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Interaction between *cochleata* and *stipule-reduced* mutations results in exstipulate hypertrophied leaves in *Pisum sativum* L.

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In the wild type *P. sativum*, each of the adult plant stem nodes, bears a pair of sessile foliaceous stipules and a petiolated unipinnately compound leaf of 4 to 6 leaflets and 7-9 tendrils. The *stipule-reduced* (*st*) and *cochleata* (*coch*) single *null* mutants and *coch st* double null mutant differ fom the wild type in respectively having sessile stipules of much reduced size, petiolated simple and/or compound leaf-like stipules and no stipules. It is also known that *coch* leaves are somewhat bigger than *st* and wild type leaves. Here, pleiotropic phenotype of *coch st* double mutant was investigated. The morphologies of stipules and leaf were quantified in the field grown plants and microcultured shoots, latter in the presence and absence of gibberellic acid and N-1-naphthylphthalamic acid. The observations showed that as compared to the corresponding plants or shoots of *COCH ST* (WT) genotype, (a) *coch st* plants bore leaves in which all the organs were hypertrophied; (b) full complement of leaflets and 3-5 tendrils were formed on leaf; (c) the microcultured *coch st* shoots were taller despite lower number of nodes, and (d) they also produced leaves in which all the organs were bigger and the ratio of leaflets/tendrils was higher. It was concluded that in *coch st* double mutant (a) ST function is essential for stipule primordium differentiation, in the absence of COCH function and (b) absence of negative feedback loops between simple stipules and compound leaf for metabolite utilization allows hypertrophied growth in leaves.

Keywords: Biomass allocation, Compound leaf, Compound stipule, Phytomere regulation, Simple stipule, *UNIFOLIATA*

Pisum sativum L., garden pea crop plant (2n = 14; 4300 Mbp), is a model leguminous species[1] for the analyses of gene regulatory networks and mechanisms of differentiation of the determinate plant organs stipule, leaf, inflorescence and flower and suborgans leaflet, tendril, bracteole and flower whorls[2-15]. A wild type pea plant bears on its primary stem a small number of embryonic and vegetative phase phytomeres and many reproductive phase phytomeres. All the adult plant phytomeres bear at their node a unipinnately compound leaf, 2 to 3 pairs of leaflets, 7-9 tendrils and a pair of stipules. The reproductive phase phytomeres also bear a secondary inflorescence. Leaflets and stipules are laminated; they are structurally and functionally similar and comprise the major sites of photosynthesis[15,16]. Stipules simultaneously possess properties of entire and lobed simple leaves[14].

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The development of wild type *P. sativum* leaf primordium into compound leaf is promoted by the master regulator gene *UNIFOLIATA* (*UNI*), also called as *UNIFOLIATA TENDRILLED ACACIA* (*UNI-TAC*), an ortholog of the ubiquitously present plant gene *LEAFY* (*LFY*)[2,4,5]. In the differentiating leaf primordia, *UNI* acts together with its coregulators *STAMINA PISTILLOIDA* (*STP*)[17], *AFILA* (*AF*), *INSECATUS* (*INS*), *MULTIFOLIATE-PINNA* (*MFP*) and *TENDRIL-LESS* (*TL*)[12,18-23], which themselves are positively regulated by *UNI*[9]. The null mutants (*uni*), and promoter mutants (*uni-tac*) of *UNI* and null *stp* mutants respectively produce simple and compound leaves of low complexity[2,6,7,9,12,15,17,24-28]. *AF*, *INS*, *MFP* and *TL* are weak repressors of *UNI*[9,12]. The *af*, *ins* and *mfp* mutants produce leaves of higher than wild type complexity[9,21-23]. In *tl* mutants tendrils are replaced by leaflets and in *af* mutants leaflets are replaced by compound tendrils[7,27,28]. The *af tl*, *af mfp*, *tl mfp* and *af tl mfp* double and triple mutants produce leaves in which some pinnae are bi-, tri- or quadri- pinnate[9,12]. The leaf dissection in mutants is positively correlated with *UNI* gene expression in their leaf primordia[15,26].

The *UNI* led pathway for the development of compound organ (leaf) is not expressed in the stipule primordia of wild type *P. sativum*. *UNI* activity is repressed by *COCHLEATA* gene in stipule primordia[4,15]. However, in *coch* null mutants, *UNI* activity differentiates stipule primordia into leaf-like compound stipules. Wild type stipule primordia are initiated and differentiated by *COCH* and its coregulator gene *STIPULE-REDUCED* (*ST*)[4,5,11,29-33]. The *st* mutants produce simple *COCH*-like stipules of very small size. Large majority of phytomeres are stipule-less in *coch st* double mutants[11]. Stipule primordium is initiated but not differentiated unless *COCH* and *ST* activities are together available, as in wild type or *uni* plants[11]. Compound stipule differentiation in stipule primordia does not occur in *coch* mutants, unless *UNI* and *ST* activities are simultaneously available[11].

The *coch* mutants display pleiotropic phenotype. Their leaves are larger, secondary inflorescences are bracteolated and flowers bear supernumerary organs in all of the whorls[13-15]. These phenotypes are correlated with higher than normal expression of *UNI* in differentiating leaves, inflorescences and flowers[15]. *COCH* activity strongly represses *UNI* in differentiating stipule and weakly represses (downregulates) *UNI* in differentiating leaves, inflorescences and flowers[15]. On account of the pleiotropy, *coch* plants have been observed to hyperaccumulate biomass in leaves and stems and hypoaccumulate biomass in pods (seeds) due to their poor fertility[15]. The property of hyperaccumulation of biomass in vegetative organs in *coch* plants is shared by the wild type plants from which stipules had been removed surgically[34,15]. The leaves in *coch* plants and surgically treated stipule-less wild type plants were larger/heavier mostly on account of bigger and thicker leaflets. Leaves in such plants resembled wild type leaves in their architecture, that is they carried 2 or 3 pairs of leaflets, 3 or 4 pairs of tendrils and apical tendril.

While comparing the morphologies of the field grown post-flowering phytomers of wild type, *coch ST*, *COCH st* and *coch st* plants of identical genetic background during the 2009-2010 winter seasons, it was observed that the tendrils were scarcer in *coch st* leaves as compared to leaves of other three genotypes. Therefore the present study has been undertaken to define the vegetative phytomere phenotype of *coch st* double mutant. Microcultured shoots were used for the analyses so as to minimize the genotype × environment effects.

Materials and Methods

Plant material—The *P. sativum COCH ST* (WT), *coch ST*, *COCH st* and *coch st* lines had the same genetic background[11]. The derivation of these homozygous lines has been described earlier[11]. To quantitatively characterize their phytomeres, lines were grown in the experimental farm of NIPGR during the winter seasons of 2009-2012. The cultivation procedure has been described earlier[6]. The stipules and leaf borne on the third flowering node of primary branch were traced on the mm graph paper to estimate their areas. Samples for phenotyping were drawn from three plants per genotype.

Microcultures of *COCH ST* (WT) and *coch st* genotypes were started with their single seed. The explants formed were multiplied by sub-culturing. Sub-culturing was done with single node explants. The microculture (tissue culture) technique described below was used.

The cultures were phenotyped after six weeks of incubation, when they stopped growing. Each shoot was individually observed and photographed, using Nikon COOLPIX L24 digital camera (14 megapixels) and/or by using Nikon Digital Sight DS-Ril camera at 0.5X magnification in AZ-100 Nikon multi objectives stereaozoom microscope. Each shoot was measured for its length and number of nodes borne on it. The leaves born on second node onwards were characterized for the number of pinnae, structure of each of pinna and sizes of each of the suborgans. The sizes of organs were measured either manually as described for the field samples or by using Nikon Digital Sight DS-Ril camera at 0.5X magnification in AZ-100 Nikon multi objectives stereozoom microscope. The samples were weighed on TE64 (Sartorius, New Delhi) to determine their fresh weight. For each treatment per genotype atleast 60 leaves formed on 20 shoots were examined.

Tissue culture (microculture) technique—MS medium containing Gamborg vitamins, 11 µM 6-benzylaminopurine (both from Sigma-Aldrich, USA), 3% sucrose and 0.8% agar (both from Hi-Media Laboratories Pvt. Ltd., India) was used throughout. Any supplements were added to autoclaved medium cooled to 75 °C before dispensing it to the sterilized culture jars. Cultures were raised in 375 mL glass bottles (Allied Scientific Sales, New Delhi) which

accommodated up to 15 explants on 70 mL medium. The explants were grown at 25 °C and exposed to white light at the rate of 3000 lux for 16 h and to darkness for 8 h at same temperature. Under these conditions, explants on basal medium produced more than 10 nodes in 5 weeks time. The supplements used were the elicitors N-1-naphthylphthalamic acid (NPA) and gibberellic acid (GA_3). Their stock solutions were prepared at 100 mM in dimethyl sulphoxide (DMSO) (Qualigens, Mumbai). The concentration of NPA and GA used were 40 µM. Each treatment was repeated six times.

Gene expression studies—Transcript levels of *UNI* gene whose sequence is known was studied. The primer sequences are given in the Table 1. The apices for measuring transcript levels were resourced from 14 days old *in vitro* grown shoots. Several apices per genotype per treatment were pooled and frozen in liquid nitrogen and stored at –70 °C as a sample. From each sample total RNA was extracted by using the RNeasy plant mini kit (QIAGEN, Germany). The RNA was quantified by nanodrop-1000 spectrophotometer and its integrity was checked by resolving on 1.5% agarose gel made in 10X MOPS buffer with formaldehyde. First strand cDNAs were generated by using the oligo (dT8) primer of the Revert Aid™ H Minus first strand cDNA synthesis kit (FERMENTAS, Leutonia) according to manufacturer's instructions. Semi-quantitative PCR (RT-PCR) was performed by using 400 ng first strand cDNA and gene primers, enzyme and dNTP mix at 0.4 µM, 0.05 units/µL and 0.2 µM concentration, respectively in 25 µM final volume. The temperature profile for RT-PCR was: one 3 min cycle of initial denaturation at 95 °C, 35 cycles of 30s at 94 °C, annealing at 59 °C for 30s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min and final hold at 4 °C for infinity. PCR product was separated on 0.8% agarose gel in 1X TBE buffer containing 0.5 µL/mL ethidium bromide and quantified in 1-D-analysis tool by using Vision Works Image™ acquisition and analysis software in GelDoc-it™ imaging system (UVP, UK).

Real time PCR (qRT-PR) assays were performed with 20 ng cDNA on Step one Real Time PCR detection system (Life technologies, formerly Applied Biosystem, USA) according to manufacturer's instructions. The relative expression levels of *UNI* between genotypes were compared by calculating the relative quantity values (RQ) by use of comparative Ct method also referred to as the $2^{-\Delta\Delta CT}$ method. The pea *ACTIN9* gene was used as the internal control. Two biological and three technical replicates were used for qRT-PCR.

Statistical analysis—The *t*-tests were performed using the Graphpad software at 95% confidence interval to evaluate the significance of difference.

Results

coch st double mutations increase the leaf and leaflet sizes and proportion of leaflets to tendrils—Vegetative organs borne on the third flowering node of the primary stem of the field grown plants of *COCH ST*, *coch ST*, *COCH st* and *coch st* genotypes are presented in the Fig. 1. The area measurements on leaves and stipules of the four genotypes are given in the Table 2. The leaf, stipules and leaf plus stipules area-wise the genotypes could be respectively arranged in the following order: *coch st* (168 cm^2) > *coch ST* (106 cm^2) > *COCH st* (76 cm^2) and *COCH ST* (WT) (75 cm^2); *COCH ST* (WT) (48 cm^2) > *coch ST* (21 cm^2) > *COCH st* (3 cm^2) > *coch st* (0); and *coch st* (168 cm^2) > *coch ST* (127 cm^2) > *COCH ST* (WT) (122 cm^2) > *COCH st* (79 cm^2). These observations revealed that the nodes of mature *coch st* plants produced leaf whose area was larger than the joint area of *COCH ST* (WT) leaf and its associated stipules. The *coch ST* leaf and leaf plus stipules produced at a node also possessed areas that were larger than the areas of corresponding

Table 1 a—Primer sequences for the semi-quantitative PCR (RT-PCR) assays:

Gene	Forward primer	Reverse primer
UNIFOLIATA (UNI)	CTACGCGGTTACCCCTACAA	ATTTCTCACCGCGCTCTTTA
ACTIN-9	ATGGTTGGAATGGGACAAAA	GCAGTTTCCAACTCCTGCTC

UNI = Gene bank accession no. AF0101902; *ACTIN-9* = Gene bank accession no. U81047

Table 1 b—Primer sequences for the quantitative real time PCR (qRT-PCR) assays:

Gene	Forward primer	Reverse primer
UNIFOLIATA (UNI)	CAACCGCCCCGATG	CCTCCAAGCCTCCTAGTTCTCTT
ACTIN-9	TTGTAGCACCACCAGAGAGGAA	TTGCAATCCACATCTGTTGGA

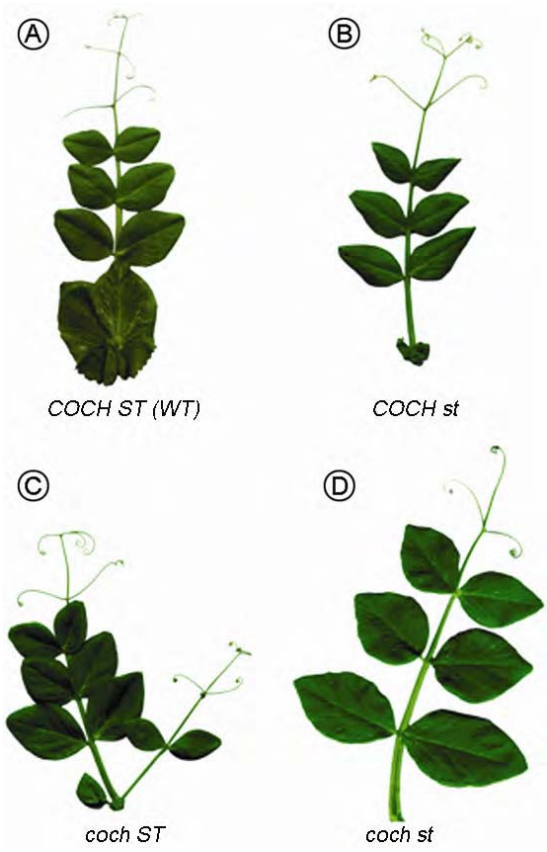

Fig. 1—Wildtype (WT, *COCH ST*), *stipule reduced* (*st*), *cochleata* (*coch*) and *st coch* leaves and stipules in *Pisum sativum.* The leaves/stipules belong to nodes immediately above the first flowering node from lines of largely similar (constant) genetic background. It may be noted that stipules are absent from the *coch st* double mutant.

organs on *COCH ST*. These results showed that *coch* mutation caused enlargement in the leaf size. The leaf size enhancement was greater in the *coch st* double mutant. The effect of *coch* and *st* mutations on the leaf size increase was synergistic.

Another pronounced effect of the simultaneous presence of *coch* and *st* mutations was on the leaf architecture. The *coch st* leaves possessed lesser number of tendrils in their distal domain as compared to the *COCH ST* (WT) leaves (Fig. 1). Whereas at least three pairs of tendrils were present in the distal domain of *COCH ST* (WT) [8.3 tendrils in distal domain (DD) and apical domain (AD)], *COCH st* (8.6 in DD and AD) and *coch ST* (7.8 in DD and AD) leaves, large majority of the *coch st* leaves had only one pair of tendrils in their distal domain [3.4 tendrils in DD and AD; (Fig. 1; Table 2)].

The effect of simultaneous presence of *coch* and *st* mutations on leaf morphology was investigated further in the microcultured shoots of *COCH ST* (WT) and *coch st* genotypes. Single node explants of *COCH ST* (WT) and *coch st* genotypes were cultured to obtain shoots on media containing GA, NPA or no elicitor. The observations on fresh weight of one leaf and one leaflet and area of one leaflet in shoots of the two genotypes microcultured in the presence and absence of GA are given in the Table 3. The fresh weight of *coch st* leaf was 1.6 fold higher than that of *COCH ST* (WT) leaf. In the shoots grown in presence of GA, fresh weight of *coch st* leaf was 1.9 times more than that of *COCH ST* (WT) leaf. Leaf fresh weight of both the genotypes was lower in shoots

Table 2—Leaf and stipules sizes and proportion of laminated organs/suborgans and tendrils in the reproductive phase phytomers of field grown plants of *COCH ST* (WT), *coch ST*, *COCH st* and *coch st* genotypes in *P. sativum*

Serial no.	Genotype[a,b]	Leaf (cm^2)	Stipules (cm^2)	Leaf+Stipules (cm^2)	Laminated organs/suborgans:Tendrils in a leaf[b]	stipule[c]	phytomere
1	*COCH ST* (WT)	74.83±3.86[d]	47.51±3.63	122.34[d]	6.0±8.4±0.3[e]	2.0±0:0	8.0:8.3
2	*coch ST*	105.89±17.76	21.11±1.72	127.00[d]	6.0±7.8±0.3[e]	1.6±0.2:2.6±0.7	7.6:10.4
3	*COCH st*	76.33±6.43[d]	2.61±0.22	78.94	6.0±8.6±0.3[e]	2.0±0:0	8.0:8.5
4	*coch st*	167.85±10.97	0[g]	167.84	6.0±3.4±0.3[f]	0:0[g]	6.0:3.4

a = Significance of differences between genotypes for leaf area, stipule area and leaf +stipule area were tested by applying '*t*'-test. The values having the superscript d were not different, all others were different. Except for leaf size difference between *COCH ST* and *coch ST* which was significant at 5% level of probability, all others were significant at 1% level of probability;

b = '*t*' test was used for testing the significance of difference between number of tendrils in leaves of the genotypes. The values having the same superscript are not different. The *coch st* was different from the other three genotypes in the expression of laminated organs/suborgans:tendrils;

c = Here the stipules, being simple laminated organs, have been treated at par with simple leaflets for the purposes of comparing the laminated versus tendriller structures at nodes in the genotypes studied;

g = Stipules were absent in *coch st* plants.

Table 3—Effect of *coch st* mutations on the parameters of leaf growth observed in shoots grown *in vitro* in the presence and absence of gibberellic acid (GA_3)

Serial no.	Genotype	GA concentration (μM)	Fresh weight of one leaf (mg)	Fresh weight of one leaflet (mg)	Area of one leaflet (cm^2)
1	*COCH ST* (WT)	0 - c	5.85±0.07[a]	1.66±0.02[a]	0.25±0.01[a]
2	*coch st*	0	8.80±0.10[b]	2.48±0.06[b]	0.43±0.04[b]
3	*COCH ST* (WT)	40	2.27±0.12[b]	0.93±0.04[b]	0.15±0.03[b]
4	*coch st*	40	4.32±0.09[b]	1.19±0.05[b]	0.27±0.05[b]

a, b = For a statistic/trait, the values of treatment effects that carry the letter as b superscript are different from the control;

c = Control for all of three other treatments.

microcultured in the presence of GA than in shoots cultured without GA. The fresh weight and area of individual leaflet were higher in *coch st* shoots than in *COCH ST* (WT) shoots, irrespective of GA.

This aspect was studied in greater detail in terms of leaf architecture. The microcultured shoots of *COCH ST* (WT) and *coch st* grown in the presence of GA and NPA separately and absence of any elicitor were characterized for the suborgan (leaflets and tendrils) composition of their leaves and the results are given in the Table 4. On an average basis the leaves of *coch st* and *COCH ST* (WT) shoots formed on plain medium without addition of elicitors produced suborgans in equal numbers (column XVI). However, *coch st* shoots formed leaves that were richer in leaflets (columns III to VII and Fig. 2b, c and Fig. 3b-d, f-i) as compared *COCH ST* (WT) shoots which formed leaves comparatively richer in tendrils (Fig. 1a, f and g; Fig 2a). It was observed that GA reduced the complexity of *COCH ST* (WT) leaves but not of the *coch st* leaves. Presence of NPA in the medium, reduced the complexity of leaves formed on shoots of both *COCH ST* (WT) and *coch st* genotypes. The negative effect of NPA on the leaf complexity was greater in *COCH ST* (WT) shoots than in *coch st* shoots. Considering the three treatments together, it was observed that the ratio (a) *coch st*/*COCH ST* (WT) for all the pinnae, leaflets and tendrils was 1.2, 1.3 and 1.1 respectively and (b) leaflets/tendrils was 1.8 and 1.5 for *coch st* and *COCH ST* (WT) genotypes, respectively. The above results altogether showed that *coch st* leaves had heavier leaves with more pinnae and the pinnae were richly leafleted and poorly tendrilled as compared to *COCH ST* (WT) leaves.

coch st *shoots produce leaves of larger size but lesser in number as compared to COCH ST* (WT) *shoots*—The observations on some features of the *COCH ST* (WT) and *coch st* microcultured shoots are summarized in the Table 5. The *coch st* shoots were about 1.2 fold taller than *COCH ST* (WT) shoots. However, the number of nodes was about 1.5 fold higher in *COCH ST* (WT) shoots as compared to *coch st* shoots. This was so because internode length was about 1.9 times larger in *coch st* shoots than in *COCH ST* (WT) shoots. In the *coch st* shoots, leaves possessed bigger petiole, leaflets as well tendrils. Thus all the suborgans of *coch st* leaves were of larger size than in *COCH ST* (WT) leaves.

Exogenous GA induced sporadic stipule formation in coch st microcultured shoots—The microcultured *coch st* shoots were generally stipule-less, like the field grown plants of the genotype. When GA was added in the growth medium, occasional nodes produced stipules. Frequency of stipule emergence was 0.133±0.044 (Fig. 4). The GA stimulated stipules formed were simple or compound. At some nodes one stipule was simple and the other compound (Fig. 4d). Stipulated nodes were not seen in shoots grown in presence of NPA or in absence of NPA and GA.

Overexpression of UNI gene in coch st shoots—The *UNI* gene transcript levels were estimated by RT-PCR and qRT-PCR, in the apices of the *COCH ST* (WT) and *coch st* young microcultured shoots. The results show that *UNI* expression was 1.4 fold (RT-PCR) to 4.5 fold (qRT-PCR) or about 3 fold (average of RT-PCR and qRT-PCR) higher in the apices of *coch st* shoots as compared to *COCH ST* (WT) shoots (Fig. 5). These results suggested that the leaf morphology differences between *coch st* and *COCH ST* (WT) were related to the differences in the *UNI* gene expression in the differentiating leaves of the two genotypes.

Table 4—Effect of *coch st* mutations on leaf complexity

Genotype	Treatment	Frequency of leaves possessing from petiole side onwards												Sum of number of suborgans × frequency
		leaflet pair + leaflet pair+ tendril	leaflet pair+ leaflet pair+ leaflet	leaflet pair + tendril/ leaflet + tendril	leaflet pair + tendril and leaflet + tendril	leaflet pair + tendril pair+ leaflet	leaflet pair + tendril pair	leaflet pair+ leaflet and tendril	leaflet pair+ leaflet	leaflet pair + tendril	Leaflet pair+ tendriled leaflets	leaflet pair	simple leaf*	
		Fig. 2b	Fig. 3c	Fig. 2c	Fig. 2d	Fig. 3b	Fig.2f	Fig. 2g	Fig. 3f	Fig.2h and 3e	Fig. 3d	Fig. 3g	Fig. 3h and i	
I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
COCH ST (WT)	c	0[a]	0[a]	0[a]	0[a]	0[a]	0.650± 0.061[a]	0.183± 0.048[a]	0[a]	0[a]	0.167± 0.047[a]	0[a]	0[a]	0[a]	4.483
	GA (40µM)	0[a]	0[a]	0[a]	0[a]	0[a]	0.133± 0.044[b]	0.05± 0.028[b]	0[a]	0.066± 0.061[b]	0.667± 0.061[b]	0[a]	0.083± 0.035[a]	0[a]	3.230
	NPA (40µM)	0[a]	0[a]	0[a]	0[a]	0.033± 0.023[a]	0.117± 0.041[b]	0.017± 0.017[b]	0.033± 0.023[b]	0.250± 0.056[b]	0.183± 0.050[b]	0[a]	0.133± 0.044[b]	0.183± 0.050[b]	2.698
coch st	c	0.150± 0.046[a]	0[a]	0.033± 0.020[a]	0.050± 0.020[a]	0[a]	0.45± 0.060[a]	0.033± 0.020[a]	0.066± 0.030[a]	0[a]	0.220± 0.050[b]	0[a]	0a	0[a]	4.471
	GA (40µM)	0.183± 0.050[b]	0[a]	0.117± 0.041[b]	0.200± 0.052[b]	0[a]	0.167± 0.04[b]	0[b]	0.083± 0.035[b]	0[a]	0.233± 0.054[b]	0[a]	0.050± 0.030[b]	0[a]	4.466
	NPA (40µM)	0.100± 0.038[b]	0.083± 0.035[b]	0.083± 0.035[b]	0.067± 0.032[b]	0.083± 0.034[b]	0.100± 0.038[b]	0[b]	0.067± 0.032[a]	0.100± 0.038[b]	0.083± 0.035[b]	0.100± 0.038[b]	0.083± 0.035[b]	0.050± 0.027[b]	3.913

a, b = For a genotype and parameter, the values of treatment effects that carry the same letter as superscript are not different from the respective control;
c = No supplement;
* = This class included bifid lamina bearing leaves.

Discussion

The results quantitatively confirmed the already known phenotypes of the wild type and *st* and *coch* mutant leaves and stipules in *P. sativum*. The new findings concern the *coch st* double mutant. The distinctive leaf and stipule properties of *coch st* and their significance are discussed below.

A phytomere, in both field grown plants and microcultured shoots, of the *coch st* double mutant comprised of an internode, a node and a leaf attached to the node via leaf's petiole. The phytomeres of *coch st* were bereft of stipules. However, simple and/or compound stipules were formed occasionally on the nodes of the *coch st* shoots microcultured in the presence of exogenously supplied GA. Microcultured shoots grown in presence of NPA and in absence of both the elicitors did not produce observable stipules. It is known that field grown *coch* plants produce five kinds of phytomeres with respect of stipule: phytomeres that bear (a) two compound leaf-like stipules; (b) one compound stipule and one simple stipule; (c) one compound stipule; (d) one simple stipule; and (e) no stipule[4,5,11]. Thus the phenotype of stipules formed on *coch st* GA supplemented microcultured shoots conformed to the *coch* stipular phenotype. These observations imply that stipule primordia are separated at *coch st* nodes; among them some that maintain their integrity differentiate and develop into stipules upon GA supplementation. The *coch* and *st* mutants are known to produce stipules at their nodes, former produces petiolated compound or simple stipules. This means that in both *coch* and *st* mutants, stipule primordia are formed, except that some of the *coch* stipule primordia fail to differentiate. Interaction of the gene regulatory network of *UNI*-led organ development pathway and ST function must be responsible for the formation of petiolated leaf-like stipules on *coch* stem nodes[9,11]. Therefore, it is the absence of ST function that must restrict the stipule primordia differentiation in *coch st* nodes. The *st* stipules are known to be several fold smaller in size than *COCH ST* (WT) and *coch ST* stipules[35]. Therefore, ST function may be involved in the cellulation of early stages of the differentiating *ST* stipule primordia causing enlargement of stem cell population, irrespective of the status of *COCH* and *UNI* genes. Present results implicate GA supplementation as a partial substitute of ST function in the rescue of *coch st* stipule primordia, that are destined to abort in the absence of ST function.

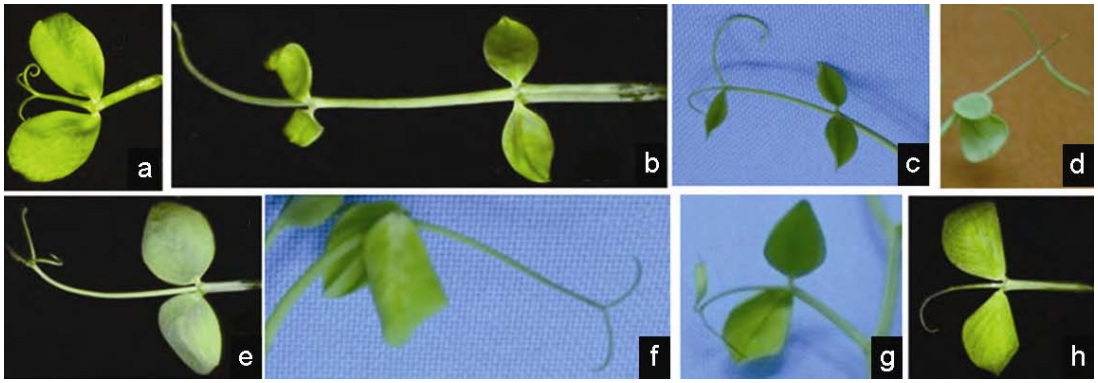

Fig. 2—Leaf morphologies seen on the shoots of WT (a) and (b-h) *coch st* genotype grown *in vitro* on bed of basal medium. The leaf morphology descriptions and frequencies of their occurrence on WT and *coch st* genotypes are given in Table 4.

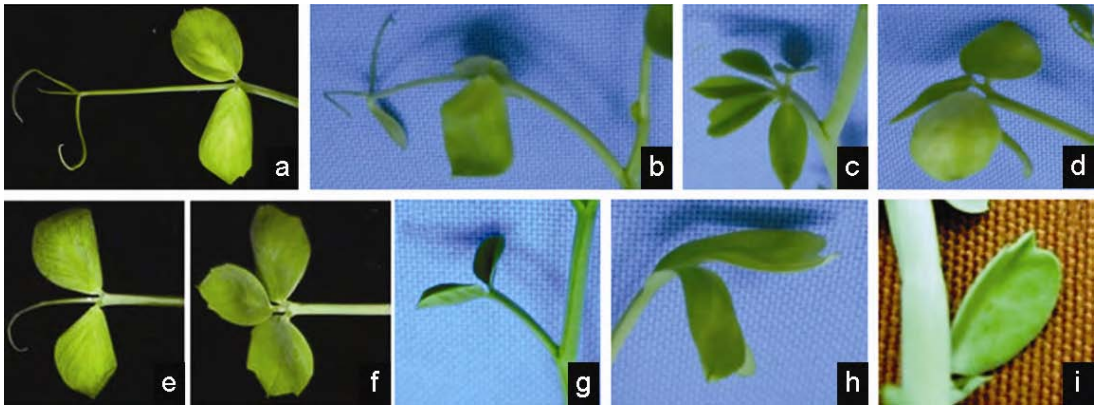

Fig. 3—Leaf morphologies seen on the shoots of *COCH ST* (WT) (a) and *coch st* (b-i) grown *in vitro* in the presence of the auxin transport inhibitor 1-N- naphthylpthalamic acid (NPA). The leaf morphology descriptions and frequencies of their occurrence on WT and *coch st* genotypes are given in Table 4.

Table 5—Effect of *coch st* mutations on the parameters of growth in shoots grown *in vitro* on basal medium

Genotype	Shoot length (cm)	Internode length (cm)	Number of leaves	Number of pinnae in leaf in the form of		Leaf petiole length (cm)	Leaf rachis length (cm)	Leaflet length (cm)	Leaflet width	Tendril length
				leaflets	tendrils	A	B			
COCH ST (WT)	10.0±0.4[a]	0.7±0.02[a]	12.0±0.08[a]	2.0±0.00[a]	2.8±0.03[a]	0.4±0.02[a]	0.2±0.01[a]	0.5±0.03[a]	0.4±0.01[a]	0.4±0.06[a]
coch st	11.8±0.1[b]	1.3±0.08[b]	8.9±0.09[b]	2.7± 0.05[b]	1.8±0.06[b]	0.7±0.03[b]	0.5±0.03[b]	0.8±0.04[b]	0.7±0.03[b]	0.9±0.03[b]

a, b = For a parameter, the values of treatment effects that carry different letters as superscript are different.

The second phenotype of *coch st* phytomeres observed in the present study related to increased size of their leaves. This phenotype has been described earlier for the field grown exstipulate *coch st* plants and in *COCH ST* (WT) plants from which stipules had been surgically removed as soon as they were formed[11,34]. In the present study *coch st* leaves were found to be more than two fold larger in size than *COCH ST* (WT) leaves, in both field grown plants and microcultured shoots. All the suborgans, namely petiole, rachis, leaflets and tendrils were of larger size in *coch st* microcultured shoots as compared to *COCH ST* (WT) shoots. These observations indicated that *coch st* leaf primordia may be larger in size by containing more stem cells than *COCH ST* (WT) leaf primordia. Further, the microcultured *coch st* shoots were taller but comprised of lesser number of phytomeres than the corresponding *COCH ST* (WT) shoots. The field grown *coch st* plants and *COCH ST* (WT) plants from which stipules had been removed surgically have been earlier reported to produce significantly lesser number of nodes than normal *COCH ST* (WT) field grown plants[34,35]. Present observations taken together with the results of surgical

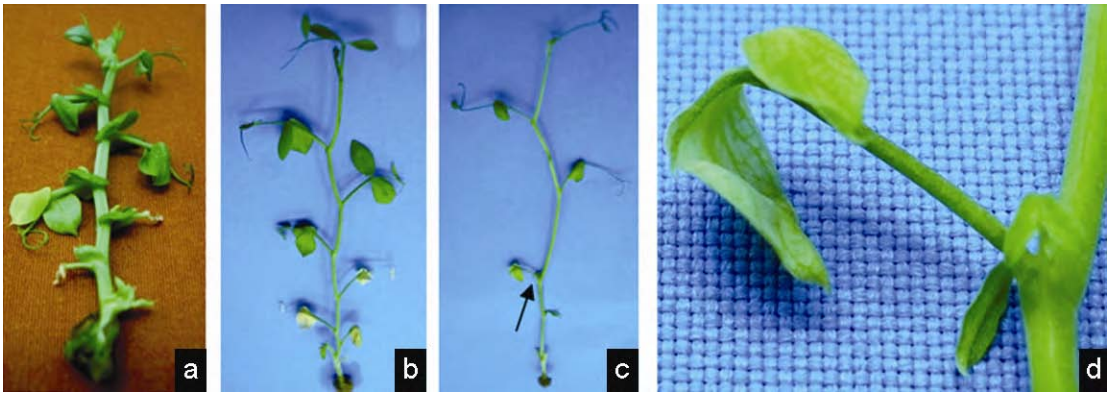

Fig. 4—Induction of stipules by gibberellic acid (GA) treatment on *in vitro* grown shoots of *coch st* genotype, a = Wild type shoot grown on basal medium showing presence of normal stipules on all nodes. b = *coch st* shoot grown on basal medium showing absence of stipules on all nodes. c = *coch st* shoot grown on GA medium showing absence of stipules on all nodes except one node. d = Enlargement of the node from c on which stipules were formed. In the stipule pair, one is simple and other is binnate. The frequency of stipulation following GA treatment was 0.133 ± 0.044.

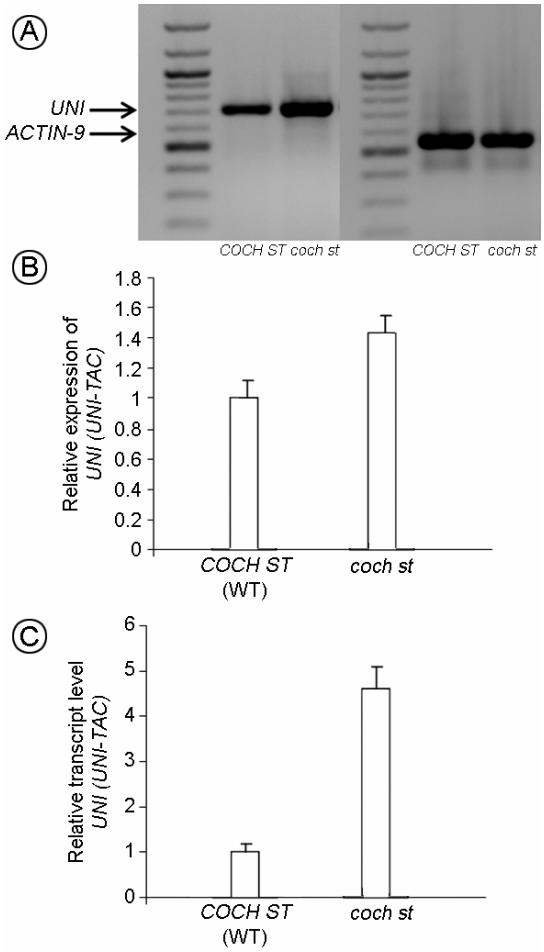

Fig. 5—Transcript levels of *ACTIN-9* and *UNI (UNI-TAC)* genes in the apices of wild type (*COCH ST*) and *coch st* mutant shoots grown *in vitro* on the bed of basal MS medium (salts+vitamins+ kinetin). A=RT-PCR bands of *UNI* and *ACTIN-9* in *COCH ST* (WT) and *coch st* shoots; B=Relative levels of *UNI* transcripts in *COCH ST* (WT) and *coch st* shoots based on A; C=Relative *UNI* transcript levels in *COCH ST* (WT) and *coch st* shoots estimated by use of qRT-PCR.

experiment indicate that there may be inordinately strong feedback stimulation for cellulation and cell expansion in growing leaves that are not associated with stipules. Differentiating leaves in which epidermal, mesophyll parenchymatous and veinal tissues have formed are expected to photosynthesize. It is thought that in *coch st*, the leaf synthesized growth promoting metabolites and signal compounds may be expended for leaf and internode growth in phytomeres already formed, at the expense of growth at apex to separate more phytomeric primordia. This phenomenon in the field grown plants is reflected both in reduced growth at the apex and lower partitioning of biomass to the reproductive organs (pods and seeds)[34,35]. The observed hyper-expression of *UNI* gene in the apices of microcultured *coch st* shoots was in agreement with the larger size of leaves in this genotype as compared to *COCH ST* (WT) genotype.

The novel phenotype of *coch st* phytomeres being reported in this study is high leaflets/tendrils ratio in leaves, observed in both field grown plants and microculured shoots, with and without exogenous supply of GA and NPA. In *COCH ST* (WT) plants, the leaf rachis meristem of adult plants separates three pairs of pinna primordia for leaflets proximal to petiole, four pairs of pinna primordia for leaflets distal to petiole and consumes itself in the formation of apical primodium for the terminal tendril[9,36]. Presumably the pinna primordia for leaflets are allocated more stem cells than the pinna primordia for tendrils. Comparatively the *coch st* leaves possess three pairs of leaflets proximal to petiole and mostly three tendrils in the distal and apical part of leaf. It is thought that in the *coch st*, pinna primordia proximal

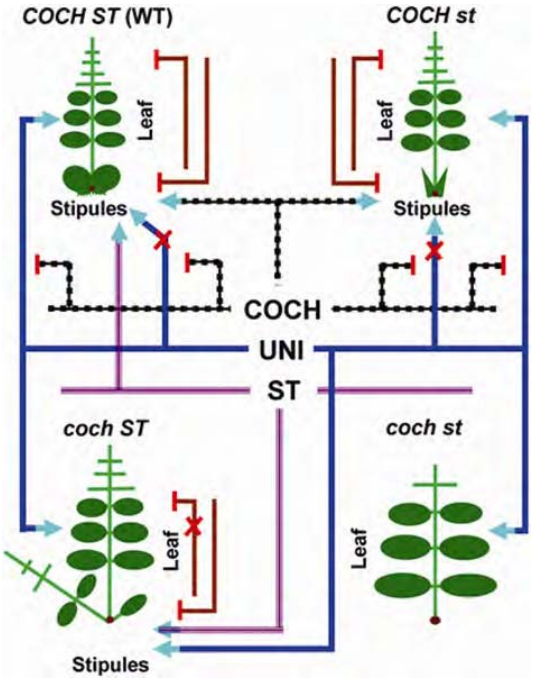

Fig. 6—Diagrammatic representation of the regulatory network for differentiation and growth of leaf and stipules in the phytomeres of *COCH ST* (WT), *coch ST*, *COCH st* and *coch st* genotypes in *P. sativum*, the model plant for lateral organ morphogenesis. The regulatory roles of *COCH*, *ST* and *UNI* genes and photosynthetically produced metabolites in the control of differentiation and expansion of the organs are shown. The roles of COCH, UNI and ST are respectively shown in black, blue and violet colours and that of metabolites in brown. Red bars indicate repression and green arrows activation.

to petiole that are to form leaflets get allocated stem cells in larger numbers than normal. Consequent stem cell depletion in the rachis meristem permits separation of pinna primordia, formed distal to petiole for tendrils, in smaller than normal numbers.

The figure 6 summarizes the *coch st* phytomere phenotype and its significance. The *coch st* mutants do not differentiate their stipule primordia on account of the absence of ST function. Their leaf primordium is larger. The *coch st* primordia for leaflets are abnormally larger, therefore primordia for tendrils are formed in lesser number. In the *coch st* leaves the control over photosynthetic/metabolite feedback for leaf growth is missing in the absence of stipules.

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