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An analysis of vascular system in the compound tendrilled *afila* leaf in *Pisum sativum*

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Recent work on the venation patterning and morphogenesis of leaf/leaflet has posed the question how different are these in tendrils, which are another type of vegetative lateral organ. Here, the venation patterns of leaflets, stipules and tendrils were compared in the model species, *P. sativum*. Unlike reticulated venation in leaflets and stipules, venation in tendrils comprised of one or more primary veins. A few secondaries were attached to a primary vein, mostly distally. Bilaterally symmetrical secondary veins were rare. The primary veins in tendrils were daughter strands from dichotomously divided mother veins in rachis, connected finally to vascular strands in stem. A tendril received primary vein from one or more mother strands. Some mother strands contributed primary veins to proximal, distal and terminal domain tendrils of *af* leaf. The tendrils shared the multi-primary vein character with stipules. Vein redundancy provided a mechanism for survival of tendril/leaf against injury to some of the veins/mother veins. The presence of aborted primary veins that did not reach apex, rows of cambium cells attached to primary vein(s) at apex, the pattern of attachment of primary veins to mother veins and cessation of vein growth in apical direction in aborted tendrils of *af lld* genotype indicated that the growth of primary veins and tendril was acropetal. Loss-of-function of *AF* extended the repression of *TL* and *MFP* genes on leaflet development from distal and apical domains to proximal domain of leaves in *af* mutants.

Keywords: *afila,* Dichotomous vein-divisions, Leaflet-development, Primary veins, Tendril, Vein redundancy

Tendril is a chlorophyllous (photosynthetic) organ of shoot which coils around or clasps other structures and thereby helps the plant to climb up. Its presence helps the plant to maximise capture of light by its foliaceous organs. Shoot organs such as stipules and leaves are known to occur in the form of tendrils[1-3]. Whereas leaves have been extensively investigated for their pattern(s) of venation[4-6], such analyses of tendrils have received much less attention.

Garden pea *Pisum sativum* of papilionoideae (2n=14, haploid genome=4300Mb) is proving to be a useful model system for studying the development of lateral organs- stipules, leaf, inflorescence, bracteoles and flowers[2,7-15]. Wild type adult leaf of *P. sativum* consists of a petiole extended into a rachis which bears upto 15 pinnae (pinna=leaflet or tendril): 3 pairs of leaflets proximal to petiole (proximal domain), 4 pairs of tendrils distal to petiole (distal domain) and an apical tendril (apical/terminal domain). On either side of the site of attachment of petiole to stem node, a foliaceous stipule is attached such that together the two stipules form a peltate structure. Several leaf mutants are known in *Pisum sativum.* In one of these called *afila* (*af*), all the pinnae are tendrils. *af* is a natural mutation inherited as a Mendelian recessive factor[8,11,16-18]. In another recessive mutant called *leaflet development* (*lld*), pinnae abort at different stages of development. *lld* is an induced mutant. Individual tendrils stop at different stages of development in the *lld af* double mutant[19]. The wild type, *af* and *af lld* lines in *P. sativum* provide opportunity for a comparative study of venation in leaflets, stipules and tendrils. Here, some hitherto unknown features of tendriller venation pattern are reported.

Materials and Methods

Three genotypes of *Pisum sativum* were used, namely wild type, *af af* and *af af lld lld.* The plants of the three lines were propagated in the field as well as on synthetic medium under laboratory conditions.

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For the latter, starting from a seed each of these lines, micropropagated cultures were developed. Single nodes were inoculated to obtain serial cultures of the genotypes. The cultures were grown 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)[20]. The cultures were incubated at 25 °C under white light of 3000 lux for 16 h each day (16 h light: 8 h dark cycle). Well developed leaves along with stipules (*af af* and *af af lld lld)* and leaflets (wild type) were taken from the *in vitro* grown shoots of 4 weeks age and fixed in rectified sprit. They were clarified using a mixture of phenol : lactic acid : glycerol : water :: 1 : 1 : 1 : 1 incubated at 90 ºC for 15 min. Cleared organs were stained with dilute safranine (20%), washed in 5% alcohol and mounted in 25% glycerol on slides. Transverse sections were cut with hand held razor, stained in dilute safranine (5%), mounted in dilute glycerine and examined. Photographs were taken at 4X, 10X and 40X magnifications, using Nikon E100 microscope and Nikon 8400 digital camera. For the estimation of venation density, the magnified pictures were printed on mm graph papers. The vein lengths were measured in a mm^2 area by superimposing thread on each primary, secondary, tertiary or higher level vein. The thread length that covered a vein length was then measured using a mm scale. The magnification factor was divided from the final length to get the actual length. In the pictures of tendrilled leaves the movement of different vascular strands was traced from end to end across the entire leaf. Each strand was given a different colour. Statistical analysis was done according to Panse and Sukhatme.

Results

Tendrils had isobilateral architecture unlike bifacial morphologies of leaflet and stipule (Fig 1). Leaflets and stipules had several layers of cells between dorsal and ventral epidermis (Fig. 2k-n). In these organs, below the dorsal epidermis was present a layer of tightly arranged palisade cells. There were several layers of loosely arranged spongy parenchyma between the palisade layer and ventral epidermis. The tendrils did not have palisade parenchymatous tissues (Fig 2c, i and j). Whereas vascular bundles were present between palisade and spongy parenchyma tissues in leaves and stipules (Fig. 2l and m), they were arranged in the cortical parenchyma in tendrils (Fig. 2c). The venation was reticulate in leaflets where there was a midvein to which were connected several lateral veins on either side (Fig. 2k). The laterals were fused at margins. Each compartment so formed was full of veins of tertiary, quaternary and of higher order complexity. In stipules, venation was characterized by presence of more than one primary vein (Fig. 2m). All of them demonstrated reticulate venation. While the main primary vein traversed from the base to the tip of stipule the others covered the lobes in the lower stem proximal part of stipule. Venation per mm^2 was 5867±397, 5300±360 and 4839±952 µm respectively in leaflet, stipule and tendril (Table 1).

It was observed (Fig. 3b) that tendrils were centrally traversed by up to three major veins. Although the veins were slender yet they differed in their width, some veins were unusually narrow. The veins were branches of thicker veins that were visualized in the rachis to which tendrils were attached. Not all the major veins in tendril extended from base to tip, a few got terminated along the course. There were some lateral veins seen attached to one or the other major veins in the tendril. These were produced more frequently in the distal part of tendril as compared to the proximal part. Usually the laterals were scattered (Fig. 2d; Fig. 3b), but in some tendrils they occurred serially at the apex, such that biggest one was distal most and smallest one at the apical

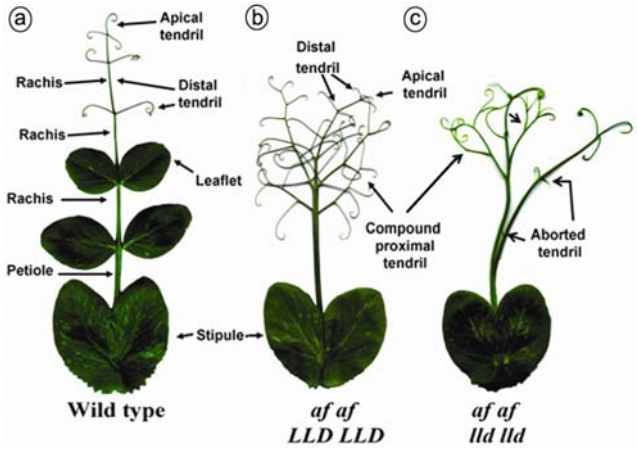

Fig. 1—Stipulate leaves of wild type, *af af* and *af af lld lld* lines of *Pisum sativum*. The three lines have similar stipules but differ in leaf morphology. a = Wild type leaf has proximal (to petiole) two pairs of pinnae in the form of leaflets, three pairs of distal pinnae and apical pinna as tendril(s). b = All pinnae are tendriller, proximal are compound and distal and apical are simple in *af af*. c = Leaf has the *af af* morphology but some tendrils are aborted in *af af lld lld* (shown by arrows). Scale bar = 2 cm.

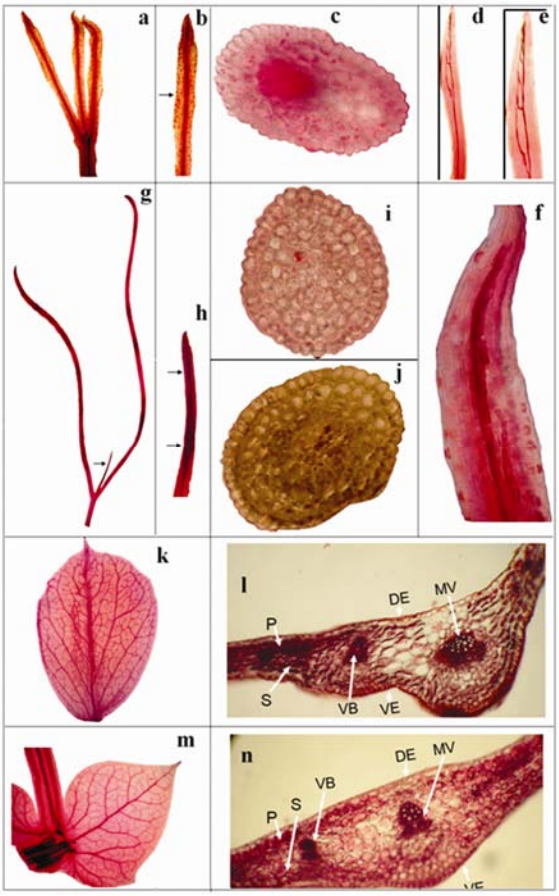

Fig. 2—Histological properties of tendrils, leaflets and stipules in *Pisum sativum*. a-j = Tendril(s); k and l = Leaflet and m and n = Stipule. a = A part of a proximal compound *af af* tendriller pinna; b = A tendril; c = Transverse section (TS) of the tendril at b in the sub-apical region, Section of a vascular strand is seen surrounded by parenchymatous cells bounded by epidermis. d-f = Apical part of a tendril (d), a vascular strand is seen reticulated near to the apex (e) and apex has cambium cells placed in straight lines (f), indicating acropetal growth of the vascular strand. g-j = A part of a proximal compound *af af lld lld* tendriller pinna in which central tendril has aborted. The aborted tendril has poorly developed vasculature at the apex as well as in sub-apical region, demonstrating cessation of acropetal growth due to loss of vascular strand. k = Reticulated venation fused at the margins in a leaflet. l = TS of a leaflet has typical histological features of a eudicot leaf. The lamina is bounded dorsally and ventrally by epidermis. Immediately blow the dorsal epidermis a layer of palisade mesophyll parenchymatous cells. The rest of space is filled by layers of spongy mesophyll parenchymatous cells. Vascular bundles are placed between the palisade and spongy parenchymatous tissues. m = Venation pattern in a stipule. There are several primary veins. The main primary or mid-vein is reticulated like in leaflet. The other primary veins are at the stem proximal side, in the region where in stipule is lobed. These are also reticulated. The lateral veins arising from different primaries are fused at margins. n = TS of a stipule. The histology of stipule is similar to that of the leaflet. DE = dorsal epidermis; VE = ventral epidermis; MV = mid-vein; VB = vascular bundle; P = palisade parenchyma; S = spongy parenchyma. Scale bar for a-e, g-n = 200 µm and for f = 100 µm.

position (Fig. 2e). The lateral veins were rarely bilateral. Tendrils at the apex appeared to carry strands of cambium cells indicating that the growth of veins was acropetal in tendrils (Fig. 2f). These cambial strands appeared to be in line with the major veins. All the above observations indicated that the major veins in tendrils were equivalent to primary vein(s) in leaflets and stipules. The tendril had features of palmate pinnae by having more than one primary vein.

The examination of the venation in whole of a leaf (Fig. 3a and b) in relation to the vascular strands (mother veins) in rachis end of petiole showed that there were in all seven mother vascular strands. One of these strands aborted in the rachis of left proximal pinna and did not contribute veins to any of the tendrils. The other six contributed major veins to 9 proximal tendrils, 2 distal tendrils and the terminal tendril. The mother strands were observed to invariably divide into daughter strands dichotomously, in rachis. The major veins that went to tendrils came either from the same mother strand or from two mother strands. Tendrils produced on the same proximal rachis often shared the veins branched from a mother strand. However, some mother strands contributed veins to tendrils borne on both left and right branches of proximal rachises. There were mother strands that contributed major veins to both distal and proximal tendrils. A major vein of the terminal tendril was from a mother strand that contributed major veins to both of distal tendrils. Pattern of venation such as above was common to all of the *af af* leaves examined.

The pattern of venation in the aborted tendrils of *af lld* (Fig. 1c and Fig 2g-j) leaves showed that the abortion of tendril development was related to cessation of primary venation. In the aborted tendrils, whereas the major vein was well developed proximally it was ill-developed apically. This observation and the pattern of venation origin in normal tendrils (Fig. 3b) and occurrence of rows of procambium/cambium cells in attachment with main vein at the apex of growing tendrils indicated that the primary veins and tendrils grew acropetally.

Discussion

The experiments here have revealed some important features of the venation in *af* tendrilled leaves of *Pisum sativum* (Fig. 3) which are briefly discussed below.

Table 1—Venation density in leaflets, stipules and tendrils of wild type and stipules and tendrils of *af af* in *Pisum sativum*

[Values arre mean ± ??]

Serial no.	Leaf sub-organ	Position in relation to stem node	Venation density (µm/mm^2)			
			Primary	Secondary	Higher order	Total
1.	Tendril [a]	Apical	2063±0	938±492	0	3001
2.		Middle (distal)	5156±0	169±377	0	5325
3.		Basal (proximal)	6188±0	0	0	6188
4.	Leaflet	Apical	1022±15	1052±45	4348±710	6422
5.		Middle	1031±0	861±309	4189±1055	6082
6.		Basal	1031±0	1246±448	2821±379	5098
7.	Stipule [a]	Apical	1031±0	1105±377	3681±969	5817
8.		Middle	1031±0	845±491	3598±1199	5475
9.		Basal	1198±186	852±563	2556±781	4606

a = Average of samples taken from wild type and *af af* genotypes.

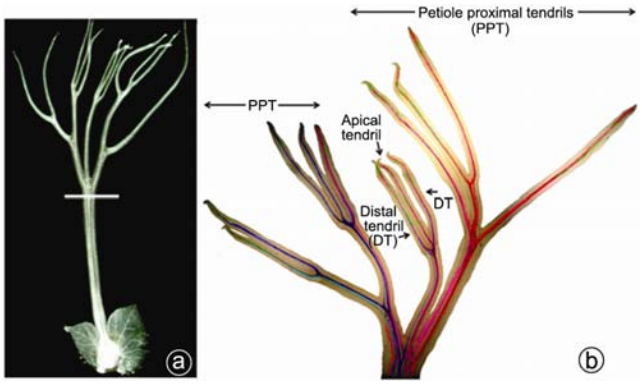

Fig. 3—The *af af* tendrilled leaf and pattern of its venation in *Pisum sativum*. a = A leaf that has compound tendril pairs proximal to petiole, a pair of simple distal tendril and a simple apical tendril. b = Venation pattern of the leaf as seen after clearing and safranine staining. The vascular strands have been traced from the rachis-petiole boundary to the apex of the tendril (shown by the white line in a). Different strands have been filled with different colours. The compound leaf received in its petiole 7 thick bundles of veins (vascular strands). Their course in the petiole is overall parallel but they are seen intermingled. Each strand is seen dividing dichotomously in rachis, for example a strand gives rise to three strands by two dichotomous divisions of the original strand. A tendril receives one to three strands. A mother vascular strand sends one each of its daughter strands to adjacent tendrils. When two strands are present in a tendril they usually come from different parental strands and sometime from the same mother strand. One of the strands in a tendril may not traverse the entire length of tendril. Secondary veins are seen only in the apical and middle region of the tendril and these are depicted with fluorescent green colour. The positions of tendrils in the petiole proximal, petiole distal and apical domains of leafblade are identified. Scale bar a and b = 200 µm.

Tendrils were often supplied with more than one primary veins. Each of these vein was an acropetal extension of a division product of a mother vein. Several tendrils were served their primary veins from the same mother vein by its dichotomous divisions. Tendrils with multiple veins were served their primary veins either from the same mother vein or different mother veins. Each mother vein served primary veins to several tendrils of the proximal domain, tendrils of proximal and distal domain or tendrils of proximal, distal and terminal domains. A number of mother veins derived from stem served the entire leaf. This mechanism ensured that if a mother vein of a leaf or a primary vein of a tendril got injured, the leaf will survive.

The venation of tendrils is largely linear and not reticulate like that of leaflets and stipules. The *af* leaf therefore as a whole grows acropetally unlike acropetal growth in rachis and tendrils and basipetal growth in leaflets of wild type leaf.

The above properties that differentiate *af* leaf from *AF* leaf result from loss-of-function mutation in a single gene, *AF*. It is implied that *AF* function controls vein patterning and thereby positively regulates development of laminated leaflets in the proximal domain of wild type leaves of *P. sativum*. There is evidence that tendrils in the proximal domain are replaced by leaflets when *af* shoots are grown in the presence of 1-N-naphthylpthalmic acid (NPA) an auxin transport inhibitor[22]. This observation means that high auxin concentration in the growing *af* leaf primordium prevents formation of leaflets, or presence of auxin in excessive concentration prevents differentiation of adaxial-abaxial and medio-lateral polarities in pinnae such that they develop into tendrils. In *af* leaves, the normal pathway of pinna development to form leaflets is circumvented by a different pathway of pinna development which is normally used in the

distal and terminal domains of wild type *P. sativum* leaf for tendril development.

In the wild type leaf, *TENDRIL-LESS* (*TL*) and *MULTIFOLIATE-PINNA* (*MFP*) prevent leaflet development in the distal and terminal domains[11,23]. *AF* is the repressor of rachis/tendril growth and activator of leaflet growth. Loss of AF function, allows *TL* and *MFP* mediated repression of pinna development as leaflets to become operational in the proximal domain. Thus tendrils are formed in all the domains of *af* leaf[3,11,17,22-24].

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