COCHLEATA controls leaf size and secondary inflorescence architecture via negative regulation of *UNIFOLIATA* (*LEAFY* ortholog) gene in garden pea *Pisum sativum*

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UNIFOLIATA [(*UNI*) or *UNIFOLIATA-TENDRILLED ACACIA* (*UNI-TAC*)] expression is known to be negatively regulated by *COCHLEATA* (*COCH*) in the differentiating stipules and flowers of *Pisum sativum*. In this study, additional roles of *UNI* and *COCH* in *P. sativum* were investigated. Comparative phenotyping revealed pleiotropic differences between *COCH* (*UNI-TAC* and *uni-tac*) and *coch* (*UNI-TAC* and *uni-tac*) genotypes of common genetic background. Secondary inflorescences were bracteole-less and bracteolated in *COCH* and *coch* genotypes, respectively. In comparison to the leaves and corresponding sub-organs and tissues produced on *COCH* plants, *coch* plants produced leaves of 1.5-fold higher biomass, 1.5-fold broader petioles and leaflets that were 1.8-fold larger in span and 1.2-fold dorso-ventrally thicker. *coch* leaflets possessed epidermal cells 1.3-fold larger in number and size, 1.4-fold larger spongy parenchyma cells and primary vascular bundles with 1.2-fold larger diameter . The transcript levels of *UNI* were at least 2-fold higher in *coch* leaves and secondary inflorescences than the corresponding *COCH* organs. It was concluded that *COCH* negatively regulated *UNI* in the differentiating leaves and secondary inflorescences and thereby controlled their sizes and/or structures. It was also surmised that *COCH* and *UNI* (*LFY* homolog) occur together widely in stipulate flowering plants.

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1. Introduction

Generally, in flowering plant species, competence for photosynthesis and reproduction is provided by lateral organs formed on the stem nodes. In the course of their life cycle, plants produce several types of lateral organs that differ in structure and function (Steeves and Sussex 1989; Poethig 2003); these arise from primordia that are separated in a regulated manner from the periphery of a mass of undifferentiated and proliferative cells within the stem-borne shoot apical meristem (Moyroud *et al*. 2009). In the vegetative phase of life cycle, the principal lateral organs formed are leaves and meristems for branches in the axils of leaves. After the onset of flowering, flowers or flower-bearing secondary inflorescences are formed on the nodes of primary inflorescence stem directly or in the axils of leaves (or leaf-like bracts). Some plant species produce a pair of stipules, in addition to leaf, on each of their vegetative and flowering nodes (Tyler 1897; Bell and Bryan 2008). In view of the dependence of plant's fitness on lateral organs, unraveling of the regulatory mechanisms that underlie the formation of various types of lateral organs is fundamental, especially in terms of applications in plant breeding. The regulation of lateral organ development has been studied in several models and many other plant species. These studies have revealed that the transcription factor *LEAFY* (*LFY*), which is ubiquitously present in one or very few copies in angiosperms (Kelly *et al*. 1995; Weigel and Nilsson 1995; William *et al*. 2004; Maizel *et al*. 2005; Shistukawa *et al*. 2006; Benlloch *et al*. 2007; Bosch *et al*. 2008; Ma *et al*.

Keywords. Bracteole differentiation; *COCHLEATA* gene; garden pea; leaf differentiation; *LEAFY* ortholog; leaf size; *Pisum sativum*; secondary inflorescence; stub histology; *UNIFOLIATA* gene; *UNIFOLIATA* repression; *UNIFOLIATA-TENDRILLED ACACIA* gene

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2008; Moyroud *et al*. 2009 and 2010; Prenner *et al*. 2010; Sooda *et al*. 2011; Liu *et al*. 2011; Hirai *et al*. 2012; Jinghua *et al*. 2012), is the principal determinant of meristematic activity in lateral organs such as flower, branched inflorescence and compound leaf (Weigel and Nilsson 1995; Kelly *et al*. 1995; Molinero-Rosales *et al*. 1999; Bomblies *et al*. 2003; Rao *et al*. 2008; Souer *et al*. 2008; Wang *et al*. 2008). Progress in the analysis of genetic circuitry involving *LFY* in the model plant *Arabidopsis thaliana* has led to the identification of genes that are present upstream and downstream of *LFY* and which work in concert with *LFY* in the process of flower development (Liu *et al*. 2009; Yamaguchi *et al*. 2009; Huijser and Schmid 2011; Moyroud *et al*. 2011; Winter *et al*. 2011). *Pisum sativum* and related fabaceae plants are proving to be useful models for the characterization of *LFY*-related gene network involved in the regulation of flower, secondary inflorescence, compound leaf and stipule (Hofer *et al*. 1997; Gourlay *et al*. 2000; Fawole 2001; Yaxley *et al*. 2001; Prajapati and Kumar 2002; DeMason 2005; Wang *et al*. 2008; Mishra *et al*. 2009; Kumar *et al*. 2009a and 2010; Kumar *et al*. 2011).

The *A. thaliana* shoot comprises indeterminate racemose inflorescence of bractless flowers, subtended by a small number of cauline leaves that possess primary stem like racemose inflorescence in their axils (secondary branches) and root proximal phytomers that bear rosette leaves. Flowering is induced by environmental and endogenous cues that enable high level expression of *LFY* in the flank of shoot apical meristem; thereupon floral meristem and, subsequently, floral primordium get produced (Jack 2004; Krizek and Fletcher 2005; Michaels *et al*. 2005; Parcy 2005; Kobayashi and Weigel 2007). *LFY* is expressed at low levels in rosette leaves (Weigel *et al*. 1992; Blazquez *et al*. 1997; Hempel *et al*. 1997). However, post flowering, *LFY* expression promotes production of secondary branches from the axils of cauline leaves (Blazquez *et al*. 1997; Benlloch *et al*. 2007; Moyroud *et al*. 2010). In the floral meristem, *LFY* is directly activated by the microRNA-targeted SBP-box transcription factor SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3). The MADS-box transcription factors *APETALA 1* (*AP1*) and *FRUITFUL* (*FUL*; *AP1* paralog) are also activated in parallel by *SPL3*. LFY protein directly promotes expression of the transcription factor *APETALA1* (*AP1*) and its redundant homolog *CAULIFLOWER* (*CAL*; *AP1* paralog) (Parcy *et al*. 1998; Wagner *et al*. 1999; William *et al*. 2004; Pastore *et al*. 2011). Thereafter, LFY is maintained at high level in the flower primordium by activation of *LFY* transcription by AP1 and CAL (Bowman *et al*. 1993; Liljegren *et al*. 1999). Besides, several other MADS-box genes, *AGAMOUS-LIKE 24* (*AGL24*), *SHORT VEGETATIVE PHASE* (*SVP*) and *SUPPRESSOR OF CONSTANS 1* (*SOC1*), in an overlapping manner regulate transcription of their targets in flower primordium. These repress *SEP3* in the emerging floral meristems (Liu *et al*. 2008 and 2009). *AP1*, *UNUSUAL FLORAL ORGANS* (*UFO*) and *SEPALLATA 3* (*SEP3*) products form functional complex(es) with LFY (Lee *et al*. 1997; Ng and Yanofsky 2001; Irish 2010; Winter *et al*. 2011). *AP1* and *SEP3* expression is directly induced by *LFY* in flower primordia. *LFY* and *SEP3* together induce *APETALA3* (*AP3*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) transcription factor genes that specify the identity of reproductive/flower organs (Liu *et al*. 2009). The *svp agl24 soc1* triple mutants produce flowers in which chimeric organs are present, the number of organs being less than normal and each flower is subtended by a bract (Liu *et al*. 2009). *LFY* also participates in the regulation of flower's pedicel length; LFY acts in pedicel development by directly activating the *ASYMMETRIC LEAVES 2* transcription factor, which in turn represses the KNOX gene *BREVIPEDICELLUS* (Yamaguchi *et al*. 2012)

Although the floral primordium contains a floral meristem and a bract meristem, the normal flower is not supported by a bract because of the suppression of its development by the combined action of several genes, *LFY*, *UFO*, *PUCHI* and redundant *BLADE-ON-PETIOLE 1* and *2* (Levin and Meyerowitz 1995; Wilkinson and Haughn 1995; Hepworth *et al*. 2005 and 2006; Karim *et al*. 2009). A cryptic bract is produced on flowering nodes in *puchi* and *ufo* mutants (Karim *et al*. 2009). The *lfy*, *ufo* and *puchi* plants have roughly similar plant architectural modification: partial conversion of flowers into branch-like structures, and the constitutive expression of *LFY*, *AP1* or *UFO* is sufficient to convert branches into flowers, indicating that these genes play essential roles in the specification of floral meristem identity (Schultz and Haughn 1991; Huala and Sussex 1992; Mandel *et al*. 1992; Weigel *et al*. 1992; Bowman *et al*. 1993; Weigel and Nilsson 1995; Parcy *et al*. 1998; Hepworth *et al*. 2005, Blazquez *et al*. 2006; Kobayashi and Weigel 2007; Karim *et al*. 2009). *EMBRYONIC FLOWER 1* (*EMF1*), which prevents premature activation of floral homeotic genes such as *AP3*, *AG* and *PI*, is directly repressed by *LFY* (Calonje *et al*. 2008). Indeterminacy of the apical inflorescence meristem is maintained by the expression of *TERMINAL FLOWER 1 (TFL1)*. In *tfl1* mutants, inflorescence is determinate; inflorescence has reduced number of flowers and the shoot apex is replaced by a terminal flower (Shannon and Meeks-Wagner 1991; Alvarez *et al*. 1992; Bradley *et al*. 1997; Parcy *et al*. 2002; Hanano and Goto 2011). *LFY* directs the meristematic activity for development of the normal flower by first activating the synthesis of proteins with which it complexes and then acting together with them in regulation of the downstream gene network. *LFY, UFO* and *TFL1* interconnect the regulation of plant's transition from vegetative to reproductive phase, indeterminacy and morphology of inflorescence and flower structure.

Homologs of *LFY*, *UFO* and *TFL1* are present in the genome of *P. sativum*. In *P. sativum*, the shoot in its vegetative phase comprises of phytomers that bear compound leaves of increasing complexity upwards from cotyledons. The leaves formed at the time of onset of flowering are bigger in size and more complex by having up to 15 pinnae. Such a leaf consists of a petiole extended into the rachis, which ends in a tendril (apical domain of leaf). The rachis bears 3 pairs of leaflets towards the petiole (proximal domain of leaf) and 4 pairs of tendrils towards the terminal tendril (distal domain of leaf). On either side of the site of attachment of the petiole to the stem, a foliaceous (simple) stipule is attached directly to the stem. The inflorescence is indeterminate raceme. Each node of raceme bears two stipules, a leaf and a secondary inflorescence in the axil of leaf. The stipule axils are barren. The secondary inflorescence consists of two flowers and ends into a stub. The secondary and higher order branches reiterate the structure of the reproductive phase primary shoot. *UNIFOLIATA* [(*UNI*) synonymously called *UNIFOLIATA-TENDRILLED ACACIA* (*UNI-TAC*)], ortholog of the *A. thaliana LFY* gene, and *STAMINA-PISTILLOIDA* (*STP*), ortholog of *UFO* of *A. thaliana*, are essential for the flowering and normal determinate development of compound leaf and flower (Monti and Devreux 1969; Hofer *et al*. 1997; Ferrandiz *et al*. 1999; Gourlay *et al*. 2000; Taylor *et al*. 2001; Yaxley *et al*. 2001). The *uni* (*loss-of-function* null) mutants produce leaves of single pinna (Lamprecht 1933; Hofer *et al*. 1997; DeMason and Schmidt 2001). The *uni-tac* mutants (hypomorphic allele of *UNI*) and *stp* (null) mutants produce leaves of reduced complexity; in *uni-tac* mutant the most complex leaf has three pairs of leaflets in the proximal domain, two pairs of leaflets in the distal domain and a leaflet and not a tendril in the apical domain (Sharma and Kumar 1981; Marx 1986; DeMason and Schmidt 2001; Prajapati and Kumar 2002; DeMason and Chawla 2004a and b; DeMason 2005; Mishra *et al.* 2009; Kumar *et al*. 2010). In the loss-of-function *uni*- and *stp*- plants, the flowers are sterile by possessing supernumerary carpels and lacking petals and stamens and produce supernumerary flowers in the axils of sepals (Hofer *et al*. 1997; Ferrandiz *et al*. 1999). The *uni-tac* flowers are fertile, although some of the flowers have less than the typical number of petals and stamens (Kumar *et al*. 2011). These observations mean that *UNI* directs floral development such that all the 4 whorls of organs are formed in flowers. The stipules are of normal morphology in *uni* (null), *uni-tac* and *stp* plants. The *DETERMINATE* (*DET*), ortholog of *UFO* of *A. thaliana*, acts to maintain indeterminacy of apical meristem during reproductive phase; in *det* mutants the inflorescence turns determinate (Swiecicki 1987; Singer *et al*. 1999; Foucher *et al*. 2003). Mutants of *unifoliata* leaf morphology are also known in the leguminous plants *Phaseolus vulgaris* (Myers and Basett 1993), *Vigna unguiculata* (Fawole 2001) and *Medicago truncatula* (Wang *et al*. 2008).

Besides *UNI* (*UNI-TAC*) and *STP*, several other genes such as *AFILA* (*AF*), *INSECATUS* (*INS*), *MULTIFOLIATE-PINNA* (*MFP*) and *TENDRIL–LESS* (*TL*) (de Vilmorin and Bateson 1911; Lamprecht 1959; Goldenberg 1965; Hofer *et al*. 2001; Smirnova 2002; Kumar *et al*. 2004) control meristematic activity in the rachis primordium and subprimordia for pinnae in the compound leaf differentiation in *P. sativum*. *UNI* gene mediates meristematic activity that grows rachis in proximodistal and mediolateral directions (Hofer *et al*. 1997; DeMason and Schmidt 2001; Gourlay *et al*. 2000; Yaxley *et al*. 2001; Prajapati and Kumar 2002; DeMason 2005; Mishra *et al*. 2009; Kumar *et al*. 2010); these activities are respectively made determinate by *AF* and *INS* and *TL* and *MFP* (DeMason and Schmidt 2001; DeMason and Chawla 2004a and b; Mishra *et al*. 2009; Kumar *et al*. 2010). The proximal, distal and terminal domains of leaf are delimited by interactions among *AF*, *INS*, *MFP* and *TL* (Mishra *et al*. 2009; Kumar *et al*. 2010). The nature of genetic interactions between *uni-tac, af, ins, mfp* and *tl* have indicated that *UNI* is an activator for all the other genes, of which *TL* has been observed to encode a transcription factor (Hofer *et al*., 2009). Stipule differentiation is regulated by *COCHLEATA* (*COCH*) and *STIPULE-REDUCED* (*ST*) (Pellew and Sverdrup 1923; Blixt 1967; Nougarede and Rondet 1973; Sharma 1981; Marx 1987; Gourlay *et al*. 2000; Yaxley *et al*. 2001; Kumar *et al*. 2009b). *COCH* is involved in the initiation of simple stipule; *ST* and *COCH* synergistically promote stipule differentiation (Kumar *et al*. 2009b). The stipule is simple and of small size in *st* (loss of function) mutants (Pellew and Sverdrup 1923). Many stipules are leaf-like in *coch* (loss of function) mutants (Marx, 1987; Gourlay *et al*. 2000; Yaxley *et al*. 2001; Kumar *et al*. 2009b). *UNI* directs leaf development and stipule development when COCH function has been lost as in *coch* mutants. *UNI* (*UNI-TAC*) expression is negatively regulated by *COCH* in stipule primordia (Gourlay *et al*. 2000). *COCH* stipules are simple because of the repression of *UNI*, *STP*, *AF*, *INS*, *MFP* and *TL*-pathway of compound leaf differentiation in the stipule domain (Gourlay *et al*. 2000; Kumar *et al*. 2009b, 2010). Many leguminous and non-leguminous plants are known to bear stipules that are ochreate, intrapetiolar, opposite and inter-petiolar (Kumar *et al.* 2012). All these kinds of stipules were observed in the shoots of *COCH ST* grown *in vitro* in the presence of the auxin transport inhibitor 1-*N*-naphthylphthalamic acid (Kumar *et al.* 2012). The flowers of *st* mutants are fertile and normal while those of *coch* mutants are grossly defective, much like in *uni* (null) mutants (Pellew and Sverdrup 1923; Yaxley *et al*. 2001; Kumar *et al*. 2011). The *coch* flowers are poorly fertile because of the presence of supernumerary organs in one or more floral whorls (Yaxley *et al*. 2001; Kumar *et al*. 2011).

The effects are partially rescued by *uni-tac* mutation in *coch uni-tac* double mutants (Kumar *et al*. 2011). Optimum expression of *UNI*-imparted meristematic activity in flower primordium achieved by the negative control of *COCH* on *UNI* is responsible for the normal differentiation of flowers in *P. sativum* (Kumar *et al*. 2011). Thus, in *P. sativum*, the developmental regulation of flowering, primary inflorescence architecture, and flower, stipule and leaf morphogenesis is interconnected by *UNI*, *STP*, *DET* and *COCH* and in the vegetative phase *UNI*, *STP* and *COCH* interconnect stipule and leaf morphogenesis.

Like in *P. sativum*, expression of *LFY* or its homologs has been observed in primordia of organs other than flowers in different plant species. *LFY* expression has been observed in the inflorescence axis of *Ionopsidium acaule* (Bosch *et al*. 2008). The expression of *LFY* homolog *REL* has been shown in spikelets and the panicle bearing them (Rao *et al*. 2008). *DFL* the homolog of *LFY* in *Dendranthema lavandulifolium* has been reported to express in both single flowers and whole inflorescence (Ma *et al*. 2008). These and the facts about the role of *LFY* in the flower and pedicel development in *A. thaliana* and the roles of *UNI* and *COCH* in *P. sativum* enumerated above have led us to hypothesize the following. In *P. sativum COCH* down-regulates *UNI* expression in primordia of leaves as well as secondary inflorescences, in addition to that in stipules and flowers. Or, *COCH* negatively regulates *UNI* in all the lateral organs: stipules, leaves and bracteole and flower bearing secondary inflorescences. We report here the results of quantitative comparisons of the biomass of various organs, morphology of secondary inflorescence, histology of leaf and *UNI* transcript levels of differentiating secondary inflorescences and leaves among *UNI-TAC COCH*, *UNI-TAC coch* and/or *uni-tac COCH* and *uni-tac coch* genotypes. The results demonstrate that *COCH* controls/regulates leaf size and secondary inflorescence complexity via repressive effect on *UNI*-led differentiation of compound leaf and secondary inflorescence.

2. Materials and methods

The homozygous genotypes *UNI-TAC COCH* and *uni-tac COCH* were already available in the genetic background of SKP1 (Kumar *et al*. 2004). SKP1 bears wild-type leaf and stipules and has been used in our work as a parent in crosses. These were crossed with *coch* homozygous line A109 (Sharma 1981) carrying a null (loss-of-function) *coch* allele of Blixt (1972) collection. The *uni-tac coch* and *UNI-TAC coch* F_2 segregants were backcrossed to the parental lines for three generations to obtain *uni-tac coch* and *UNI-TAC coch* homozygous lines. It is to be noted that *uni-tac* mutation in *UNI* gene is hypomorphic (causes partial loss-of-activity/function on account of lower expression of gene) unlike amorphy (total loss-of-function) caused by *uni*-null alleles in *UNI* gene (Sharma and Kumar 1981; Hofer *et al*. 1997; Gourlay *et al*. 2000; DeMason and Schmidt 2001; DeMason and Villani 2001; Prajapati and Kumar 2002; DeMason and Chawla 2004a, b). The phenotypes of stipules and leaves of *COCH UNI-TAC*, *COCH uni-tac*, *coch UNI-TAC* and *coch uni-tac* are shown in the figure 1.

The plant growth in genotypes was characterized by measuring the dry weight of organs, since dry biomass is a standard/stable measure of the sizes of a whole plant and its individual organs. For their characterization, the genotypes were grown in the experimental farm of our institute at New Delhi during

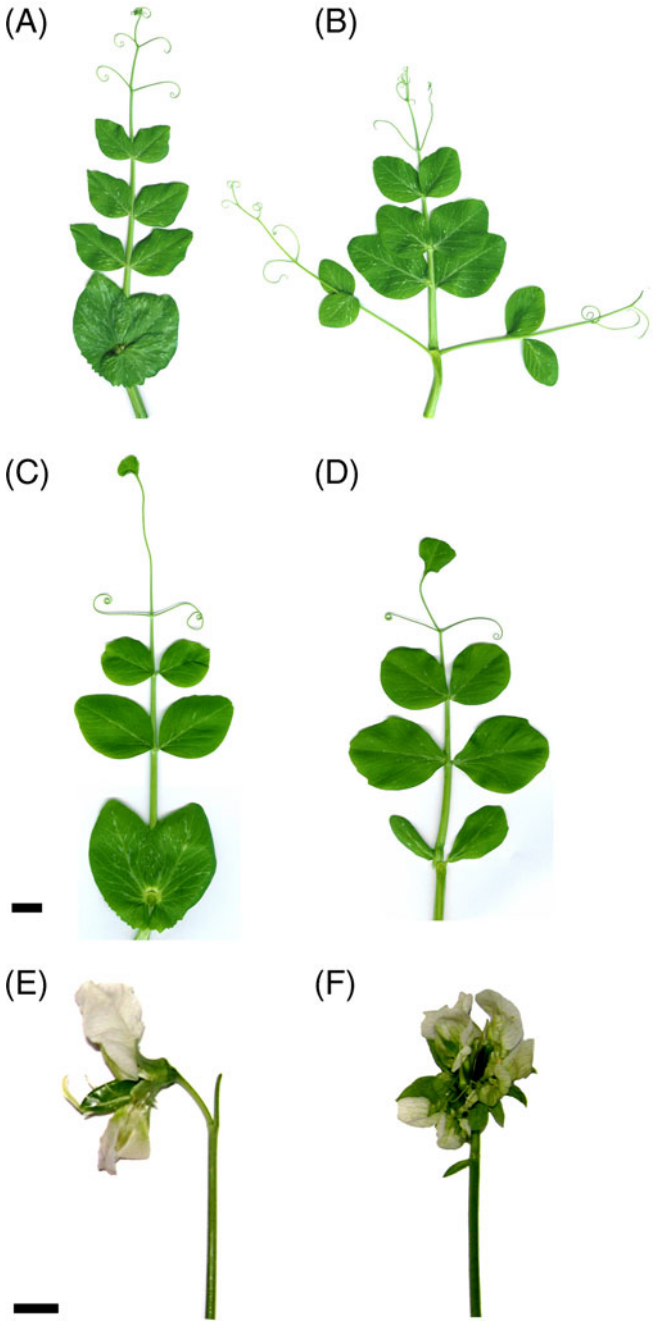

Figure 1. Morphologies of stipules and leaves of the homozygous *COCH UNI-TAC* (**A**), *coch UNI-TAC* (**B**), *COCH uni-tac* (**C**) and *coch uni-tac* (**D**) and flower-bearing segments of secondary inflorescences of *COCH UNI-TAC* (**E**) and *coch UNI-TAC* (**F**) plants of *Pisum sativum*. The leaves were taken from the first flowering node of each genotype. The *COCH* stipule pairs (**A** and **C**) demonstrate their wild-type structure. The stipules are attached to the stem, one on either side of the site of attachment of leaf petiole to the stem node. They peltately overlap each other around the stem node. Each stipule has a foliaceous sessile lamina which is entire on the side proximal to leaf and is lobed and toothed on the opposite side. Unlike leaflets, which have one primary vein, the stipule has several primary veins. The mutant *coch* stipules are compound leaf-like in *coch UNI-TAC* genotype (**B**). They are less complex in architecture than their counterparts. The mutant *coch* stipules are simple sessile and of small size with one primary vein in *coch uni-tac* genotype (**D**). The *UNI-TAC* leaves (**A** and **B**) demonstrate their wild-type structure. The leaf petiole extends into the rachis, which bears three pairs of leaflets proximal to petiole, three pairs of tendrils distal to petiole and an apical tendril. The mutant *uni-tac* leaves (**C** and **D**) demonstrate lower level of complexity than *UNI-TAC* leaves. Their rachis bears two pairs of proximal leaflets, one pair of distal tendrils and a terminal leaflet. The leaf of *COCH UNI-TAC* is smaller than that of *coch UNI-TAC*, and leaf of *COCH uni-tac* is relatively smaller than that of *coch uni-tac*. The pedicel of *COCH UNI-TAC* has on it only one bilaterally symmetrical flower in which only one carpel/developing pod is present (**E**). The majority of pedicels bear one flower. Occasional pedicels bear more than one flower. On the pedicel of *coch UNI-TAC* shown here three flowers are present, each has a carpel; the main flower has supernumerary and chimeric organs (**F**). The scale bar of (**A**–**D**)=2 cm and for (**E**) and (**F**) =1 cm.

winter seasons (October/November–March/April) of the years 2009 to 2012. The field plot was solarized before use and has sandy loam soil. The seed storage and crop cultivation and protection conditions standardized earlier were used (Kumar and Sharma 1986; Prajapati and Kumar 2002; Mishra *et al*. 2009; Kumar *et al*. 2009a, b). Ten seeds were sown in a 1-m-long row, and row-to-row distance was 75 cm. The leaf size of the genotypes was studied during the 2009–2010 season. The genotype rows were replicated five times and all the rows were completely randomized, using the random number tables (Cochran and Cox 1992). When the crop was 14 weeks of age, 5 plants per genotype per replication were uprooted as samples. Each uprooted plant was scored for the number of nodes. Then the plants were pooled genotype- and replication-wise. Root, stem, stipules, leaves and pods (along with pedicels) were separated, placed in paper bags, heated at 80°C for 90 min were then dried at 37°C; before placing them in bags, the roots had been washed with running water to remove the adhering soil. Since the organs/sub-organs, including leaves, stipules, secondary inflorescence stem (stalk)-borne pods, varied in their size over nodes, average dry weight of each type of organ was considered a reliable measure of organ size in genotypes. The dried materials in 200 paper packets were weighed. The data were statistically analysed to obtain genotype-wise estimates of mean and standard error. Variance analysis as per completely randomized design was used to test the significance of differences between genotype means and means of sets of genotypes and to obtain critical difference estimates for the differentiation of significantly different genotypes (Cochran and Cox 1992). In the 2010–2011 season, plants of the four genotypes were sampled for the sixth leaf from cotyledons for histological examination of petiole at a site most proximal to leaflets and the leaflets most proximal to petiole. The genotypes were also surveyed for recording observations on the secondary inflorescence features, in the 2010–2011 and 2011–2012 seasons. For each genotype, 10 plants were sampled for morphological-cum-quantitative examination and 3 to 10 plants were sampled for histological examination of appendages, other than flowers, borne on secondary inflorescence axis.

The plant organs sampled for histological examination were fixed in acetic acid : alcohol 1 : 3 and transferred to 70% alcohol. The organs were cleared by incubation at 90°C for 15 min to 1 h in phenol : lactic acid : glycerol : water 1 : 1 : 1 : 1 mixture and stored in 20% glycerol. Cleared organs were stained with dilute safranine (20%), washed in 5% alcohol and mounted in 25% glycerol on slides and examined microscopically at 40, 100 and/or 400× magnification using Nikon E100 microscope. The qualitative observations were recorded, and for quantitative description a Nikon 8400 digital camera was used to take pictures of cleared leaflets, sections of petiole and leaflets and a micrometer, which were printed on millimeter graph papers; cell sizes were arrived at by counting the square millimeters covered by their images on graph paper and dividing by the concerned magnification factor. The leaflet area was estimated by scanning it on a graph paper using the hp psc 750 scanner. For their sectioning, organs were held between splits of radish and sectioned transversely using hand-held razor. The safranine (2%)-stained sections were microscopically examined for qualitative and quantitative observations as described above.

UNI mRNA expression was measured semiquantitatively by reverse transcription (RT) polymerase chain reaction (PCR) and/or quantitatively by quantitative (q) RT-PCR. Three sets of experiments were carried out. In an experiment, biologically replicated three times, *UNI* expression was measured in leaves taken from two or three apical nodes of vegetative phase plants of two genotypes. In the second experiment biologically replicated twice, the *UNI* expression was measured, in shoots apices comprising 2 or 3 apical nodes from which flower buds had been dissected out, in four genotypes. Thirdly, the *UNI* expression was measured, separately in leaves and whole secondary inflorescences taken from the top 2 or 3 nodes, in two genotypes. Each sample comprised specific organ(s)/sub-organ(s) taken from 5 plants of a genotype and frozen in liquid nitrogen. Total RNA was isolated sample-wise from the frozen material using the RNeasy

plant mini Kit (QIAGEN). The process involved treatment of RNA with DNAse on the mini spin column according to the manufacturer's instructions to eliminate any contaminating genomic DNA. The RNA was quantified by Nanodrop-1000 spectrophotometer, version 3.7.0, and run on 1.5% agarose gel made in 10X MOPS [3-(*N*-morpholino) propane sulphonic acid] buffer with formaldehyde. Reaction mixture of RNA was made in 10X MOPS buffer with formaldehyde, formamide and ethidium bromide (EtBr). First-strand cDNAs were generated using a 20 µL reaction volume containing 4.5 µg total RNA, 1 µL (200 U) RevertAid™ H Minus M-MuLV Reverse Transcriptase, 4 µL 5X reaction buffer, 2 µL 10 mM dNTP mix, 1 µL oligo$(dT)_{18}$ primer, and 20 U (1 µL) Ribolock™ RNase inhibitor (FERMENTAS). Synthesis time for incubation was 1 h at 42°C and for termination it was 5 min at 70°C.

For semiquantitative PCR analysis, PCR was performed using a 25 µL reaction containing 2.5 µL 10X reaction buffer with $MgCl_2$, 0.25 µL (0.05 U/µL) Taq DNA polymerase (Sigma-Aldrich), 0.2 mM dNTP, 5 pM of each specific primer. The PCR reaction parameters were: one 5 min cycle of initial denaturation at 95°C, 35 cycles of 30 s at 94°C, 30 s annealing at 59°C and 1 min elongation at 72°C and final extension for 10 min at 72°C. PCR product was resolved on 0.8% agarose gels in 1X TBE buffer containing 0.5 µL/ mL EtBr and quantified in 1D analysis by using Vision Works Image TM acquisition LS and Analysis Software in GelDoc-it^{TM} Imaging System (UVP, United Kingdom). The primers used were: *UNI* gene (Gene Bank accession no. AF010190.2), UNI-F: 5′-CTACGCGGTTACCCCTACAA-3′, UNI-R; 5′-ATTTCTCACCGCGCTCTTTA-3′; ACTIN 9 gene (Gene Bank accession no. U81047.1), ACTIN-F: 5′-ATGGTTGGAATGGGACAAAA-3′, and ACTIN-R: 5′-GCAGTTTCCAACTCCTGCTC-3′. These were designed using the Primer 3 output software.

The primers used in the qRT-PCR were: ACTIN 9-F: 5′-TTGTAGCACCACCAGAGAGG-3′ and ACTIN 9-R: 5′-TTGCAATCCACATCTGTTGG-3′; UNI-F: 5′-CAACCGCCCCGATG-3′ and UNI-R 5′-CCTCCAAGCCTCCTAGTTCTCTT-3′. These were designed using the primer express, version 3 software of ABI (Life Technologies, formerly Applied Biosystem, USA). qRT-PCR was performed in a total volume of 20 µL with 20 ng (2 µL) of cDNA, 6.2 µL DEPC (diethyl pyrocarbonate, Sigma) treated water, 900 nM gene specific primers (0.9 µL each) and 10 µL KAPA™ SYBER Fast qPCR Master Mix (2x) ABI Prism™ on Step one Real Time PCR Detection System (Life Technologies, USA) according to manufacturer's instructions. All primers were annealed at 60°C and run for 42 cycles. The pea *ACTIN 9* gene served as the internal control. The determination of PCR efficiency and calculation of mRNA transcript levels were done using the Step ONE Version 1.O (ABI System). The relative expression levels of *UNI* between genotypes/organs were compared by calculating the relative quantity values (RQ) by use of comparative Ct method also referred to as the $2^{-\Delta\Delta C}{}_{T}$ method (Schmittgen and Livak 2008). There were two qRT-PCR replications for each sample.

3. Results

3.1 *COCH leaves are smaller than coch leaves*

The *uni-tac* mutation in *UNI* (*UNI-TAC*) is proven to decrease the transcription of *UNI* and consequently reduce the availability of UNI protein. As a result, the compound leaves of *uni-tac* plants are less complex in architecture than those of *UNI-TAC* plants (figure 1), at all the nodes. Further, the interactions between *uni-tac*, *af*, *ins*, *tl* and *mfp* mutations have shown that growth is promoted by UNI in both proximodistal and mediolateral directions of the compound *UNI-TAC* leaves, in leaflets of the petiole-proximal domain, tendrils of the domain distal to petiole and tendril of the apical domain. The dry weight of whole leaf is a stable measure of size/growth in all the three domains of leaf. Therefore, the effect of *coch* mutation on leaf growth was studied by comparing the size (dry weight/biomass) of leaf in *UNI-TAC* and *uni-tac* genotypes. Since leaves demonstrate heteroblasty, average dry weight of leaves borne on a plant was treated as a reliable estimate of leaf growth on the plant. The average dry weight of leaf was arrived at by dividing the total dry weight of all leaves by the total number of nodes or leaves. To compare the biomass accumulation in leaves versus that in other organs, the corresponding dry weights of root, stem, stipules and inflorescences (inflorescence stem or stalk, pedicels, bracteoles and developing and mature pods=pods) were also estimated. Table 1 gives primary observations and calculated dry weights of a leaf and a stipule pair genotype-wise, group-wise for *COCH* and *coch* genotypes and for all the genotypes together. It is seen that the studied genotypes accumulated biomass in the organs of their single plants on average basis as follows: 0.19 g in root system, 5.36 g in stem, 1.22 g in stipules, 4.95 g in leaves and 10.88 g in pods or respectively 0.9%, 23.7%, 5.4%, 21.9% and 48.1% of the total dry matter (22.6 g). Plants of all the genotypes accumulated about the same amount of total biomass (the differences were statistically insignificant). The variation between the genotypes for the stipules, stem and pod biomass and node number per plant was significant but that for the biomass of leaves per plant was not significant. In respect of the node number, stipules biomass and biomass of stem, *COCH* genotypes demonstrated significantly higher level of growth than *coch* genotypes. The stipules biomass of *COCH* plants was 4.3-fold higher than that of *coch* plants. The node number and

Table 1. The *cochleata* (*coch*) mutation increases leaf size (dry weight) in *Pisum sativum*: Effect of interactions between *COCH* and*UNIFOLIATA-TENDRILLED ACACIA* [(*UNI-TAC* or *UNIFOLIATA* (*UNI*)] on leaf biomass relative to that on biomass of other plant organs

Serial no.	Genotype		No. of nodes	Weight[e] (g)						
	COCH	*UNI-TAC*		Whole plant	Stipules	Leaves	Stem	Pods	One stipule pair	One leaf
1.	+	+	56±3.0[a]	28.08±1.63	2.12±0.12	4.92±0.28	6.94±0.37	13.82±0.96	0.04±0.0004	0.09±0.002
2.	−	+	34±1.7	21.48±1.20	0.38±0.03	4.64±0.37	3.80±0.26	12.50±0.70	0.01±0.001	0.14±0.01
3.	+	−	48±1.7	20.62±1.95	1.84±0.09	4.42±0.43	5.72±0.19	8.46±1.28	0.04±0.001	0.09±0.01
4.	−	−	44±2.1	20.20±1.97	0.54±0.05	5.80±0.41	4.96±0.33	8.74±1.24	0.01±0.001	0.13±0.004
CD $t_{0.01,16}$[b]			9.12	7.11	0.33	1.55	1.22	4.42	0.003	0.02
$F_{3,\ 16}$[c]			16.2**	4.6	121.1**	2.6	20.0**	6.3**	458.6**	20.6**
5. Mean of all genotypes			46±4.6	22.60±1.85	1.22±0.44	4.95±0.30	5.36±0.66	10.88±1.34	0.03±0.01	0.11±0.01
6. Mean of all *COCH* genotypes			52±4.0	24.35±3.73	1.98±0.46	4.67±0.25	6.33±0.61	11.14±2.68	0.04±0	0.09±0
7. Mean of all *coch* genotypes			39±5.0	20.84±0.64	0.46±0.08	5.22±0.58	4.38±0.58	10.62±1.88	0.01±0	0.14±0.005
$F_{1,\ 16}$[d]			32.6**	4.2	355.4**	2.1	43.6**	0.2	1373.9**	61.0**

a=Each value is average of 5 replications and is provided with its standard error.

b=Critical difference (CD) value at 1% level of significance and 16 degrees of freedom (df).

c=F value, ratio of treatment variance at 3 df and error variance at 16 df.

d=F value, ratio of treatment variance at 1 df and error variance at 16 df.

e=Weight of root system=Weight of whole plant-weights of stipules, leaves, stem and pods.

**=Significant at≤1% level.

stem biomass were about 1.4-fold lower in *coch* plants than in *COCH* plants. These observations indicated that higher levels of growth in leaves and pods compensated for lower levels of growth in stem and stipules in *coch* genotypes. Since stipule pairs and leaves are formed on every plant node and node numbers had been recorded, it was possible to calculate for single plants the average biomass values of single stipule pair and single leaf. The biomass of single stipule pair (0.04 g) of *COCH UNI-TAC* and *COCH uni-tac* genotypes was observed to be four fold higher than that (0.01 g) of *coch UNI-TAC* and *coch uni-tac* genotypes. At least two properties of *coch* stipules may account for the observed low biomass of stipule pair in *coch* genotypes. One, stipules formed on *coch uni-tac* are of small size and have simple lamina carrying one primary vein as compared to the proleptic simple but lobed and toothed stipules of larger size having several primary veins in *COCH* genotypes (figure 1A, C and D). Secondly, heterostipulation in *coch* genotypes. Several to many nodes of *coch* plants produce stipules of varying sizes (figure 1C and D); often one stipule is simple and other compound in the nodes of *COCH UNI-TAC* plants (Yaxley *et al.* 2001; Kumar *et al.* 2009b; this study). Contrary to the low stipule pair biomass, the biomass of single leaf (0.14 g) of *coch* genotypes (*coch UNI-TAC* and *coch uni-tac*) was about 1.5-fold higher than the single leaf biomass (0.09 g) in *COCH* genotypes (*COCH UNI-TAC* and *COCH uni-tac*). These results showed that *coch* increased the growth/size/biomass of leaves by a factor of about 1.5 (figure 2). The results also indicated that *COCH* and *coch* differentially affected the growth/size of secondary inflorescences (given the name pods because pod bearing mature plants were investigated here).

3.2 *Increase in size (biomass) is related to increase in cellulation in coch leaves*

Measurements of biomass of stem, leaves and other organs and thereby estimated biomass of single leaf, in single plants of *COCH* and *coch* genotypes reported above, revealed that *coch* plants produced leaves of larger size in smaller numbers as compared to *COCH* plants. To reveal the mechanism (s) of increased biomass in *coch* leaves, the petiole and the largest leaflets of sixth leaf from vegetative phase plants of *coch* and *COCH* genotypes in *UNI-TAC* and *uni-tac* backgrounds were compared for tissue characteristics (table 2; figure 3). The total numbers and areas of cells and stomata in the dorsal epidermis, sizes of palisade and spongy parenchyma cells of mesophyll tissue, number of cell layers between upper (dorsal) epidermis and lower (ventral) epidermis and sizes of vascular bundles of primary (mid-rib) and secondary veins were estimated from cleared whole leaflets as well as transverse sections of leaflets. The cross diameters and cell layer numbers were estimated from transverse sections of petioles. *COCH* and *coch* leaves differed significantly in the following traits: cross-sectional area of petiole, leaflet area, size and number of pavement cells and stomata in adaxial (upper epidermis), adaxial-abaxial thickness and size of spongy mesophyll cells and primary vascular bundles. All these traits were expressed at higher levels in *coch* leaves than in *COCH* leaves. *COCH* and *coch* leaves did not differ significantly in their traits relating to stomata size, number of cell layers in petiole, number of cell layers in the mesophyll palisade and spongy parenchyma of leaflets and size of palisade parenchyma cells and secondary veins in leaflets. The *coch* petioles were 1.45-fold thicker (figure 3E–H) and

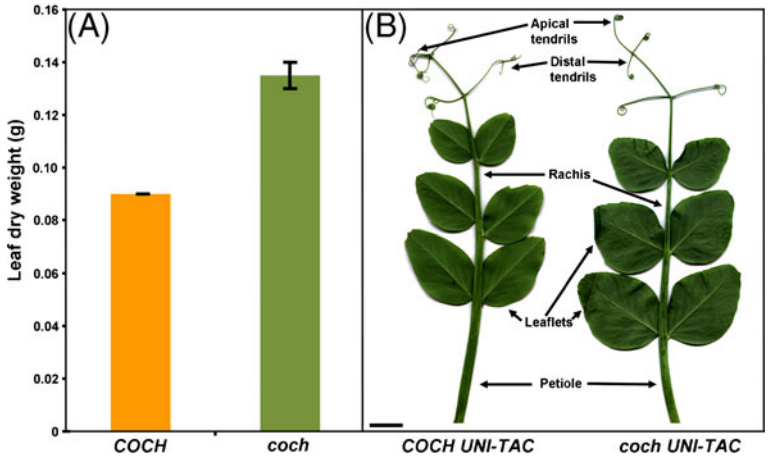

Figure 2. The *cochleata* (*coch*) mutation increases leaf biomass (dry weight) in *Pisum sativum*. (**A**) Average dry weight of leaf in the plants of *COCH* genotypes is smaller than that in plants of *coch* genotypes. (**B**) Leaf of *COCH* genotype is smaller than that of *coch*. The leaf parts tendrils, leaflets, rachis and petiole are labelled. The architecture of *COCH* and *coch* leaves is identical. Scale bar for (**B**)=2 cm.

Table 2. The *cochleata* (*coch*) mutation increases cellulation (cell mass or cell number and size) in leaflets: Effect of interactions between *COCH* and*UNIFOLIATA-TENDRILLED ACACIA*, [(*UNI-TAC* or *UNIFOLIATA* (*UNI*)] on the leaflet histology in *Pisum sativum*

Genotype		Adaxial epidermal cell layer[x]			Stomata in the adaxial epidermis[x]			Leaflet area[x]
COCH	*UNI-TAC*	Size of cell (μm^2) (df=8)[y] (a)	Total number of cells (df=2) (b)	Area occupied by epidermal cells (mm^2) (df=2) (c=a×b)	Size of a stomata (μm^2) (df=8) (d)	Total number of stomata (df=2) (e)	Area occupied by the stomata (mm^2) (df=2) (f=d×e)	mm^2 (df=2) (c+f)
+	+	2998±224	226549±14	679±26	411±13	60864±18	25±5	704±22
-	+	4460±292	336144±10	1499±63	460±20	114432±5	53±3	1552±62
+	-	3227±247	202400±5	653±13	442±15	60720±9	27±3	680±10
-	-	3608±177	236096±18	852±58	431±16	74069±11	32±4	884±54
Mean of all genotypes		3573±321	250297±29480	921±198	436±10	77521.25±12695	34±7	954.97±204
Mean of *COCH* genotypes		3113±114	214475±12076	666±13	426±16	60792±72	26±1	692.11±12
Mean of *coch* genotypes		4034±426	286120±50031	1176±324	446±15	94250±20184	42±10	1217.84±334
t test values (*COCH* vs *coch*)		t_{34}=3.6** (P=0.001)	t_{10}=2.5* (P=0.03)	t_{10}=3.4** (P=0.007)	t_{34}=1.2 (P=0.25)	t_{10}=2.8* (P=0.02)	t_{10}=2.8* (P=0.02)	t_{10}=3.4** (P=0.007)

Genotype		Petiole proximal to first pair of leaflets		Leaflet most proximal to petiole						
				Adaxial-abaxial thickness (μm) of lamina next to		Vascular bundle size (μm^2) of		Palisade mesophyll parenchyma		Spongy mesophyll parenchyma
COCH	*UNI-TAC*	Cross sectional diameter (μm) (df=2)	Number of cell layers (df=2)	Primary vein (df=2)	Secondary vein (df=2)	primary vein (df=2)	secondary vein (df=2)	Number of layers (df=2)	Transectional cell area (df=5)	Number of layers (df=2)	Transectional cell area (df=5)
+	+	1297.9±24.03	8.0±0	60.1±0.72	24.2±0.83	36640.6±1669.0	2492.4±8.5	1±0	1369.3±189.30	6.0±0	188.95±67.01
-	+	1973.4±186.74	7.5±0.5	70.0±0.72	28.8±0.72	44688.2±2435.4	7779.2±4220	1±0	1026.6±69.80	6.5±0.5	230.06±92.18
+	-	1898.4±13.53	7.5±0.5	61.9±2.53	19.6±0.83	28671.9±947.2	5578.4±582	1±0	794.5±67.11	6.0±0	163.38±34.48
-	-	2648.4±22.55	9.0±0	72.1±0.41	25.4±1.82	36093.8±4341.3	6494.1±1402	1±0	970.0±97	7.0±0	266.03±65.53
Mean of all genotypes		1954.6±276.31	8.0±0.4	66.0±2.97	24.5±1.90	36523.8±3272.4	5585.8±1125.7	1±0	1040.25±120.19	6.4±0.2	212.00±22.68
Mean of *COCH* genotypes		1598.2±300.31	7.8±0.3	61.0±0.94	21.9±2.30	32656.5±3985.1	4035.4±1543.2	1±0	1082.12±287.04	6.0±0	176.01±13.00
Mean of *coch* genotypes		2310.9±337.55	8.3±0.8	71.04±1.04	27.1±1.67	40391.3±4297.7	7136.5±642.6	1±0	998.50±28.50	6.8±0.3	248.03±18.02
t test values (*COCH* vs *coch*)		t_{10}=3.3** (P=0.009)	t_{10}=1.3 (P=0.24)	t_{10}=7.3** (P=0.0001)	t_{10}=3.2** (P=0.0095)	t_{10}=2.2* (P=0.05)	t_{10}=1.5 (P=0.18)	NA	t_{22}=0.6 (P=0.6)	t_{10}=0.9 (P=0.40)	t_{22}=2.63* (P=0.015)

X = The observations pertain to the leaflets most proximal to petiole in sixth leaf from cotyledons;

Y = Degrees of freedom (df) on which the standard errors are based.

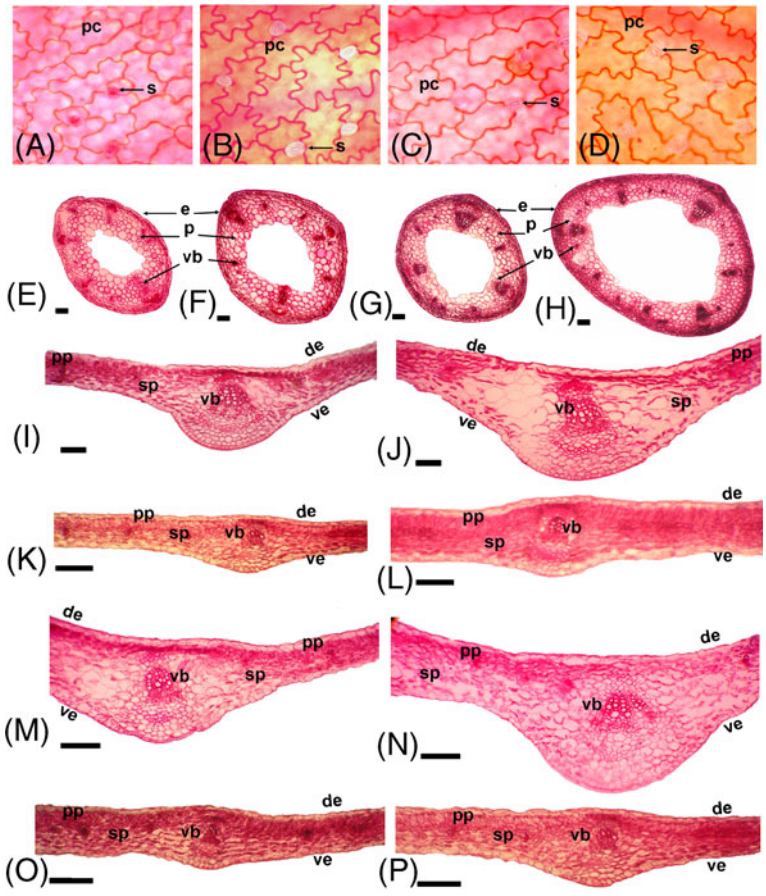

Figure 3. The *cochleata* (*coch*) mutation enhances cellulation in the petioles and leaflets of compound leaves of *Pisum sativum*. The petioles and petiole proximal leaflets of the sixth leaf from cotyledons of *COCH UNI-TAC* (**A**, **E**, **I** and **K**), *coch UNI-TAC* (**B**, **F**, **J** and **L**), *COCH uni-tac* (**C**, **G**, **M** and **O**) and *coch uni-tac* (**D**, **H**, **N** and **P**) plants were compared for their histology. The petioles of *coch* leaves are larger in transverse diameter and therefore contain more cells than the counterpart *COCH* petioles. The *coch* leaflets possess more cells in proximodistal and mediolateral directions as compared to counterpart *COCH* leaflets. The adaxial-abaxial thickness is higher in *coch* leaflets as compared to *COCH* leaflets. The size of primary and secondary vascular bundles is also larger in *coch* leaflets. The adaxial epidermis and mesophyll parenchyma tissues comprise cells larger in size in *coch* mutant leaflets as compared to *COCH* leaflets. (**A**–**D**) Adaxial epidermis consisting of pavement cells (pc) and stomata (s) visualized in cleared leaflets. (**E**–**H**) Transverse section (TS) of petiole adjacent to first pair of leaflets; epidermis (e) and parenchyma (p) and vascular bundles (vb) are seen. (**I**, **J**, **M** and **N**) TSs across central vein in the middle of leaflets; palisade mesophyll parenchyma (pp) is seen below dorsal epidermis (de); spongy mesophyll parenchyma (sp) is seen between pp and ventral epidermis (ve); also seen are a major and minor vascular bundles (vb). (**K**, **L**, **O** and **P**) TSs across secondary vein in the basal region of leaflets; de, ve, pp, sp and vb are seen. The quantitative observations on the various tissues are summarized in table 2. Each of the panel (**A**) to (**D**) covers 0.25 mm^2 of the lamina area of leaflet. Scale bar for (**E**) to (**P**)=200 μm.

leaflets 1.76-fold bigger (figure 1) than the corresponding *COCH* leaf sub-organs. The adaxial (upper) epidermis of *coch* leaflets comprised of 1.33- and 1.55-fold more pavement cells and stomata, respectively, as compared to *COCH* leaflets. The size of epidermal cells in *coch* leaflets was 1.3-fold higher than in *COCH* leaflets. As compared to *COCH* epidermal cells, those in *coch* possessed highly enlarged perimeter (figure 3A–D). The lamina was about 1.2 times thicker in *coch* leaflets because of larger size of spongy parenchyma cells and vascular bundles (figure 3I–P). Altogether, the histological analysis indicated that individual *coch* leaves possessed larger biomass because of elevation in their cellulation (increase in cell number), in comparison to individual *COCH* leaves.

3.3 *COCH inhibits bracteole formation in secondary inflorescence*

The biomass measurements of organs in single plants of *COCH* and *coch* genotypes (table 1) indicated that although the stems of *coch* plants produced lesser number of nodes

than *COCH* plants, the biomass of pods (or inflorescence structures with their appendages, including pods) in *coch* plants was about equal to that in *COCH* plants. These results indicated that *coch* plants produced pod bearing secondary inflorescences of larger biomass than those formed on *COCH* plants. Two major differences were observed between *COCH* and *coch* pod bearing inflorescences. One of these related to the presence of supernumerary pods on *coch* pedicels. Whereas one pod was formed per pedicel in *COCH* plants, two or three carpels/pods were formed on several of the pedicels in *coch* plants (figure 1E and F). In the latter case, the supernumerary pods were partially developed and usually sterile. Occurrence of supernumerary carpels in flowers and flowers on pedicels on *coch* plants has been reported earlier (Yaxley *et al.* 2001; Kumar *et al.* 2011). The second difference between the *COCH* and *coch* secondary inflorescences was novel and related to the presence of a bracteole below the pod-pedicel in *coch* plants, which was invariably absent in *COCH* plants. Therefore, the secondary inflorescences of *COCH* and *coch* genotypes were examined in some detail at their flowering stage of development.

A wild-type secondary inflorescence in the SKP-1 background genotypes had two flowers. From the stem that emerges from the axil of leaf (bract), two alternately placed pedicillate flowers were produced in racemose fashion (figure 4A–E). Post flowering, the inflorescence stem (stalks) ended into a structure called stub (figure 4B and C). Table 3 summarizes quantitative observations on the architectures of secondary inflorescence in *COCH* and *coch* genotypes of *UNI-TAC* and *uni-tac* backgrounds. It was observed that the flowers in the secondary inflorescences of *coch UNI-TAC* and *coch uni-tac* plants were formed on the secondary inflorescence stem in the axils of small petiolated leaf-like structures or bracteoles (figure 4D and E). The bracteoles formed on *coch uni-tac* plants were simple/unipinnate (figure 4D, J and K). The *coch UNI-TAC* plants bore simple or compound bracteoles (figure 4E, L and M); in this genotype the structure-wise proportion of bracteoles was: unipinnate:bipinnate:tripinnate 1:0.37:0.74 (table 3). The middle pinna in the tripinnate bracteoles was often (with ≥90% frequency) tendril-like in morphology (table 3; figure 4M). Histologically, the laminated pinnae of *coch uni-tac* and *coch UNI-TAC* bracteoles were leaflet-like, except the cells of palisade-like morphology were not visible (figure 4T and U). The tendril-like pinnae in the compound bracteoles had tendril-laminate of somewhat compound histology (figure 4V). The stub was observed to be present in about 80% of the *COCH* and 50% of the *coch* secondary inflorescences (table 3). Stubs were present in about 95% of *COCH UNI-TAC* secondary inflorescences (table 3). Variation was observed in the stub size. Stubs of ≥5 mm size were called prominent and of smaller size were called minute. In *COCH UNI-TAC* genotypes 68% of the stubs were prominent; the average size of the stubs was 9.43± 1.23 mm. The frequency of occurrence of stubs in the secondary inflorescences of *coch UNI-TAC*, *COCH uni-tac* and *coch uni-tac* was respectively 45%, 65% and 55%. Besides being less frequent, stubs were largely minute (≤3.00 mm) in these genotypes (table 3). The coefficients of correlation between number of bracteoles on the one hand and frequency of occurrence of stubs or size of stubs on the other were negative and highly significant (table 3). Thus a negative relationship was observed between the presence and prominence of stubs and the bracteole formation. The body of the stub was observed to have stem-like histology (figure 4N, P and R). The stub apex appeared like an aborted shoot meristem (figure 4N, O and Q). The results showed that *coch* mutant plants produced bracteoles on secondary inflorescence. The COCH function blocked the formation of bracteoles.

3.4 *Expression levels of UNI are increased in coch leaves and secondary inflorescences*

The hypothesis that increased *UNI* expression is associated with (a) differentiation of *coch* leaves that are larger in size than *COCH* leaves because of hyper-cellulation and possession of bigger cells in them, (b) formation of bracteoles on *coch* secondary inflorescences, and (c) *COCH* down-regulation of *UNI* expression in leaves as well as secondary inflorescences, was experimentally tested. For this purpose, the endogenous transcript levels of *UNI* were assessed in the (i) differentiating leaves present in shoot tips of the vegetative and reproductive phase plants of *COCH UNI-TAC* and *coch UNI-TAC*, (ii) differentiating secondary inflorescences present in the shoot tips of the flowering plants of *COCH UNI-TAC* and *coch UNI-TAC* genotypes, and (iii) *COCH UNI-TAC*, *coch UNI-TAC*, *COCH uni-tac* and *coch uni-tac* flowering phase shoot tips in which stipules, leaves and inflorescence stems and any other appendages of secondary inflorescences were intact but flower buds had been removed. The shoot tips evaluated for *UNI* gene comprised 2 or 3 nascent phytomeres in which lateral organ primordia were undergoing growth, differentiation and development. The results of semiquantitative RT-PCR (RT-PCR) and quantitative real time RT-PCR (qRT-PCR) assays for *UNI* transcripts are presented in the figure 5. The *UNI* transcripts were significantly higher in both vegetative phase and reproductive phase leaves of *coch UNI-TAC* plants than in comparable leaves of *COCH UNI-TAC* plants (figure 5A, B and E). The RT-PCR and qRT-PCR assays showed 1.3- and 5.2-fold (mean=3.2) higher levels of *UNI* transcripts in the vegetative phase *coch UNI-TAC* leaves than vegetative phase *COCH UNI-TAC* leaves. The qRT-PCR assays on the reproductive phase leaves showed that *UNI* transcripts were about 5.2-fold higher in *coch UNI-TAC* leaves than in

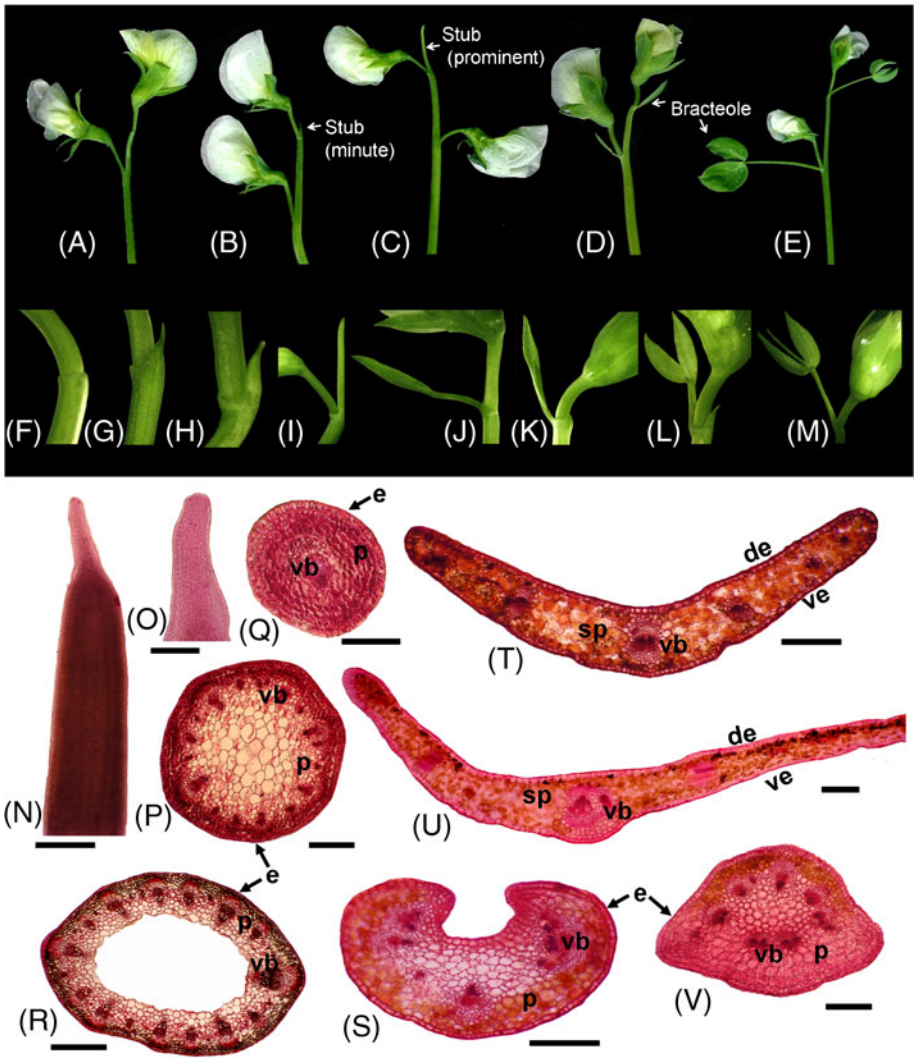

Figure 4. Effect of *coch* and *uni-tac* mutations on the architecture of secondary inflorescence in *Pisum sativum*. The secondary inflorescence of *COCH UNI-TAC* (**A** and **C**) and *COCH uni-tac* (**B**) genotypes is normal: it comprises of two pedicillate flowers borne on inflorescence stem in racemose fashion and the latter often ending in a stub of variable size (**A**, **B** and **C**). The flowers on secondary inflorescence are produced in the axils of petiolated unipinnate (simple) and unipinnate or bipinnate and tripinnate (compound) miniature leafy bracteoles in *coch uni-tac* (**D**) and *coch UNI-TAC* (**E**) genotypes, respectively. Stub morphology: (**F**–**I**) (*COCH UNI-TAC*)=Highly reduced to prominent stub; (**J**) and (**K**) (*coch uni-tac*)=unipinnate bracteole without (**J**) and with (**K**) stub; (**L**) (*coch UNI-TAC*)=bipinnate bracteole with prominent stub and (**M**) (*coch UNI-TAC*)=tripinnate bracteole without stub. Stub histology (**N**) Terminally aborted stub; (**O**) aborting terminus of stub; (**P**) transverse section (TS) of the stub body; (**Q**) TS of the stub immediately below the terminus [epidermal (e) and parenchymatous (p) tissues and vascular bundle(s) (vb) are seen]. Bracteole histology: (**R**) TS of inflorescence stem below the bracteole; (**S**) TS of the petiole of a bracteole; (**T**) TS of unipinnate-bracteole lamina; (**U**) TS of a laminated pinna of a tripinnate bracteole; (**V**) TS of tendrilled central pinna of a tripinnate bracteole [dorsal epidermis (de), ventral epidermis (ve) and parenchyma (p) or spongy parenchyma (sp) tissues and vascular bundle(s) (vb) are seen]. Scale bar for (**N**) to (**V**)=200 μm.

COCH UNI-TAC leaves. The qRT-PCR assays of *UNI* transcripts on secondary inflorescences (figure 1E) demonstrated that *UNI* transcripts were present at about (a) 3.3-fold higher level in *coch UNI-TAC* inflorescences than in *COCH UNI-TAC* inflorescences, 1.4-fold higher level in inflorescences than in leaves of *COCH UNI-TAC* plants, and (c) same level in the leaves and inflorescences of *coch UNI-TAC* plants. The RT-PCR and qRT-PCR assays of *UNI* transcripts on shoot tips devoid of flower buds (Figure 1C and D), respectively, showed that *UNI* transcript levels were 3- and 4-fold lower in *COCH uni-tac* plants than in *COCH UNI-TAC* plants. This confirmed the earlier observations that *uni-tac* allele of *UNI* (*UNI-TAC*) negatively affected transcription of the structurally intact gene (Hofer *et al*. 1997; Gourlay *et al*.

Table 3. *COCHLEATA* (*COCH*) gene blocks the formation of bracteoles at the site of attachment of flower to secondary inflorescence stem: The interaction between *COCH* and *UNIFOLIATA* [(*UNI* or *UNIFOLIATA-TENDRILLED ACACIA* (*UNI-TAC*)] genes in the morphogenesis of secondary inflorescence in *Pisum sativum*

Genotype[a]		Stub (df=19)[b]				Bracteoles (df=19)[b]			
		Frequency of occurrence	Structure-wise relative frequency when present		Size (mm)	Number	Structure-wise frequency of occurrence		
							Simple	Compound	
COCH	*UNI-TAC*	(A)	Prominent	Minute	(B)	(C)	Unipinnate	Bipinnate	Tripinnate
+	+	0.95±0.05	0.65±0.12	0.30±0.11	9.43±1.23	0	0	0	0
−	+	0.45±0.11	0	0.45±0.11	1.75±0.49	2±0	0.95±0.17	0.35±0.59	0.70±0.66[c]
+	−	0.65±0.11	0.15±0.08	0.50±0.11	2.90±0.96	0	0	0	0
−	−	0.55±0.11	0.15±0.08	0.40±0.11	3.00±1.04	1.95±0.11	1.95±0.11	0	0

a=The secondary inflorescence of all the genotypes bore two pedicels that terminated in flowers. About 25% of pedicels produced on *coch* plants bore compounded flowers (more than one flower).

b=Pearson's coefficient of correlation (*r*) between A and B, A and C and B and C respectively were: 0.604 (P=0.000), −0.318 (P=0.004) and −0.385 (P=0.000).

c=The frequency of occurrence of tendril-like structure in the tripinnate bracteole was about 90%.

2000; DeMason and Schmidt 2001; DeMason and Chawla 2004a, b). *UNI* transcript levels were about 12- and 3.5-fold (mean=7.7) higher in the *coch UNI-TAC* shoot tips as compared to *COCH UNI-TAC* shoot tips in the RT-PCR and qRT-PCR assays, respectively. Even in the background of *uni-tac*, *UNI* transcript levels were higher in shoot tips of *coch* plants as compared to *COCH* plants. Altogether *UNI* transcript assays showed that *UNI* transcription occured at significantly higher levels in the leaves and secondary inflorescences of the *coch UNI-TAC* plants as compared to *COCH UNI-TAC* plants. *COCH* down-regulated the expression of *UNI* both in leaves and secondary inflorescences. Increased expression of *UNI* in *coch* leaves led to increase in cell size and cell number in them and therefore increase in their size/biomass. Higher than normal expression of *UNI* in inflorescence led to the development of bracteoles on secondary inflorescences.

4. Discussion

Each node of a reproductive phase *P. sativum* plant bears a pair of stipules, a leaf and a racemose secondary inflorescence of two or more flowers. The previous work has shown that for the normal progression of differentiation of stipules and flowers, *UNI* expression occurs under the control of *COCH* antagonism (Hofer *et al*. 1997; Gourlay *et al*. 2000; Yaxley *et al*. 2001; Kumar *et al*. 2009b and 2011). Thus, it seemed plausible that at each node *COCH* controls *UNI* expression not only in stipules and flowers but also in leaf and secondary inflorescence stem. Therefore, leaf size, inflorescence architecture and *UNI* expression were observed in *COCH* and *coch* genotypes. Among the available mutants in the *UNI* gene, the *uni-tac* mutations are known to express active *UNI* albeit at much reduced levels presumably due to defect in transcription initiation site (DeMason and Schmidt 2001; DeMason and Chawla 2004a, b). Presumably, the lesion in the *uni-tac* allele affects interaction of *UNI* transcription initiation region with its transcription factors. Unlike the *uni*-null mutants, *uni-tac* mutant plants produce fertile flowers on secondary inflorescences of normal (wild type) architecture, normal stipules and compound leaves (of less than normal complexity) (Sharma and Kumar 1981; Mishra *et al*. 2009; Kumar *et al*. 2009b and 2011). Therefore, *COCH* and *coch* mutations were recombined with *UNI-TAC* and *uni-tac* alleles in a constant genetic background to understand the nature and effects of epistasis between *COCH* and *UNI*.

Using biometrical techniques on the four genotypes, it was possible to show that plants of *coch* genotypes bore leaves of significantly larger size than those borne on plants of *COCH* genotypes. Based on anatomical analysis of petioles and leaflets of the four genotypes, it was inferred that *coch* leaves were bigger than *COCH* leaves because the former contained more cells, and in certain tissues cells of larger dimensions were found. Morphological and anatomical analysis of secondary inflorescences of the genotypes showed that *coch* secondary inflorescence stems formed each flower in the axil of a leaf-like bracteole and *COCH* inflorescence stems were barren of bracteoles. The bracteoles of *coch UNI-TAC* were mostly compound mini-leaves, and contrastingly, the bracteoles on *coch uni-tac* plants were simple mini-leaves. This was consistent with the relative less complexity of *uni-tac* compound leaves

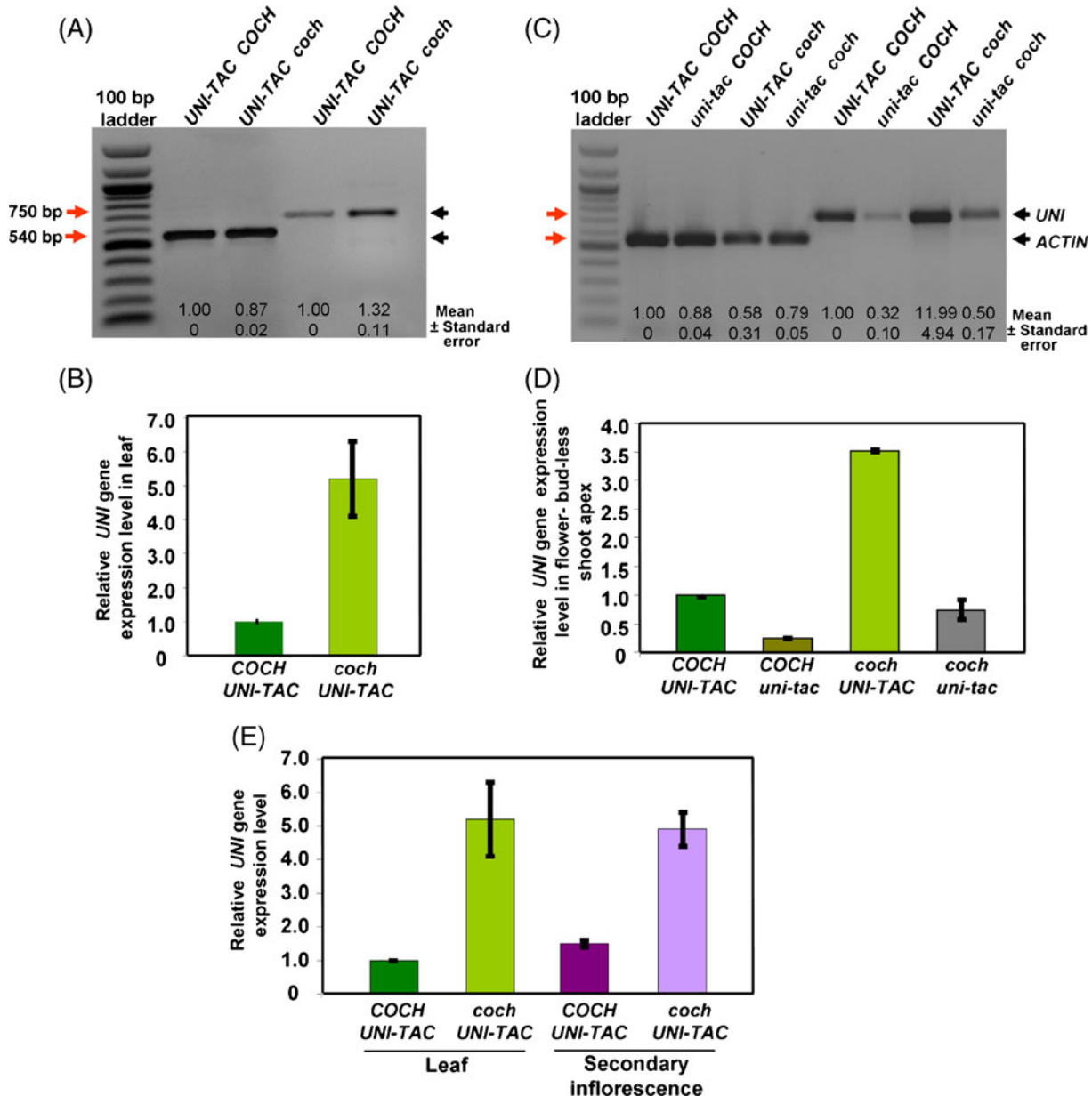

Figure 5. Enhanced expression of *UNIFOLIATA* [*UNI* or *UNIFOLIATA-TENDRILLED ACACIA* (*UNI-TAC*)] gene in the absence of *COCHLEATA* (*COCH*) gene function in *Pisum sativum*. The *UNI* mRNA transcript levels determined in apical leaves of vegetative phase plants by RT-PCR (**A**) and qRT-PCR (**B**), in shoot apices from which flower buds had been removed by RT-PCR (**C**) and qRT-PCR (**D**) and in leaves and secondary inflorescences (with flowers intact) of apical phytomers by qRT-PCR (**E**). In all the PCRs ACTIN served as internal control. The experiments (**A**), (**B**) and (**E**) were biologically replicated thrice and (**C**) and (**D**) twice and the expression values are averages with their standard errors. (**A** and **C**) Semi-quantitative RT-PCRs: (**A**) The relative levels of *UNI* expression in the vegetative phase apical leaves were 1.32 in *coch UNI-TAC* and 1.00 in *COCH UNI*-TAC; (**C**) in terms of the *UNI* level-of-expression in shoot apices rid of flower buds the genotypes fell in the following order: *coch UNI-TAC* (11.99)>*COCH UNI-TAC* (1.00)>*coch uni-tac* (0.50)>*COCH uni-tac* (0.32). The size of products is indicated with arrows with reference to molecular size markers in 100 bp ladder. (**B**, **D** and **E**) Quantitative RT-PCR: (**B**) The relative levels of *UNI* expression in apical leaves were 5.2±1.1 in *coch UNI-TAC* and 1.0±0 in *COCH UNI-TAC*; (**D**) in terms of the expression of *UNI* in shoot apices from which flower buds had been removed the relative order of genotypes was *coch UNI-TAC* (3.51±0.03)>*COCH UNI-TAC* (1.0±0)>*coch uni-tac* (0.74±0.17)>*COCH uni-tac* (0.24±0); (**E**) the relative order of *UNI* expression was *coch UNI-TAC* leaf (5.2±1.1)>*coch UNI-TAC* secondary inflorescence (4.9±0.5)>*COCH UNI-TAC* secondary inflorescence (1.5±0.1)>*COCH UNI-TAC* leaf (1.0±0). In each of the five experiments (**A**–**E**), two-way comparisons between genotypes were all significantly different at the 5% probability level.

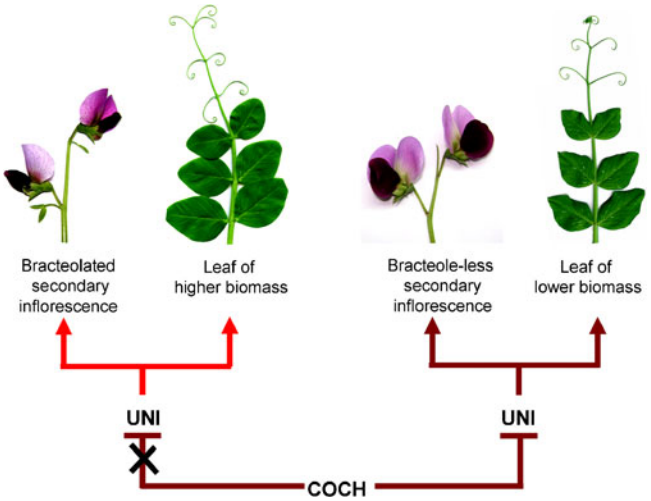

Figure 6. Suggested model of genetic interactions between *UNIFOLIATA* (*UNI*) and *COCHLEATA* (*COCH*) genes in *Pisum sativum*. The *COCH* gene down-regulates *UNI* in leaf differentiation such that compound leaf of normal size is formed and thus the formation of leaf of enlarged size is blocked. *COCH* down-regulates *UNI* in the differentiation of secondary inflorescence and thus obviates differentiation of bracteolated inflorescence.

versus full wild-type complexity of *UNI-TAC* leaves and suggested *UNI*'s mediation in the bracteole differentiation. Assays of *UNI* transcript accumulation, in the shoot tips, comprising stipules, leaves and secondary inflorescences (minus flowers) borne on nascent nodes *COCH UNI-TAC*, *COCH uni-tac*, *coch UNI-TAC* and *coch uni-tac* plants, differentiating leaves borne on top few nodes of vegetative and reproductive phase plants of *COCH UNI-TAC* and *coch UNI-TAC* plants and secondary inflorescences of nascent nodes of *COCH UNI-TAC* and *coch UNI-TAC* plants, indicated that large leaf and bracteolated flower/inflorescence phenotypes of *coch* genotypes were a consequence of up-regulated expression of *UNI* in them than in corresponding *COCH* genotypes. Several-fold lower *UNI* expression, lack of bracteoles in secondary inflorescences and leaves of smaller cellular biomass in *UNI-TAC COCH* plants on the one hand and higher *UNI* expression, presence of bracteoles and larger leaf biomass in *UNI-TAC coch* plants on the other hand appeared to be, respectively, interrelated. The presence of simple bracteoles in *coch uni-tac* plants was also in consonance with their *UNI* expression level which was lower than that in *UNI-TAC coch* plants but higher than in *uni-tac COCH* plants. Totally, the observations allowed the conclusion that *COCH* down-regulated *UNI* expression during leaf and secondary inflorescence differentiation processes so as to lead leaf and inflorescence morphogenesis to the respective wild-type pathways.

UNI of *P. sativum* is highly similar in sequence to *LFY* of *A. thaliana* (Hofer *et al*. 1997; Moyroud *et al*. 2009). The flower phenotypes of the *uni* and *lfy* mutants are also largely similar (Schultz and Haughn 1991; Huala and Sussex 1992; Weigel *et al*. 1992; Hofer *et al*. 1997; Yaxley *et al*. 2001). *LFY* is understood to first render floral specificity to meristem in the primordium laterally separated by shoot apical meristem (SAM) in the reproductive *A. thaliana* plants (Weigel *et al*. 1992; Mandel *et al*. 1992; Blazquez *et al*. 2006; Kobayashi and Weigel 2007). Later, in the flower domain already established, *LFY* activates downstream genes in order to form subprimordia for all the floral organs (Bowman *et al*. 1993; Liljegren *et al*. 1999; Lee *et al*. 2008). By analogy, it can be suggested that in *P. sativum* too *UNI* may be performing functions similar to those of *LFY* during flower differentiation. In *A. thaliana*, flowers are borne directly on the indeterminate primary inflorescence stem in the axil of cryptic/rudimentary bracts (Karim *et al*. 2009). In *P. sativum*, determinate secondary inflorescences arise in the axils of leaves (bracts) on the primary inflorescence stem; flowers are formed on the nodes of secondary inflorescence. However, in *P. sativum* plants mutated in *COCH*, flowers are subtended in the axils of bracteoles on secondary inflorescences. Considering the morphological, anatomical and molecular observations reported here, it appears that *COCH* represses *UNI* expression at the secondary inflorescence apical meristem such that the lateral meristem identity for bracteoles is not established, thus blocking bracteole development. There is phenotypic evidence that *COCH* down-regulates *UNI* activity in flowers to exercise determinacy and integrity in the formation and maintenance of subprimordia for floral organs of different whorls so that the formation of supernumerary and mosaic organs is controlled in favour of normal flower development (Ferrandiz *et al*. 1999; Yaxley *et al*. 2001; Kumar *et al*. 2011). It emerges that *COCH* reinforces determinacy on the *UNI* activated determinate morphogeneses of secondary inflorescence and flowers formed on it.

The phenotypes of mutations in *UNI*, *AF*, *INS*, *MFP* and *TL* that affect leaf morphology and observations on *UNI* expression in shoot apices of wild type and mutants have revealed a primary role of *UNI* in the differentiation of compound leaf in *P. sativum* (Hofer *et al*. 1997; Gourlay *et al*. 2000; DeMason and Schmidt 2001; Hofer *et al*. 2001; Taylor *et al*. 2001; Yaxley *et al*. 2001; Prajapati and Kumar 2002; DeMason and Chawla 2004a and b; DeMason 2005; Hofer *et al*. 2009; Mishra *et al*. 2009; Kumar *et al*. 2010). Collectively, the evidence has suggested that *UNI* maintains the meristematic activity in the main primordium for leaf rachis growth and also in the subprimordia generated on the rachis. The differentiation of the downstream primordia for leaflets and tendrils in the subdomains of leaf is regulated by *UNI* in interaction with other genes, including *AF*, *INS*, *MFP*, and *TL*, which are themselves activated by *UNI*. Further, the observations recorded in the present study have

indicated the requirement of *UNI* gene activity in the developing leaf is mandatory; *UNI* positively regulates cell division and growth in the differentiation of various tissues that comprise sub-organs of compound leaf. The negative regulation of *UNI* by *COCH* makes it possible that *UNI* threshold levels required for optimum growth of leaf are maintained, and thus the possibility of more than normal growth in leaf, as was evident in the absence of COCH function, is negated. It is possible to suggest that down-regulation of *UNI* by *COCH* during leaf differentiation limits the size of leaf as a sink for photosynthesis, within the framework of compound leaf pattern established by interactions of *UNI* and genes such as *AF*, *INS*, *MFP* and *TL*. Or, it can be surmised that *COCH* helps to direct plant resources such that reproductive fitness is increased by improvement in harvest index.

Characteristics of stipule morphology in the genotypes constructed by recombining *coch* with *uni*, *af*, *mfp*, *tl* and *ins* (Gourlay *et al*. 2000; Yaxley *et al*. 2001; Kumar *et al*. 2009b; and unpublished field observations on *coch* recombinants with *af*, *tl* and *mfp* in *ins* background) have established that *COCH* prevents development of *UNI*-led leaf-like compound stipules or antagonizes/represses *UNI* expression in stipules and promotes meristem identity for differentiation of simple stipules. It appears that *UNI* is dispensable in the development of stipules but is essential for the differentiation of compound leaves and normal flower bearing compound inflorescences. Because all the nodes in pea plant bear stipules and compound leaves, *UNI* and *COCH* must be continuously expressed right form seed germination to cessation of flowering by natural senescence. Or ,*UNI* is under negative control of *COCH* in all the lateral organs at all the nodes, albeit the degree of *COCH* repression on *UNI* varies by organ, and stages of organ differentiation.

The results described above and discussion are schematically diagrammed in figure 6. *LFY* (and its homologs) has been described as a master regulator for its role in activation of gene networks involved in lateral organ development in plants (Moyroud *et al*. 2010). Since *COCH* has been identified as a repressor of *UNI* (*LFY* ortholog) expression, *COCH* indeed performs the function of a regulator of master regulator in *P. sativum*. This conclusion and ubiquitous presence of *LFY* (*UNI* ortholog) led to the expectation that *COCH* orthologs may be present in other leguminous and non-leguminous plants. Examination of the lateral organs of the leguminous species of the flora of Delhi revealed that *Caesalpinia bonduc* and *Delonix regia* have the *coch* phenotype of *P. sativum*. They produce compound leaves, leaf-like compound stipules and bracteolated secondary inflorescences (Sharma *et al.* 2012). A literature survey revealed that leaf-like compound stipules occur in non-leguminous species (Charlton 1991; Sattler and Rutishauser 1997; Rutishauser 1999; Rutishauser *et al.* 2008; Condit *et al.* 2010, Sharma *et al*. 2012). These observations have prompted the hypothesis that *COCH* control over *UNI* (*LFY*) in the lateral organ gene regulatory network is a common property of the stipulate, if not all of the flowering plants.

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