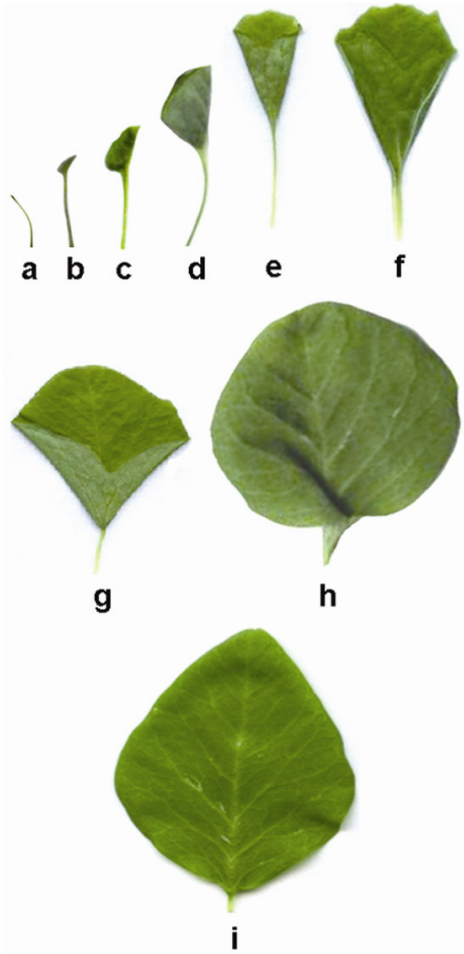


Fig. 2—*lld* leaflets arrested/aborted in their development at different stages of morphogenesis in the field grown *tendril-less* (*tl*) *leaflet development* (*lld*) double mutant line (*tl lld*) of *Pisum sativum*. a-h = aborted leaflets shaped like trumpet, cup or lotus; i = fully developed leaflet.

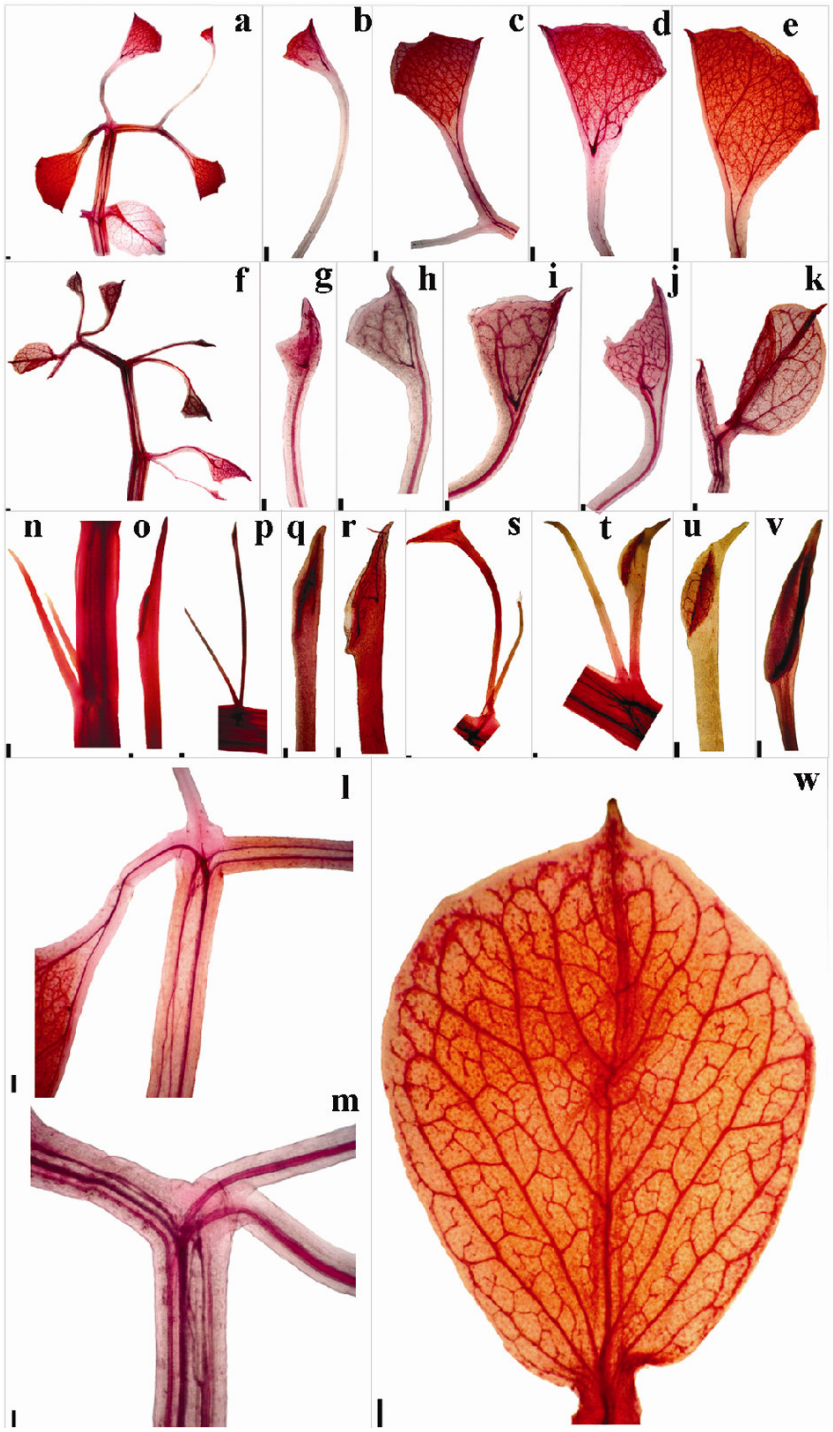


Fig. 3—Leaflets aborted at different stages of development and a normal leaflet in *tl lld* double mutant leaves on shoots grown *in vitro*. a-e and l = a leaf bearing 4 aborted leaflets and 1 normal leaflet (a); enlargements of its aborted leaflets (b-e): enlargement of the junction of aborted leaflets b and e with rachis (l). It will be seen that whereas the safranine stainable vascular connection of b with rachis is broken (a, d and l), that of e with rachis is intact (a, b and l). f-k and m= another leaf bearing several aborted leaflets (f), their enlargements (g-k) and enlargement of the junction of g and i with vasculature in rachis (m). The vascular connection of the aborted g and i leaflets with vasculature in rachis was normal (f, g, i and m).n-v = morphologies of 13 aborted leaflets are shown. It will be seen that several aborted leaflets bear errors in their vascular (vein) development. w = cleared and safranine stained normal leaflet showing its normal vein network. Magnification bar = 200µm.

by staining with safranine revealed the vein (vascular) network of the leaf/leaflets (Figs. 3 and 4). Vein (vascular) internal structure was visible in the transverse sections stained with safranine (Fig. 5). Safranine is known to deeply stain the lignified cell walls in xylem tissue[71]. In the transverse sections, xylem tissue was observed to be relatively more heavily stained by safranine than other kinds of tissues in leaflet vascular bundles (Fig. 5o). The aborted leaflets were roughly divisible into two classes. In one of these, a very small slit or from a very small cup/bell to such a structure of large size was observed in the apical/distal region of the aborted leaflet. These structures (slit on a needle or cup/bell on a stem) took up more safranine stain than the other distal and proximal parts of the aborted leaflets (Fig. 3 a-k, q-v and needles in k, p and s). Most of the stain was taken up by the vein network in these structures. The other group included needles

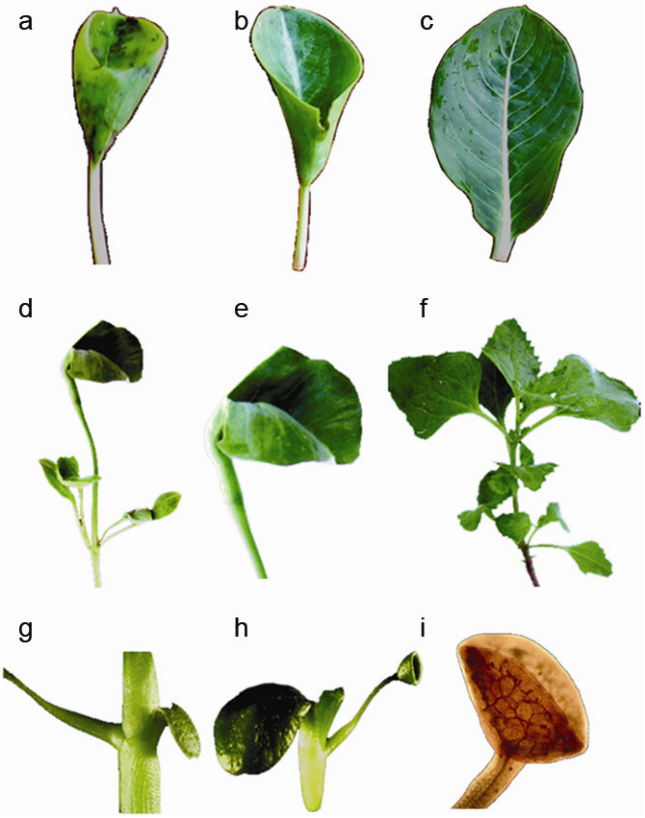
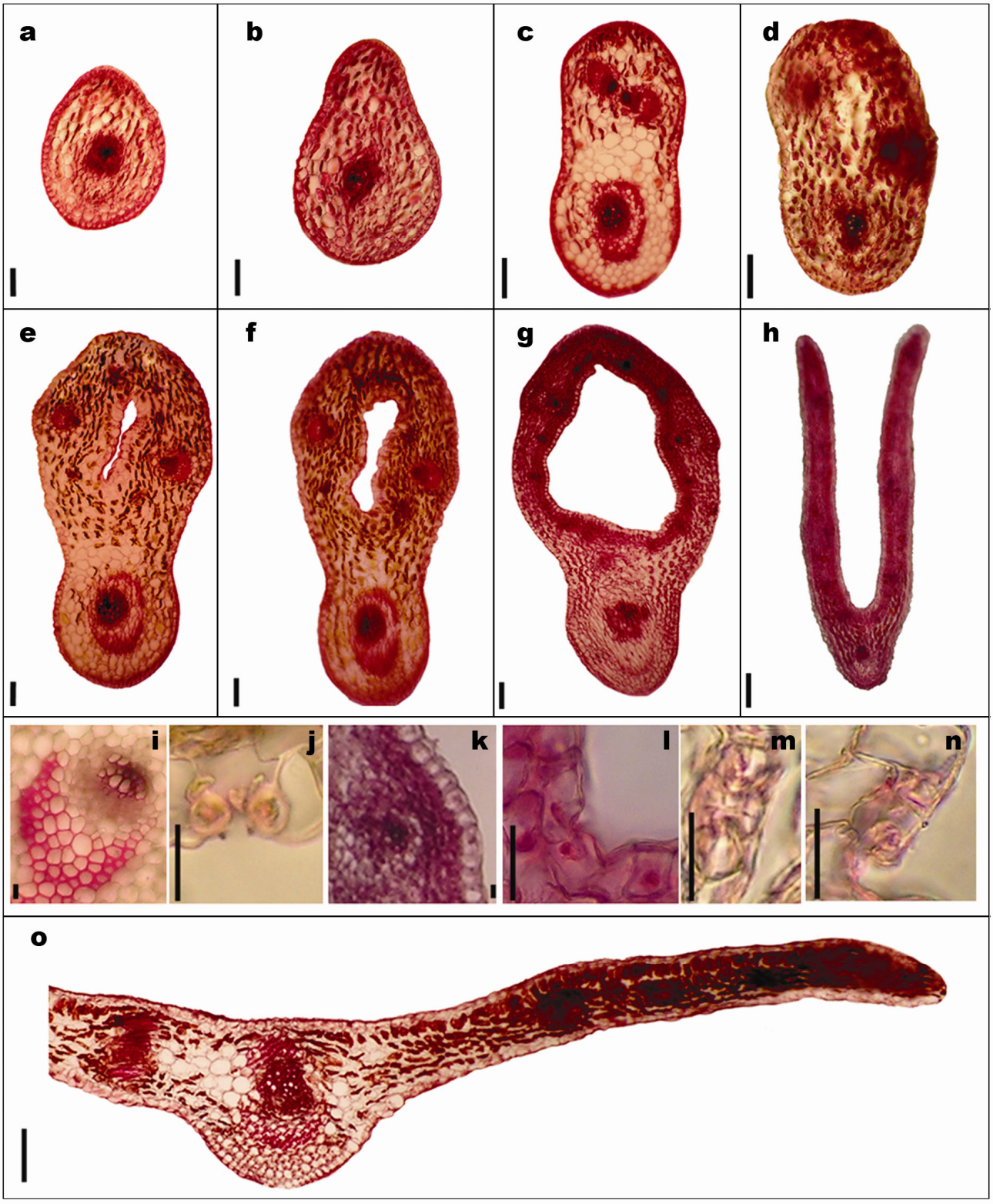


Fig. 4—Trumpet, cup and lotus shaped leaves seen in three plant species. a-c = *Catharanthus roseus*, a and b are aborted leaves and c is normal leaf; d-f = *Amaranthus spinosus*, d shows a plant bearing several aborted leaves, e is enlargement of an aborted leaf and f shows a plant bearing normal leaves; g and h = two nodes of *Bacopa monnieri* are shown bearing aborted leaves; i = cleared and safranine stained cup-shaped aborted leaf in *B. monnieri*.

that did not carry the darkly stained apical slit (Fig. 3n and needles in Fig. 3p and s). The needles on the whole were relatively less stained. Formation of slit, cups, bells and trumpets of different sizes was indicative of segmental adaxial-abaxial and mediolateral polarity development in the leaflet primordia that produced them. The developmental process for the establishment of polarity terminated prematurely, very early in the slitted needles (Fig. 3k, p-v) and at different stages of its progression in other kinds of aborted leaflets, proportionately reflected in the size of lamina present in the aborted bell/cup shaped leaflets (Fig. 3g, b, h, d and e). The lamina present in the bells/cups was adaxially-abaxially polarized and mediolaterally, proximo-distally and adaxially-abaxially expanded. It also possessed a well developed vein network. For example the bell at Fig. 3e possessed two pairs of lateral (secondary veins) closed at the margins and connected to midvein which in turn was connected to a mother vein in rachis (Fig. 3a, e and m). The secondary veins were connected to tertiary veins. There were quaternary and higher order veins also present in this bell shaped lamina, much like that in the distal part of normal leaflet (Fig. 3w). Venation system was observed to be often faulty in the aborted leaflets. For example in aborted leaflets shown in (Fig. 3d) venation system although well developed in the bell shaped lamina appeared to be disconnected from its mother vein in rachis (Fig. a, d and l). The venation bearing apical slits in the needle shaped aborted leaflets appeared to be having similarly disconnected venation system (Fig. 3o and q-u). It is possible that venation system was intact in these aborted leaflets in an altered form; xylem tissue may not have taken safranine stain in absence of its adequate lignification. Briefly, it appears that in *tl lld*, the leaflet primordia got initiated and proceeded in the developmental pathway for leaflet development. But in some of them, development got arrested at different stages giving rise to needles, cup, bell, lotus and trumpet shaped aborted leaflets of different sizes.

Morphological correspondence of lld *aborted leaflets of* P. sativum *with aborted leaves noted in heterologous eudicot species*—Since the isolation of *lld* mutant in *P. sativum*[66], different species of plants growing around the NIPGR experimental farm were searched for the presence of leaves having the morphology of aborted leaflets of the *lld* mutant.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Cup/trumpet shaped leaves were noted in the wild plants of *Amaranthus spinosus* and *Bacopa monnieri* and the cultivar called 'Self Pollinating' of the medicinal-cum-ornamental plant *Catharanthus roseus*. The progeny raised with the seeds of a *A. spinosus* plant bearing trumpet shaped leaves bred true for this trait. *B. monnieri* plant that formed cup-shaped leaves was microcultured. The microcultured shoots of *B. monnieri* formed cup/trumpet shaped leaves frequently. Fig. 4 shows occurrence of cup/bell/trumpet shaped leaves in *Amaranthus spinosus*, *B. monnieri* and *C. roseus*. Occurrence of these structures in diverse eudicot species demonstrated correspondence in the pathways of leaflet development in *P. sativum* and leaf development in heterologous species.

Histology of the aborted lld *leaflets and scheme of the leaflet development process*—The aborted leaflets of different sizes taken from the field grown *tl lld* plants were sectioned transversely, stained with safranine and examined microscopically. Aborted leaflets were cut at apex, within and below their cup or bell. Representative sections of the aborted leaflets are compared with distally cut transverse section of a normal leaflet (Fig. 5). The histological observations on aborted leaflets identified several features of the leaflet development process. Transversely, the leaf primordium at early stage of its development appeared to be a circular structure with a centrally placed vascular bundle (midvein). Except for epidermis and midvein region, the cells appeared to be similar in size and stainability (Fig. 5a). The leaf primordium was noted to grow in size laterally in one direction (Fig. 5b and c). The next recognizable stage was appearance of a pair of vascular bundles, oppositely placed in the middle of the lateral growth (Fig. 5c). In these vascular bundles xylem faced the centre, xylem tissues of the opposite vascular bundles faced each other. The primordium grew laterally and in girth. It produced a clearing at the centre of lateral growth in between the new vascular bundles (Fig. 5d). The growth in the primordium seemed to be intercalary, due to participation of all the cells in the background of the vascular bundle(s). The primordium produced a cavity (Fig. 5e-g). The origin of this cavity appeared to be related to death of cells at the centre of the primordium (Fig. 5e) where clearing had occurred earlier (Fig. 5d). The inner side of primordium was separated from the cavity by a layer of cells that were structured like epidermal cells (Fig. 5e and f). The lateral growth appeared like a bifacial oval outgrowth from the original circular midvein containing primary body (Fig. 5e-g). The outgrowth had an internal epidermis, an outer epidermis, several intervening layers of similar looking cells that were centrally interspersed with vascular bundles of varying diameters (Fig. 5f and g). In all these vascular bundles the xylem faced the internal cavity. In the next stage, there was appearance of a layer of darkly stained cells next to the internal epidermis. These cells were of much larger size than the cells in the background.. The newly differentiated cells were future palisade parenchyma cells (Fig. 5g). By this stage the inner epidermis had differentiated stomata (Fig. 5k-n). The histological structure of one side of the lateral outgrowth (Fig. 5g) was much like a lateral span of normal leaflet (Fig. 5o). The lateral outgrowth was cleaved at the outer midline, allowing formation of two lamina spans out of the lateral growth (Fig. 5h).

Fig. 5—Histological analysis of leaflet development in *tl lld Pisum sativum*. a-h = transverse sections of growing lld leaflet primordia at different stages of development are shown. The sections a-d represent early leaflet primordium states and e to h late stages of primordium development. a = early primordium has a central vascular bundle (cvb) and cells surrounding it are bounded by an outer epidermal layer of cells; b = the primordium has begun growing laterally; c = the originally present vascular bundle (cvb) has undergone further differentiation. Two smaller vascular bundles placed opposite to each other have appeared in the lateral outgrowth which has further extended to increase the size of primordium in lateral direction; d = in between the vascular bundles in the lateral outgrowth a narrow clearing is seen, perhaps reflecting an outcome of programmed cell death; e = The lateral growth has further expanded in both the directions. It now has 4 pairs of vascular bundles, besides the cvb. In between the vascular bundles a cavity has appeared which is bounded by a layer of epidermis-like cells, f = the primordium has further increased in size. Size of the internal cavity has also increased; g = the girth of the primordium outgrowth has further increased. the cavity has widened. the epidermis cell layer bounding the cavity has inside of it denser cells or cells differentiating as palisade layer of cells; h = The cavity has opened, the tissues that surrounded it now form the medio-lateral spans of the growing leaflet. i = a vascular bundle of the kind present present in c-h; it will be seen that xylem of the vascular bundle faces the cavity in e-g and later in h the future dorsal (adaxial) surface of the leaflet. j = outer epidermis bearing stomata as seen in f and g. k-n = the inner epidermis facing the cavity in g had stomata-like cells. o = a leaflet has taken its normal shape. It has dorsal and ventral surfaces bounded by epidermis, mid-vein in the center and smaller veins in the lateral spans, the epidermal layers contain in between them parenchymatous tissue surrounding the vascular bundles (veins). Palisade cells are seen on the dorsal side below the epidermis. Magnification bar for a-h and o = 200 µm and for i-n = 20 µm.

Discussion

The results described above of the morphological and histological study of lld aborted leaflets in the *tl lld* line of *P. sativum* have indicated a scheme by which leaflets may be developing their adaxial-abaxial and mediolateral polarities. Phenotypic homology between lld aborted leaflets and leaves of induced mutants or of wild population plants of other species indicates that the scheme being described may be generally applicable for the simple leaf development process. These aspects and a model of the mechanism by which *lld* mutation may disrupt the adaxial-abaxial polarity establishment process are discussed below.

Scheme of the leaflet development process—The scheme of leaflet primordium development in giving rise to a leaflet, proposed on the basis of histological analysis of aborted leaflets, is diagrammed in Fig. 6. The principal steps in the development of leaflet revealed by the histological analysis of aborted leaflet appear to be following: (1) Intercalary growth of leaf primordium occurs laterally on one side. It takes place by participation of large majority of cells other than those that comprise the mid vein. (2) Two secondary veins get differentiated in the lateral growth. The secondary veins are placed in the lateral growth opposite to each other. Xylem tissues of these vascular bundles face the centre of the outgrowth. Thus the tissue in between them is given the adaxial identity. (3) Death of a few cell layers placed in between the lateral (secondary) veins occurs at the centre of the lateral growth. (4) Epidermis separating the central cavity from the tissues of lateral outgrowth is formed. This critical step, in the adaxial-abaxial polarity establishment, defines the tissue in between this internal-epidermis and vascular bundles as the adaxial domain. (5) Coordinated increase of lateral growth and vascular bundles increases the size of future medio-lateral spans. (6) Differentiation of a cell layer adjacent to the internal epidermis (outlining the internal cavity) into palisade parenchyma finally establishes a typical adaxial domain structure. (7) Formation of stomata in the epidermis around the central cavity makes the adaxial domain further differentiated. (8) Internal cells, other than of epidermis palisade parenchyma and vascular tissues, differentiate into spongy parenchyma. (9) Opening up of the cavity by cleavage at midline of lateral outgrowth leads to the division of outgrowth into two lateral spans of lamina. (10) Straightening of the spans gives rise to a flat leaflet with adaxial side (formerly facing the cavity) facing the plant apex and an adaxial side (former outer side of growing primordium) facing the ground. (11) Adaxial-abaxial differentiation, growth in the x, y and z (adaxial-abaxial = z, mediolateral = y and proximodistal = x) directions and construction of the vein network occurs concurrently.

Phenotypic homology between lld aborted leaflets and cup/bell/trumpet shaped leaves observed in simple leaved species—In the present study, sporadic cup/trumpet shaped leaves were observed in *A. spinosus*, *B. monnieri* and *C. roseus*. Limited literature survey revealed that some species such as *F. krishnae* and *S. drummondii* bear all their leaves in the shape of cones/bells/trumpets. Many lines of *A. thaliana* carrying known mutations were found to bear aborted leaves of the same morphology as of the *lld* aborted leaflets. This together with the known internal structural and functional correspondence between the tissues of leaflet and simple leaf suggest congruence in the development processes of simple leaflet and simple leaf primordia. Accordingly it is suggested that the scheme of leaflet development

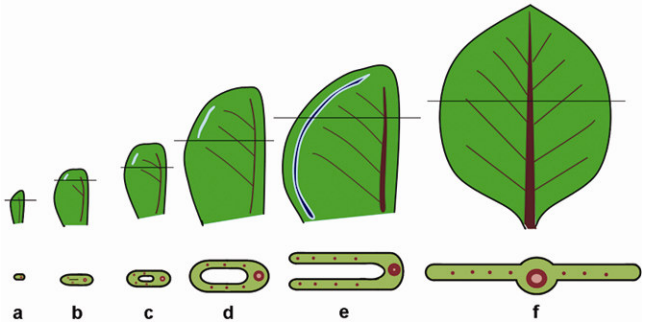

Fig. 6—Scheme of leaflet development in *P. sativum*. It is based on the histological analysis of aborted leaflets and normal leaflet. Fig. a-f correspond to the components of figure 5 as follows: Fig. 6a = Fig. 5b; Fig. 6b = Fig. 5d; Fig. 6c and d = Fig. 5e-g; Fig. 6e = Fig. 5h, and Fig. 6f = Fig. 5o. The leaflet primordium that had grown proximodistally and formed a central midvein begins to grow laterally and in girth. It produces a pair of lateral veins attached to midvein. Most of the background cells participate in enlarging the primordium laterally and in girth. In between the newly formed veins, a cavity is formed which is bounded by an epidermis which is future adaxial epidermis. Growth of the primordium continues accompanied by the formation of vein network. The cell layer next to the epidermis bounding the cavity (future adaxial epidermis) differentiates into palisade parenchyma and other internal background layers into spongy parenchyma. The future adaxial epidermis differentiates stomata. The outergrowth splits into two lamina spans by cleavage of the tissue at the outer midline of lateral growth. The flat leaflet having an adaxial and abaxial surface is formed.

outlined in the previous section is applicable to simple eudicot leaves.

Model for lld led abortion of leaflet development— In the aborted leaflets of *tl lld P. sativum* leaves the inside of the slit, cup, bell, lotus and trumpet (differentiated segment) is the adaxialized side. The outside surface of the differentiated segment of the developing primordium and stem supporting it (which attached cup etc to leaf rachis) is abaxialized. This phenotype is similar to the aborted leaf phenotype of many genetic variants described in *A. thaliana*. It will be seen from the table 1 that excepting the genotypes in which the HD-ZIP III gene family activities were over-expressed, other genotypes had the phenotypes of aborted leaves similar to phenotypes of aborted leaflets of *lld P. sativum*. This means that the disturbance of antagonism between KAN in the abaxial domain and HD-ZIP III in the adaxial domain in favour of KAN produces the lld aborted leaflet type of phenotype[38]. Leaflets in whose developing primordia HD-ZIP III activity could not be maintained at the optimum level, the adaxial-abaxial differentiation process gets prematurely interrupted. Depending upon the temporal differences in the onset of interruption of the adaxial-abaxial polarity development, from the time of initiation of development in leaf primordium, the aborted leaflets produce slit, cup, bell, lotus and trumpet of different sizes. Theoretically all genetic interventions that lead to overproduction of abaxializing gene products or underproduction of adaxializing gene products would result in abortion of leaflet during development much like in the above mentioned types of *A. thaliana* variants. Among the various possibilities, event such as deficiency of HD-ZIP III would be the cause of abortion in the development of leaf primordium[22,23]. Since HD-ZIP III synthesis/supply must be compromised in the entire cell population of adaxial domain for the cessation of polarity development, a non-cell-autonomous process could be involved. miR 165/166 have a repressive function over HD-ZIP III function via their targeting of HD-ZIP III transcripts[46,72,73]. Like other small RNAs the miR 165/166 may be diffusible among cells[5,47,74,75]. Excessive availability of miR 165/166 in the adaxial domain will abort the adaxialization process. If some cells hyper-synthesized miR 165/166 and the miRs diffused into other cells then the adaxializing cell population will cease the progression of adaxial-abaxial polarity development due to insufficiency of active HD-ZIP III proteins

The *lld* is a locus inherited in Mendelian fashion and located in the vicinity of PSPO4SG on the linkage group III of the *Pisum sativum* genetic map[66,] (unpublished observations). To explain the speculated abundant presence of miR 165/166 in the adaxializing cells of growing leaflet primordium, the following is considered a possibility. It is thought that the *lld* locus is structured such that there is a powerful promoter upstream of a transposon or IS element. The miR 165/166 gene(s) are located next to IS element, downstream to the strong promoter and IS element. Excision of IS is thought to be induced by the environment of developing leaf primordium. Loss of IS will lead to miR 165/166 genes coming under the strong promoter. If both ISs are lost there will be hyperexpression of miR 165/166 from both copies. MiRNAs being diffusible, both adaxializing and to be adaxialized cells/tissues will be enriched with miR 165/166. Consequently there will be depletion in the supply of HD-ZIP III products, due to targeting of HD-ZIP III transcripts by miR165/166. The progression of adaxial-abaxial domain differentiation will cease beyond the segment of leaflet primordium already differentiated. The variation in sizes of differentiated segment in aborted leaflets will be dependent on the time of excision of IS element from the *lld* site, which will be different in different leaflet primordia and may depend on the local environment of the concerned leaflet primordium.

Conclusion

The results presented have provided new information about the process of leaf formation. Currently the knowledge about the gene network that regulates formation of simple leaf is considerably advanced. However, the process(es) by which leaf achieves its polarized growth is little understood. Leaves of *lld* mutant of *P. sativum* bear some aborted leaflets of different sizes and shapes. The stage at which the leaflet development is aborted is reflected in the morphology and structure of each leaflet. Here, a series of leaflets that presumably aborted at different stages of development were histologically examined. Based on the observations a scheme of leaflet/leaf development was envisaged. The radially symmetrical leaflet primordium having midvein grows laterally on one side by intercalary cell division. The longitudinally and laterally growing primordium begins to develop its adaxial side, in the distal to proximal direction, within itself. Adaxial-abaxial polarity is established in the ground tissue of lateral

outgrowth by formation of lateral veins whose xylem tissues face each other. A cavity develops between the lateral veins which get bounded by epidermis (internal now and future adaxial epidermis). Growth continues and ground tissue gets supplied with higher order veins. A layer of cells below the internal epidermis differentiates into palisade parenchyma and ground tissue into spongy parenchyma. The outgrowth opens up at midline in distal to proximal direction. The lamina spans spread out to produce a flat leaflet with adaxial upper surface and lower abaxial surface. The abaxial surface served as the progenitor of adaxial surface. The leaflet has oval-obovate shape on account of the contoured restriction in the lateral growth imposed at the proximal and distal ends of the growing primordium.

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