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VEGETATIVE1 is essential for development of the compound inflorescence in pea

Ana Berbel[1,*], Cristina Ferrándiz[1,*], Valérie Hecht[2], Marion Dalmais[3], Ole S. Lund[4,†], Frances C. Sussmilch[2], Scott A. Taylor[2,5], Abdelhafid Bendahmane[3], T.H. Noel Ellis[5,6], José P. Beltrán[1], James L. Weller[2] & Francisco Madueño[1]

Unravelling the basis of variation in inflorescence architecture is important to understanding how the huge diversity in plant form has been generated. Inflorescences are divided between simple, as in Arabidopsis, with flowers directly formed at the main primary inflorescence axis, and compound, as in legumes, where they are formed at secondary or even higher order axes. The formation of secondary inflorescences predicts a novel genetic function in the development of the compound inflorescences. Here we show that in pea this function is controlled by *VEGETATIVE1* (*VEG1*), whose mutation replaces secondary inflorescences by vegetative branches. We identify *VEG1* as an *AGL79*-like MADS-box gene that specifies secondary inflorescence meristem identity. *VEG1* misexpression in meristem identity mutants causes ectopic secondary inflorescence formation, suggesting a model for compound inflorescence development based on antagonistic interactions between *VEG1* and genes conferring primary inflorescence and floral identity. Our study defines a novel mechanism to generate inflorescence complexity.

[1] Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas–Universidad Politécnica de Valencia, Valencia 46022, Spain. [2] School of Plant Science, University of Tasmania, Hobart, Tasmania 7001, Australia. [3] Unité de Recherche en Génomique Végétale, UMR INRA-CNRS, Rue Gaston Crémieux, Evry Cedex 91057, France. [4] Department of Plant Biology, Danish Institute of Agricultural Sciences, Thorvaldsensvej 40, Frederiksberg C DK-1871, Denmark. [5] John Innes Centre, Colney Lane, Norwich NR4 7UH, UK. [6] Institute of Biological, Environmental and Rural Sciences Aberystwyth University, Gogerddan Campus, Aberystwyth SY23 3EB, UK. *These authors contributed equally to this work. †Present address: Department of Agriculture and Ecology, University of Copenhagen, Taastrup 2630, Denmark. Correspondence and requests for materials should be addressed to F.M. (email: madueno@ibmcp.upv.es).

Understanding the basis of diversity in form is a major challenge in developmental biology. An important feature contributing to form diversity in angiosperms is the variation in the architecture of inflorescences, the structures that bear the flowers[1,2]. Inflorescence architecture is also important because it conditions flower and fruit production and, therefore, crop yield[3]. A process central to generation of inflorescence architecture diversity is inflorescence branching, with a major distinction between simple inflorescences, as in Arabidopsis, where flowers derive from the primary inflorescence axis (Fig. 1a,b,c), and compound inflorescences, where flowers form at secondary (or higher order) branches (Fig. 1d,e,f)[2].

Genetic control of compound inflorescence development has been best studied in grasses, particularly in rice and maize, where flowers are formed from the spikelet meristem, an inflorescence meristem that frequently derives from lateral secondary or tertiary order inflorescence branches[4,5]. This higher complexity suggests that novel genetic functions must exist for the formation of the high-order inflorescence meristems, that often are not simple reiterations of the primary inflorescence meristem. In fact, a number of genes have been characterized that control specific aspects of compound inflorescence development in grasses, such as the formation of lateral inflorescences or the determinacy of spikelet meristems[4–7]. Compound inflorescence development has also been studied in Solanaceae, where two genes that control inflorescence complexity have been isolated. However, the formation of Solanaceae compound inflorescences differs from grasses, not involving high-order inflorescence meristems[8].

Legumes (Fabaceae), the third largest angiosperm family, also have compound inflorescences[9,10], where flowers are produced on lateral secondary inflorescence branches (Fig. 1d,e,f). In this respect, legume inflorescence architecture is similar to that of grasses and different to Solanaceae. Given that legumes are only distantly related to grasses, a relevant question is whether legumes have generated secondary inflorescence meristems through a distinct mechanism involving novel specific functions. To understand the development of the compound inflorescence in legumes, we analysed the classical *vegetative1* (*veg1*) mutant from pea (*Pisum sativum*), which displays a phenotype that suggests severe defects in the formation of secondary inflorescence meristems[11,12], and, therefore, we hypothesized that it might be defective in such novel function.

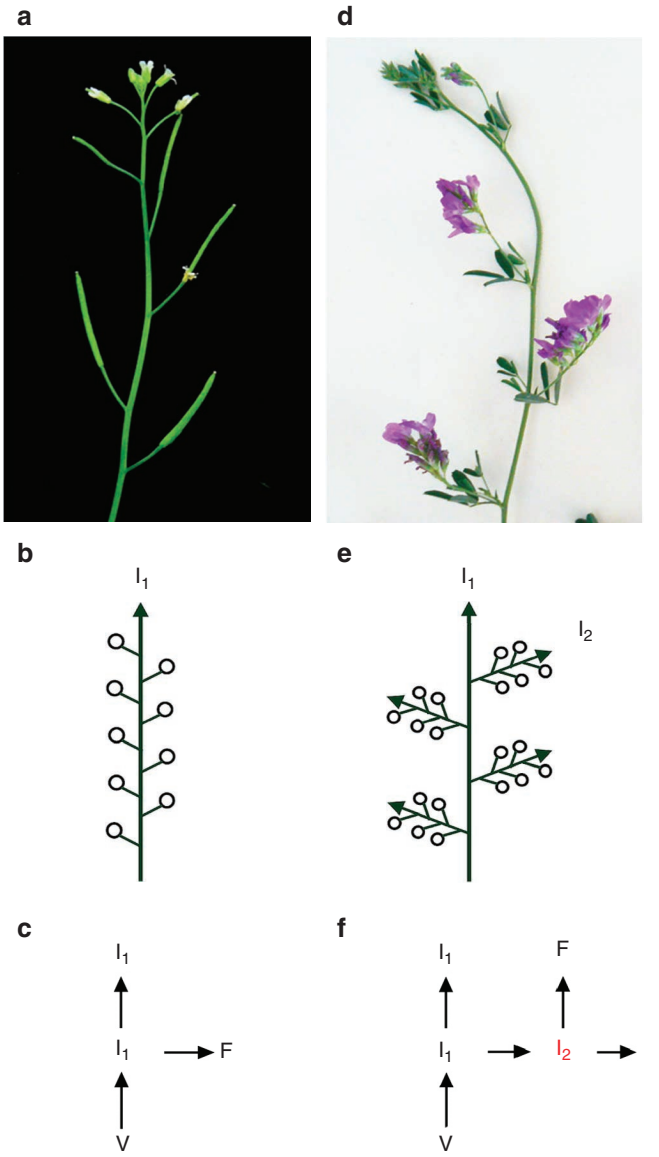

Figure 1 | Examples of simple and compound inflorescences. (**a**) Simple raceme of *Arabidopsis thaliana*. (**b**) Diagram of the architecture of the inflorescence showed in (**a**). (**c**) Schematic representation of meristem identity in the inflorescence showed in (**a**). (**d**) Compound raceme of the legume species *Medicago sativa*. (**e**) Diagram of the architecture of the inflorescence showed in (**d**). (**f**) Schematic representation of meristem identity in the inflorescence showed in (**d**). In Arabidopsis, flowers appear in the primary inflorescence stem (I_1) whereas in *M. sativa* they appear in secondary inflorescence branches (I_2). V, vegetative meristem; I_1, primary inflorescence meristem; I_2, secondary inflorescence meristem; F, floral meristem.

Results

***VEG1* is required to make secondary inflorescences**. The pea inflorescence is a compound raceme, typical of many legumes[1,10,13]. During the vegetative phase, each of the nodes produced by the vegetative shoot apical meristem (SAM) consists of a leaf with a shoot axillary meristem that generally remains dormant until the floral transition has occurred (Fig. 2a–c). At floral transition, the SAM becomes a primary inflorescence (I_1) meristem, with indeterminate growth, that produces nodes with axillary meristems that grow out immediately (Fig. 2a–c). These secondary inflorescence (I_2) meristems each produce 1–3 nodes bearing floral meristems before terminating in a stub[1,13] (Fig. 2d). Therefore, the I_2 meristem, interposed between the I_1 and the floral meristems, represents an additional level of complexity compared with the simple raceme of Arabidopsis (Fig. 1c).

The *vegetative1* (*veg1*) mutant derives from X-ray mutagenesis[11], and, among numerous flowering-related mutants in pea, it is distinctive in that it never produces flowers under any conditions (Fig. 2a), and may persist in a vegetative state for over 6 months under conditions where wild-type plants flower after several weeks[12]. During the vegetative phase, *veg1* and wild-type plants were indistinguishable, with dormant axillary meristems (Fig. 2a–c,e). However, after the floral transition, nodes equivalent to those occupied by I_2s in wild type were also released from dormancy in *veg1*, but produced only vegetative shoots (Fig. 2a,b,d,e; Supplementary Fig. S1). This suggests that *veg1* mutants undergo a phase transition but are impaired in the subsequent specification of secondary inflorescence identity.

To test this possibility, we compared the expression of inflorescence markers in wild type and *veg1*. In wild-type plants, the floral transition is associated with transcriptional induction of *DETERMINATE* (*DET*) and *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*)[14–17] (Fig. 3a), which, like their Arabidopsis orthologues *TERMINAL FLOWER 1* (*TFL1*)[18] and *APETALA 1* (*AP1*)[19], control the identity of the inflorescence and the floral meristems[14,15,17–20]. In 5-week-old plants, where *PIM* is clearly

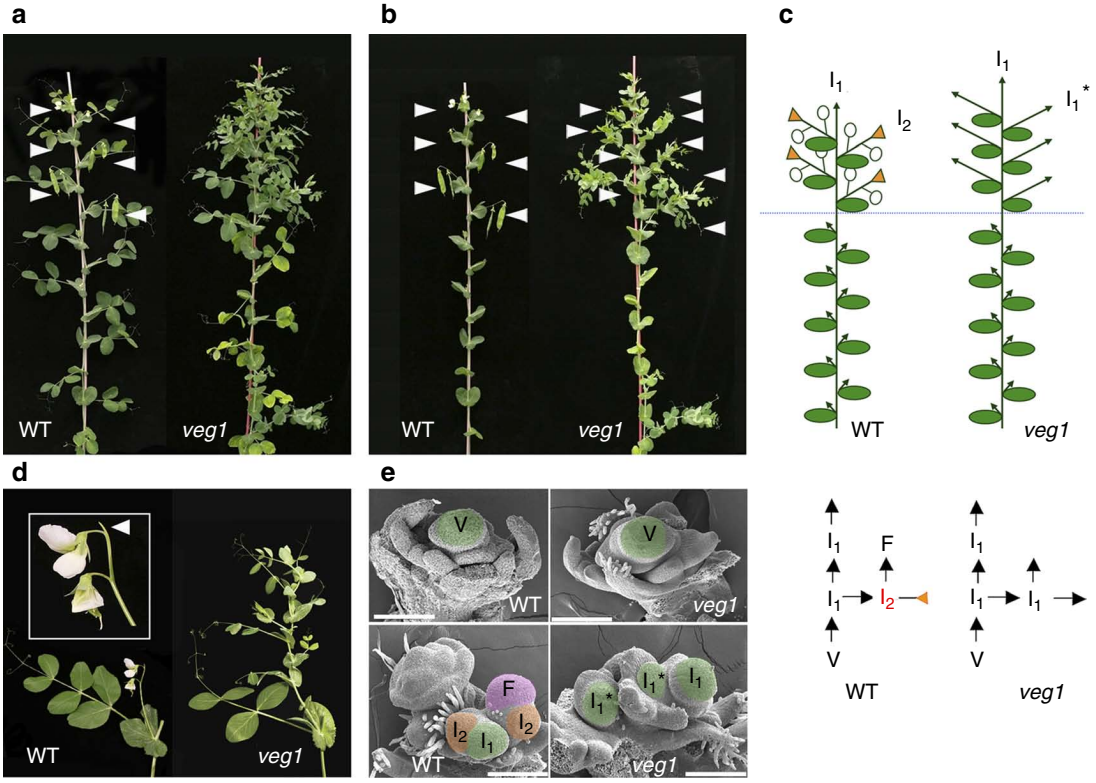

Figure 2 | Mutant secondary inflorescences replaced by vegetative branches in pea *veg1*. (**a**) Pea wild-type (WT) and *veg1* plants grown for 11 weeks. Whereas the upper nodes of the wild type contain secondary inflorescences (I_2) with flowers (pods, arrowheads), *veg1* has not produced any flower. (**b**) The same plants as in (**a**), where leaves have been removed. As in the wild type, the axillary buds of the lower nodes of *veg1* remain dormant. However, at the nodes where I_2s (arrowheads) have grown in the wild type, branches (arrowheads) have grown out in *veg1*. (**c**) Diagrams (top) and schematic representation of meristem identity (bottom) of the wild-type and *veg1* mutant plants. The vegetative meristem (V) becomes a primary inflorescence meristem (I_1) that produces secondary inflorescence meristems (I_2) that form flowers (F). In the *veg1* plants the I_2s are replaced by vegetative branches similar to I_1s (I_1*). Arrowheads, indeterminate shoots; open circles, flowers; orange triangles, stubs. (**d**) Structures formed in equivalent apical nodes of the inflorescence stems of wild-type and *veg1* plants. Whereas the wild type has produced an I_2 with two flowers and a stub (arrowhead in inset), *veg1* has produced a vegetative branch. (**e**) Scanning electron micrographs of the main shoot apex of wild type and *veg1*. During the vegetative phase (top), in both the wild type and *veg1*, the vegetative SAM (V, highlighted in green) generates leaves with dormant axillary meristems. After transition to flowering (bottom), the SAM in the wild type becomes a primary inflorescence meristem (I_1) that produces I_2 meristems (orange) with floral meristems (F, pink). At an equivalent developmental stage, the SAM of *veg1* produces leaves with axillary meristems (I_1*, green) that grow out following the same pattern as the I_1 meristem. Scale bars, 200 μm.

expressed in wild-type plants, expression was not observed in *veg1* (Fig. 3a), consistent with the absence of flowers or floral organs. However, the inflorescence marker *DET* was upregulated in *veg1* similarly to wild type. Taken together, these results support the idea that the apex of *veg1* plants go through floral transition at approximately the same time as the wild type. This implies that the extreme non-flowering phenotype of the *veg1* mutant plants does not represent a block in floral induction or a defect in timing of the floral transition, but instead reflects a failure of the lateral meristems produced by the primary 'inflorescence' apex of *veg1* to develop as secondary inflorescences. Therefore, *VEG1* is required for the pea inflorescence apex to make the I_2 meristems.

To elucidate the nature of the vegetative branches that replace the I_2s in the *veg1* mutant, we further analysed the expression of *DET*, in more detail, by *in situ* hybridization. In wild-type apices, *DET* expression was restricted to the primary inflorescence meristem (Fig. 3b,c). However, in *veg1* apices *DET* was expressed not only in the I_1 meristem but also in the lateral meristems that are formed in place of the I_2 meristems (Fig. 3d,e). This indicates that the lateral branches produced by *veg1* plants after the transition possess I_1 identity, and further supports a role for *VEG1* in the specification of I_2 identity (Fig. 2c).

Although *VEG1*, thus, seems required for the specification of I_2 meristems, a further question concerns whether it is also required for the formation of flowers. In contrast to the constitutive non-flowering phenotype of the *veg1* mutant, the *veg1 det* double mutant does produce flowers[13] (Supplementary Fig. S2a,b). However, in contrast to the *det* single mutant, where the primary inflorescence ends in a terminal I_2, the *veg1 det* primary inflorescence does not produce I_2s, and ends with the production of a terminal flower, often after producing a flower directly from the axil of a leaf at the node below[13] (Supplementary Fig. S2c-f). Consistent with that, we saw that expression of *PIM* is induced in the apex of *veg1 det* plants (Supplementary Fig. S2g,h). This again indicates that *VEG1* is required for the specification of the I_2 meristems, and shows that it is not directly required for the expression of the floral meristem identity genes or the formation of floral meristems.

***VEG1* is a MADS-box gene from the *AGL79* clade**. To identify a candidate gene for *VEG1*, we adopted a comparative mapping strategy using the related model legume *Medicago truncatula*, which has inflorescence architecture identical to pea[21]. The *VEG1* locus was initially observed to map to the bottom of pea linkage group V (top of Medicago chromosome 7), near the MADS-box gene *PsSEPAL-*

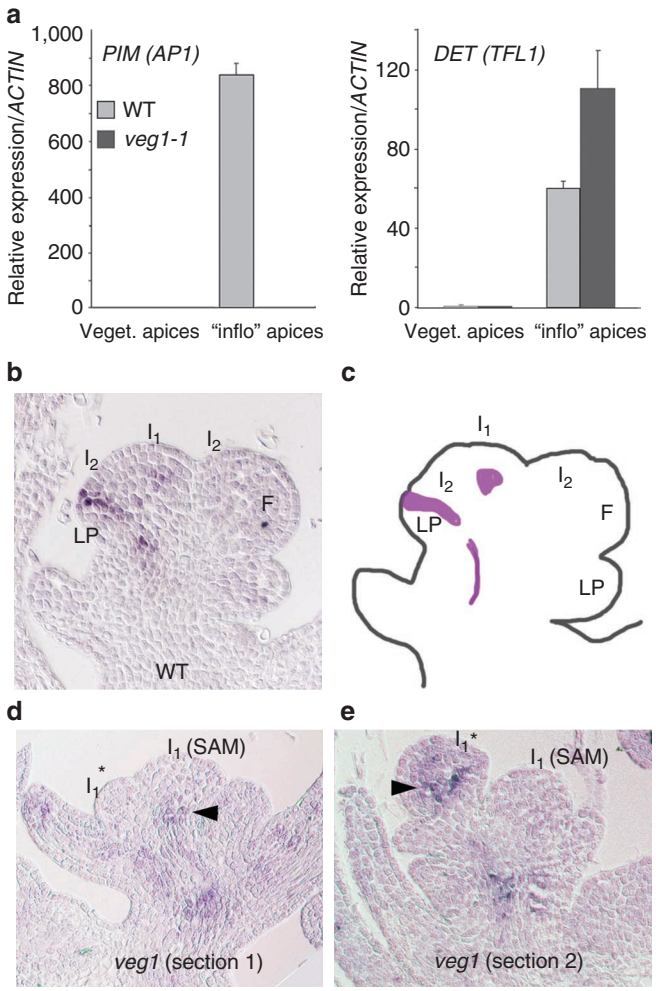

Figure 3 | Expression of meristem identity genes in the shoot apex of *veg1*. (**a**) mRNA levels of *PIM* (left) and *DET* (right) in the shoot apices of the wild type and *veg1*. Relative mRNA levels were determined by RT–qPCR. Samples were from main-shoot apices of 2-week old plants (veget. apices), before wild-type plants had gone through the floral transition, and of 5-week-old plants ('inflo' apices), when the wild type had started producing flowers. Values represent the means of two biological replicates ±s.e. (**b–e**) *In situ* hybridization of *DET* mRNA in the shoot apices of wild type and *veg1*. Samples were from the main apices of 4-week-old plants, when the wild type had gone through the floral transition. In the wild-type apex (**b**) *DET* expression was detected below the dome of the primary inflorescence meristem (I_1), in the vasculature, and in the boundary between the secondary inflorescence meristem (I_2) and the incipient leaf primordium (LP), but not in the I_2 or in the floral meristems (F), as represented in the diagram (**c**), for clarity. Similarly, in *veg1*, expression was also detected below the primary shoot meristem (I_1 SAM, arrowhead), in a section through the centre of the apex (**d**). A deeper section from the same apex (**e**) shows that in *veg1 DET* is also expressed in a lateral shoot meristem (I_1*, arrowhead).

LATA1 (*PsSEP1*)[22], and we found that this gene was deleted in *veg1*. However, consistent with the well-documented role for *SEP* genes in floral organ identity[23,24], we found that *PsSEP1* was expressed in floral but not in I_2 meristems, thus arguing against *PsSEP1* as a candidate for *VEG1*. In *M. truncatula*, a second MADS-box gene, *MtFULc*, is located on the same BAC clone as *MtSEP1* (ref. 22) (Fig. 4a). We isolated the pea orthologue of this gene (*PsFULc*), confirmed its map position very close to *PsSEP1*, and observed that it is also deleted in the *veg1* mutant (Fig. 4b). *PsFULc* and *MtFULc* belong to the *AGL79* clade of the *AP1/SQUA/FUL* genes[25,26] (Supplementary Fig. S3). Genes from the *AP1/SQUA/FUL* lineage are involved in the specification of meristem identity[20], which suggested that *PsFULc* was a good candidate for *VEG1*.

To assess whether the deletion of *PsFULc* was the cause of the *veg1* phenotype, we characterized additional mutant alleles of *PsFULc*. Forward screening of an ethyl methanesulfonate (EMS)-mutagenized population[27] identified a single M_2 plant in which secondary inflorescences were replaced by vegetative branches, as in *veg1* (Fig. 4c). Sequencing of *PsSEP1*- and *PsFULc*-coding regions in this mutant (*psfulc-2*) revealed a wild-type *PsSEP1* sequence, but identified a G-to-A mutation typical of EMS mutagenesis at the 5′-splice junction of the fourth intron in *PsFULc* (Fig. 4b). A third *PsFULc* mutant allele (*psfulc-3*), carrying a Q102STOP mutation, was identified in reverse genetic screening of an EMS-mutagenized Targeting-Induced Local Lesions in Genomes (TILLING) population[28]. Like *psfulc-2*, this mutant also showed a clear *veg1* phenotype (Fig. 4d) and carried a *PsSEP1*-coding region identical to wild type. As a third line of evidence, we used virus-induced gene silencing (VIGS)[29] to specifically suppress expression of *PsFULc*, and found that *PsFULc*-VIGS plants partly phenocopied the *veg1* phenotype. The node at which the first I_2 appeared was significantly higher in *PsFULc*-VIGS plants (14.5±1.6) than in control plants (10.7±0.9) and the intervening nodes were occupied by vegetative branches (Fig. 4e; Supplementary Fig. S1), as in *veg1* plants. In summary, the phenotype of the *PsFULc*-VIGS plants, and the defects of *psfulc-2* and *psfulc-3*, identical to those of the original *veg1* X-ray mutant, show that the *veg1* phenotype is specifically caused by the loss of *PsFULc* and that the deletion of other sequences in the X-ray *veg1* mutant does not significantly contribute to it. Therefore, we subsequently refer to *PsFULc* as *VEG1*.

***VEG1* expression marks secondary inflorescence meristems**. To assess whether the expression of *VEG1* is consistent with its proposed role in the specification of I_2 meristem identity, we examined its expression pattern in wild-type plants and in mutants either completely lacking or producing ectopic secondary inflorescences. Analysis by RT–qPCR in wild-type plants showed that *VEG1* is expressed in inflorescence apices and mature flowers (Supplementary Fig. S4a). Consistent with that, a time-course expression analysis showed that *VEG1* upregulation occurs during the floral transition, after *FTb2*, a leaf marker of floral induction[16], and before the floral meristem identity gene *PIM* (Supplementary Fig. S4b). Further analysis by *in situ* hybridization on wild-type inflorescence apices (Fig. 5a,b) showed that *VEG1* is expressed in the I_2 meristems but is not expressed in the apical meristem of the primary inflorescence (I_1) or in the young floral meristems, which show strong expression of *PIM* (Fig. 5c,d). These results further support the idea that *VEG1* specifies the identity of I_2 meristems.

Mutations in *DET* or *PIM*, homologues of *TFL1* and *AP1*, respectively, cause the conversion of other meristems of the pea inflorescence into I_2 meristems. Thus, while the primary inflorescence meristem (the inflorescence SAM) of wild-type pea plants shows indeterminate growth, producing I_2s only in lateral positions (Figs 2c and 5a), in *det* mutants, the inflorescence SAM shows determinate growth, and terminates in a typical I_2 (refs. 15,30) (Fig. 5e,f,g). On the other hand, the lateral meristems in the I_2 of *pim* mutant plants, rather than acquiring floral identity, as in the wild type (Figs 2c and 5b), retain I_2 meristem identity and themselves generate lateral meristems with I_2 identity in a reiterative manner, with some of these supernumerary I_2 meristems eventually producing flowers[17,30] (Fig. 5h,i,j). If *VEG1* specifies I_2 identity, it would be expected that the formation of the ectopic I_2 meristems in these mutants were accompanied by changes in *VEG1* expression. In fact, *in situ* hybridization showed that in the *det* mutant inflorescence

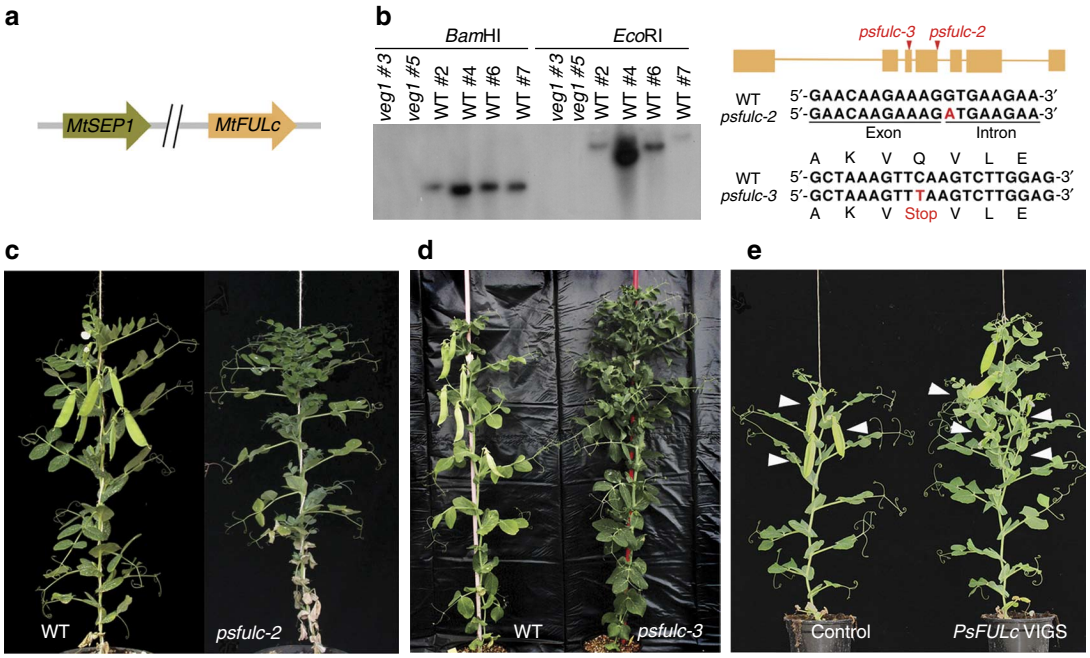

Figure 4 | Cloning of *VEG1*. (**a**) In *M. truncatula*, *MtFULc* is located 17 Kb from *MtSEP1*, whose pea homologue maps to the *VEG1* locus and is deleted in the *veg1* mutant. (**b**) Lesions in the *psfulc* mutant alleles described in this work. Left, Southern blot on DNA of plants from a F2 population segregating for *veg1*, hybridized with a *PsFULc* probe. The same DNA samples were digested with *Bam*HI and with *Eco*RI. Hybridization was observed with DNA from plants with a wild-type phenotype (2, 4, 6 and 7) but not from homozygous *veg1* plants (3 and 5), showing that *PsFULc* is deleted in *veg1*. Right, mutations in the *psfulc-2* and *psfulc-3* alleles. The nucleotide changes in the mutant sequences are indicated in red. The G-to-A mutation in *fulc-2* destroyed the splice donor site of exon 3. The C-to-T mutation in *psfulc-3* caused a Q102STOP change. (**c**,**d**) Phenotype of the *psfulc-2* and *psfulc-3* mutant plants. Both plants show a non-flowering *veg1* phenotype, where secondary inflorescences (I_2) are replaced by vegetative branches. (**e**) *veg1*-like phenotype of a *PsFULc*-VIGS plant. In the *PsFULc*-silenced plant, the I_2s appear in later nodes than in the control plant, and branches (arrowheads) develop in the nodes where I_2s (arrowheads) appear in the control plant.

VEG1 is expressed not only in the lateral I_2 meristems but also in the apical meristem (Fig. 5k), and that the supernumerary I_2 meristems in the *pim* mutant exhibit *VEG1* expression (Fig. 5l). This indicates that *DET* and *PIM* repress *VEG1* expression and restrict it to the I_2, and suggests that the ectopic expression of *VEG1* in *det* and *pim* mutants causes the conversion of I_1 and floral meristems, respectively, into I_2 meristems.

Severe mutations in the *VEGETATIVE2* (*VEG2*) or *GIGAS* genes cause a non-flowering phenotype similar to the *veg1* mutant (*gigas* only under long-day photoperiods), with no formation of secondary inflorescences[16,31]. As in *veg1*, the plants of both mutants show outgrowth of vegetative lateral branches at nodes occupied in wild-type plants by I_2s (ref. 16) (Sussmilch *et al.*, unpublished). This similarity suggests that these genes participate in the same genetic network as *VEG1* and raises the question of what is their relative position in that network. Analysis by RT–qPCR showed that although *VEG1* was upregulated in shoot apices of 4-week-old wild-type plants, its expression was not detectable in either mutant (Supplementary Fig. S4c) after 6 weeks. This indicates that *VEG1* acts downstream of *VEG2* and *GIGAS* and suggests that they participate in the activation of *VEG1* expression. This seems particularly likely for *GIGAS*[16], in view of the fact that its Arabidopsis homologue *FLOWERING LOCUS T* (*FT*) is a direct activator of the floral identity genes *AP1* and *FUL*[32–35], which are MADS-box genes from the same lineage as *VEG1* (Supplementary Fig. S3).

Discussion

Our data suggest a genetic model that explains the specification of the identity of the different meristem types formed in the pea compound inflorescence (Fig. 6a). In this model, expression of *VEG1*, which is required to specify I_2 identity, is restricted to the I_2 meristem by *DET*, which represses it in the I_1 meristem, and by *PIM*, which represses it in the floral meristem. Conversely, expression of *DET* itself is restricted to the I_1 and excluded from the I_2 through repression by *VEG1*. The simplest interpretation of the *veg1* phenotype is, therefore, that it results from ectopic *DET* expression in the lateral I_2 meristems, converting them into I_1 meristems (Fig. 6b). In addition, the formation of flowers in the *veg1 det* double mutant suggests that *DET* represses not only *VEG1* but also *PIM* expression. Finally, the fact that the flowers in *veg1 det* are directly formed from the I_1, as in simple inflorescences, suggests that the default state of the meristems in the pea inflorescence is floral identity, which is normally restricted to the lateral meristems of the I_2 by the concerted action of *DET* and *VEG1*. In this sense, *VEG1* would be required to maintain 'vegetativeness', as defined by Prusinkiewicz *et al.*[36], in the lateral meristems of the I_1.

The network of mutually repressive interactions between *DET*, *VEG1* and *PIM* resembles the simpler genetic network that controls meristem identity in the inflorescence apex of Arabidopsis (Fig. 6c), where the separation of the inflorescence and floral meristematic domains is achieved by mutual repression between *TFL1* and the floral meristem genes *AP1* and *LEAFY* (*LFY*)[20]. This similarity suggests a mechanism in which evolutionary modification of a simple raceme (for example, Arabidopsis) into a compound form may have occurred through the appearance of a new function, *VEG1*, acting between the inflorescence identity function of *DET* (*TFL1*) and the floral identity function of *PIM* (*AP1*), which leads to the formation of a new intermediate-step meristem, the I_2, and therefore to the development of a compound inflorescence.

The *VEG1* gene belongs to the *AP1/SQUA/FUL* lineage, represented in core eudicots by three distinct clades, eu*AP1*, eu*FUL* and *AGL79*, that likely arose from a common eudicot ancestor through

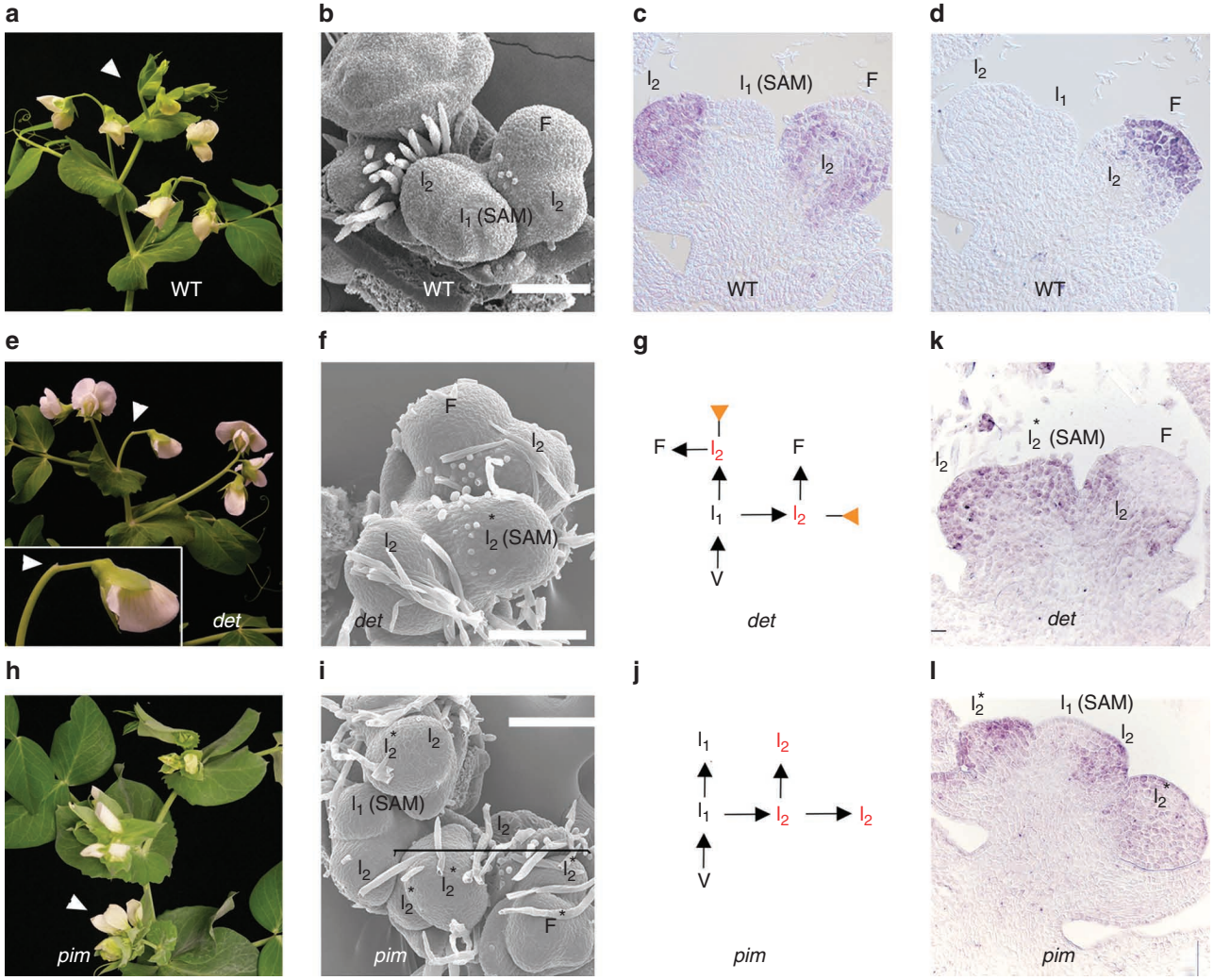

Figure 5 | *VEG1* is expressed in I_2 meristems of wild type and in ectopic I_2 meristems of pea inflorescence mutants. (**a**) Inflorescence of a pea wild-type plant. The inflorescence apex (arrowhead) exhibits indeterminate growth. (**b**) SEM image showing the different meristem types in a wild-type inflorescence. The SAM is a primary inflorescence (I_1) meristem that has produced secondary inflorescence (I_2) meristems, one of which has produced a floral meristem (F). (**c**) *In situ* hybridization of *VEG1* mRNA in the inflorescence apex of the wild type. *VEG1* is expressed in the lateral I_2 meristems, not in the I_1 neither in the floral meristem (F). (**d**) *In situ* hybridization of *PIM* mRNA in the wild-type inflorescence, in a contiguous section to that in (**c**). The *PIM* signal is observed only in the floral meristem, which does not express *VEG1*. (**e**) Inflorescence of a *det* mutant, where the apex has converted into an I_2 (arrowhead). The terminal I_2 stem subtends a flower and ends into a stub (arrowhead in inset). (**f**) SEM image of a *det* inflorescence apex. The SAM has the characteristic shape of an I_2 meristem (I_2*). (**g**) Schematic representation of meristem identity in the *det* inflorescence. V, vegetative meristem; orange triangles, stubs. (**h**) Inflorescence of a *pim* mutant, showing proliferative I_2s (arrowhead) with abnormal flowers. (**i**) SEM image of a *pim* inflorescence apex, showing I_2s that, rather than flowers, produce new I_2s (I_2*). One of the proliferative I_2s is producing a floral primordium (F*). (**j**) Schematic representation of meristem identity in the *pim* inflorescence. (**k**) *In situ* hybridization of *VEG1* mRNA in the inflorescence apex of *det*. In the *det* mutant *VEG1* is also expressed in the SAM (I_2*), which is converted into an I_2. (**l**) *In situ* hybridization of *VEG1* mRNA in the inflorescence apex of *pim*. Expression of *VEG1* is observed not only in the lateral I_2s but also in the meristem being formed by the I_2 at the right (I_2*), which is converted from a floral meristem to an I_2 meristem. Scale bars, 100 μm.

duplication[25,26]. While no functional information from mutant phenotypes is available for any *AGL79*-like gene, analysis of several *euAP1* genes and of the Arabidopsis eu*FUL* gene *FRUITFULL* (*FUL*) indicates that genes in these other sub-clades control the identity of reproductive meristems[20,37]. This suggests that this basic function was already present in the ancestor of the core-eudicot *AP1/SQUA/FUL* genes, and we speculate that *AGL79* genes, such as *VEG1*, may have sub-functionalized to specify the identity of I_2 meristems.

Is the mechanism of I_2 identity specification, through *VEG1*-like genes, also central for compound inflorescence development in species other than pea? Compound inflorescences are widespread in the Fabaceae family, suggesting a common evolutionary origin. As *VEG1* orthologues are found in several papillionoid legumes (Supplementary Fig. S3), and more widely in eudicots, it seems likely that *VEG1* function was present early in the evolution of Fabaceae and may have arisen before the origin of this group. However, no monocot orthologue exists for *VEG1/AGL79*, a core-eudicot-specific gene[25,26], and consistent with this, the genetic network controlling compound inflorescence formation in grasses is different to that in legumes and does not seem to involve a *VEG1*-related function[4–7,38]. Within the eudicots, compound inflorescences have also been studied in Solanaceae[8], but, in this group, the ontogeny of the compound inflorescence does not involve high-order inflorescence meristems[8] and is thus quite distinct from that in either legumes or

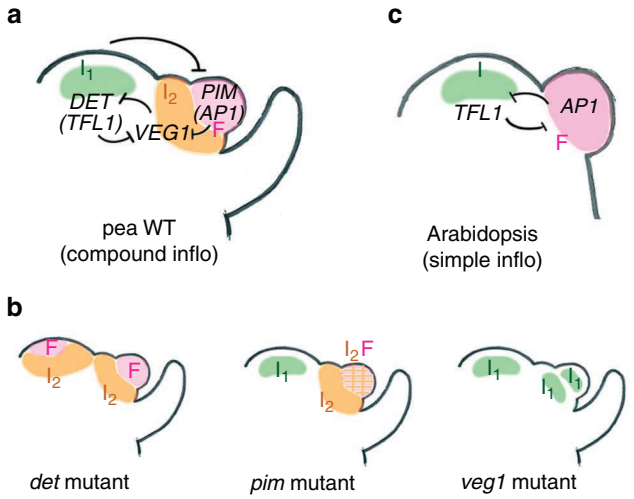

Figure 6 | Genetic model for specification of meristem identity in the compound inflorescence of pea. (**a**) In the pea compound inflorescence, expression of *DET* (orthologue of *TFL1*) in the I_1, *VEG1* in the I_2 and *PIM* (orthologue of *AP1*) in the floral meristems are required for those meristems to acquire their identity. Expression of these genes in their correct domains is maintained by a network of mutual repressive interactions. (**b**) The genetic model explains the phenotypes of the pea meristem identity mutants. The absence of *DET* in the *det* mutant allows expression of *VEG1* in the SAM, which gets converted into an I_2. The absence of *PIM* allows the expression of *VEG1* in the floral meristem, which gets converted into a proliferative I_2. In absence of *VEG1*, *DET* is expressed in all the meristems in the apex, and they get converted into I_1s. (**c**) Meristem identity in the simple inflorescence of Arabidopsis is maintained by a similar genetic network than in pea, but *VEG1* function is absent and I_2 meristem is not formed.

grasses. Accordingly, the genes known to control complexity in this family are distinct from those in legumes and grasses and also do not include a *VEG1* orthologue[8]. Thus, these groups apparently use distinct genetic networks for inflorescence complexity, suggesting that compound inflorescences have independently appeared several times during angiosperm evolution. This is consistent with the fact that compound inflorescences occur in phylogenetically distant plant families[2].

In summary, our work provides the first insight into the genetic network controlling the legume compound inflorescence. We identify a novel mechanism for generation of inflorescence complexity, distinct from that in grasses and Solanaceae, which is based on the function of the *VEG1* gene, which acts between the I_1 and floral meristem identity genes to specify formation of the I_2 meristem. The identification of *VEG1* as an *AGL79*-like MADS-box gene suggests that *VEG1* function derives from sub-functionalization of the *AGL79* clade within the eudicot *AP1/SQUA/FUL* genes. More generally, our identification of *VEG1* provides an illustration of how the expansion and functional divergence within key regulatory gene families can contribute to the evolution of morphological complexity.

Methods

Plant material and growth conditions. Plants were grown in a greenhouse at 22 °C (day) and 18 °C (night); long-day photoperiods (16 h light/8 h darkness) were maintained with supplementary lighting (400 W Phillips HDK/400 HPI (R) (N). Plants were irrigated with a Hoagland No. 1 solution supplemented with oligoelements[39]. The origins of the *veg1/psfulc-1, det-2, pim-1 and gigas-2* mutants have been previously described[11,16,17,40]. The *psfulc-2* mutant was generated from line NGB5839 by EMS mutagenesis[27]. The *psfulc-3* mutant was isolated by reverse screening in an EMS-mutagenized TILLING population of cultivar Caméor[28]. The *veg2-1* mutant was isolated from fast-neutron mutagenesis of cv. Kaliski[41]. VIGS experiments[29] were performed on the cultivar Boneville. Each mutant was analysed in comparison with its corresponding parental wild-type line.

Gene isolation and phylogenetic analysis. The *PsFULc* complementary DNA was isolated from cDNA from inflorescence apices using PCR techniques; first, a 550-bp cDNA fragment was amplified by RT–PCR with primers derived from the *M. truncatula FULc* sequence and the remaining 5′- and 3′-fragments were amplified by PCR from a pea cDNA library[14] with primers from the vector and from the *PsFULc* cDNA fragment. To analyse the sequences of the *PsFULc* and *PsSEP1* genes in the *psfulc-2* mutant, several overlapping genomic fragments from each gene were amplified from genomic DNA from the mutant and from the parental wild type. All PCR fragments were cloned in pGEM-T easy (Promega) and sequenced. For the phylogenetic tree of the AP1/SQUA/FUL genes, the deduced amino acid sequences were aligned using the CLUSTALW tool in MACVECTOR 12.0 software (MacVector http://www.macvector.com/) and further refined by hand. Pairwise Poisson genetic distances were estimated from the alignment and a neighbour joining best tree was estimated using systematic tie-breaking and rooted to AMtrAGL2, an *Amborella trichopoda* orthologue of SEPALLATA.

Mapping of *PsFULc* and *PsSEP1*. A population of 92 F_2 individuals from a cross between NGB5839 and JI1794 (Sussmilch *et al.*, unpublished) was used to map *PsFULc* and *PsSEP1* in relation to other markers on the bottom of pea linkage group V. The description of the markers used is given in Supplementary Table S1. No recombination was found between *PsFULc* and *PsSEP1*, placing them 20 cM below *COLa* and 40 cM above *FTb1* suggesting that they are less than 1 cM apart.

TILLING. To identify TILLING mutants in *PsFULc*, an EMS mutant population of 4,800 M2 families from *Pisum sativum* cultivar Cameor was screened. DNA isolation and pooling, PCR amplification and mutation detection were performed, as previously described[28]. For primer sequences in this and following sections in Methods see Supplementary Table S1.

PsFULc VIGS. Two *PsFULc*-VIGS plasmids, pCAPE2-PsFULc193 and pCAPE2-PsFULc416, were used for the VIGS experiments. The pCAPE2-PsFULc193 and pCAPE2-PsFULc416 constructs derived from two non-overlapping *PsFULc* cDNA fragments of 193 bp (positions 166–359 from ATG) and 416 bp (positions 490–906 from ATG), respectively, which were generated by PCR and separately cloned into the VIGS vector pCAPE2, using *Xba*I and *Bgl*II cloning sites present in the PCR primers. The plasmid pCAPE2-Con, containing 400 bp of the *GUS* gene, was included for comparison as the VIGS control[42]. Inoculation of plants was carried out as previously described[29] with the following modifications. In each experiment, 12 plants were inoculated which each plasmid. At day 5 after inoculation, plants were decapitated and, for each plant, a single, basal, axial shoot was allowed to proliferate into a new primary shoot. At day 50 after decapitation, the newly outgrown primary shoot of each plant was analysed by scoring the number and nodal position of secondary inflorescences and of vegetative axial shoots exceeding 1 cm in length. Similar results were obtained in different experiments with each of the two *PsFULc*-VIGS plasmids; the data presented in the text corresponds to a representative experiment with the pCAPE2-PsFULc416 plasmid.

Genotyping. To discriminate between the wild-type and *fulc-3* mutant alleles in the TILLING M3 family, we used a dCAPS marker with the primers FULc-dCF and FULc-dCR, which amplify a 228 bp fragment from the *PsFULc* gene (positions 2304–2532 from ATG at the *PsFULc* gene). FULc-dCF creates one mismatch generating a Sau3AI target site in the wild-type product (but not in *psfulc-3*), which after digestion produces two fragments of 26 bp and 202 bp. The *veg1-1* single mutants and *veg1-1 det-2* double mutants used for qPCR analysis were identified from segregating families. *veg1-1* mutants were identified by absence of PCR product from primers FULc-2F and FULc-2R, which amplify a 950-bp fragment from the *PsFULc* gene, with primers TFL1a-1F and TFL1a-Rev03 used for a positive control PCR. *det-2* mutants were determined using a CAPS marker with TFL1a-1F and TFL1a-Rev03 primers, which amplify a 764-bp fragment of the *PsTFL1a/DET* gene, which after digestion with EarI produces two fragments of 143 and 621 bp in the *det-2* mutant because of the *det-2* CAA-CGA substitution[15].

Southern analysis. 10 µg of genomic DNA were digested with restriction enzymes and separated on 0.6%. TAE 1× agarose gels run overnight at 1 V cm^{-1}. Southern Blot analysis was performed by standard methods. The probe was a 550-bp fragment, amplified by PCR from the *PsFULc* cDNA (nucleotides 221–771 from the ATG) and cloned into the pGEM-T easy vector (Promega).

RT-qPCR. Total RNA was extracted using the SV Total RNA isolation system (Promega). RNA concentrations were determined by spectrophotometer analysis using a NanoDrop 8000 (Thermo Scientific). Reverse transcription was conducted in 20 µl with 1 µg of total RNA using the MMLV high-performance reverse transcriptase (Epicenter), according to the manufacturer's instructions. RT-negative (no enzyme) controls were performed to monitor for contamination with genomic DNA. First-strand cDNA was diluted 5 times, and 2 µl was used in each real-time PCR reaction. Real-time PCR reactions using SYBR green chemistry (Sensimix, Quantace, Bioline) were set up with a CAS-1200N robotic liquid handling system

(Corbett Research) and run for 50 cycles in a Rotor-Gene RG3000 (Corbett). Two technical replicates and two-to-three biological replicates were performed for each sample. Relative transcript levels were evaluated using the reference gene *ACTIN*, as previously described[43].

***In situ* hybridization**. RNA *in situ* hybridization with digoxigenin-labelled probes was performed as described[44]. For *PSFULc/VEG1* and *DET*, RNA antisense probes were generated using as substrate a 450-bp fragment of the *PsFULc* cDNA (236–686 from ATG) or a 460-bp fragment of the *DET* cDNA (358–818 from ATG), amplified by PCR and cloned into the pGEM-T Easy vector (Promega). For *PIM*, the probe was generated from a 767-bp of the 3′-region of the *PIM* cDNA, cloned into pGEM3Zf (Promega), as described[14].

Scanning electron microscopy (SEM). Samples for SEM were prepared and analysed as previously described[45].

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Author contributions

F.M. and C.F. conceived the project and designed the experiments together with J.L.W., O.S.L., A.Ben., T.H.N.E. and J.P.B. A.Ber. and C.F. performed the experiments together with V.H., O.S.L., M.D., F.C.S. and S.A.T., F.M. wrote the paper together with J.L.W.

Additional information

Accession codes: The sequence data have been deposited in the NCBI GenBank database under accession codes JN974184 (PsFULC/VEG1 cDNA sequence) and JN974185 (PsFULC/VEG1 genomic sequence).

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Supplementary Information

VEGETATIVE1 Controls Development of the Compound Inflorescence in Pea

Ana Berbel[1,†], Cristina Ferrándiz[1,†], Valérie Hecht[2], Marion Dalmais[3], Ole S. Lund[4§], Frances C. Sussmilch[2], Scott A. Taylor[5], Abdelhafid Bendahmane[3], T.H. Noel Ellis[5,6], José P. Beltrán[1], James L. Weller[2], Francisco Madueño[1,*]

[1]Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas–Universidad Politécnica de Valencia, Valencia 46022, Spain.

[2]School of Plant Science, University of Tasmania, Hobart, Tasmania 7001, Australia

[3]Unité de Recherche en Génomique Végétale, UMR INRA-CNRS, Rue Gaston Crémieux, 91057 Evry Cedex, France.

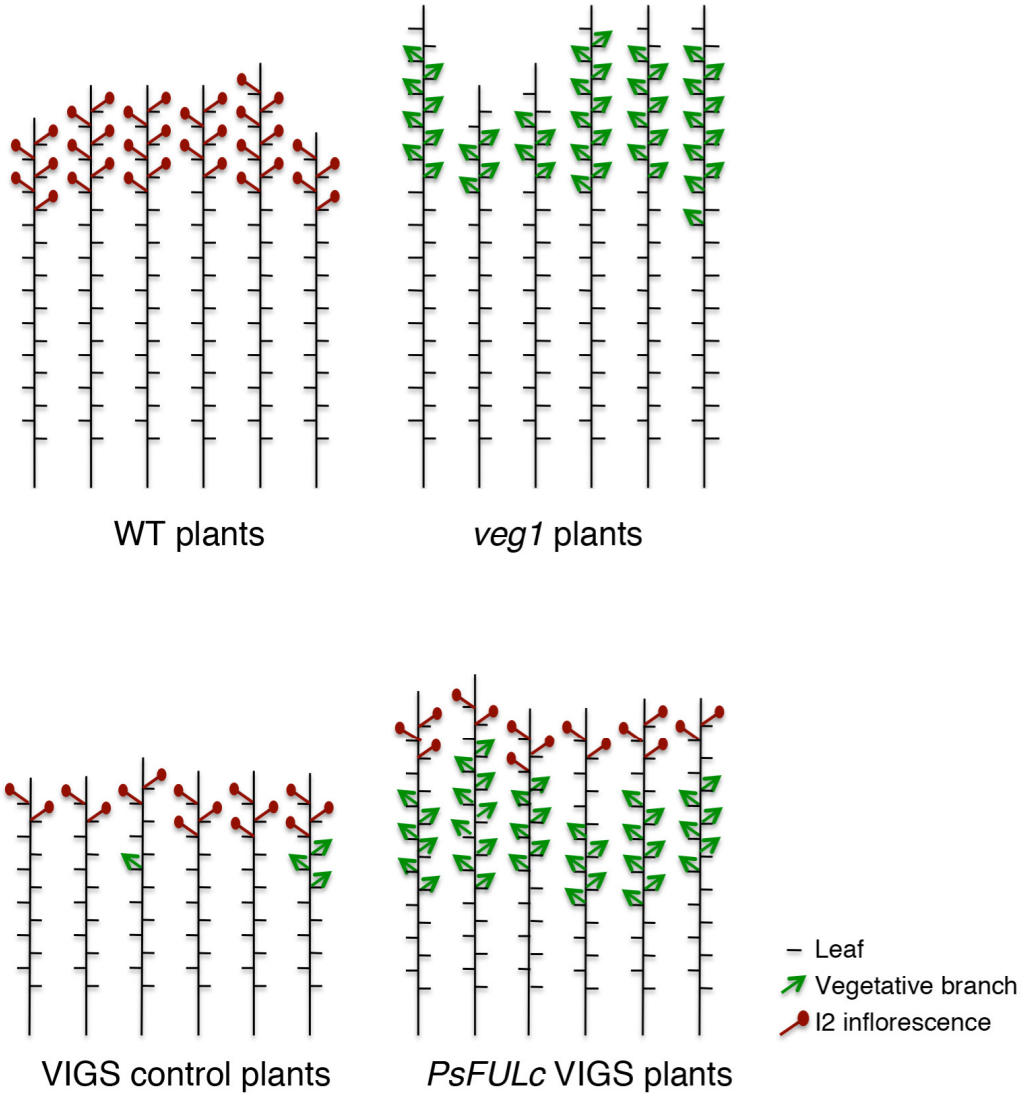
[4]Department of Plant Biology, Danish Institute of Agricultural Sciences, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

[5]John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

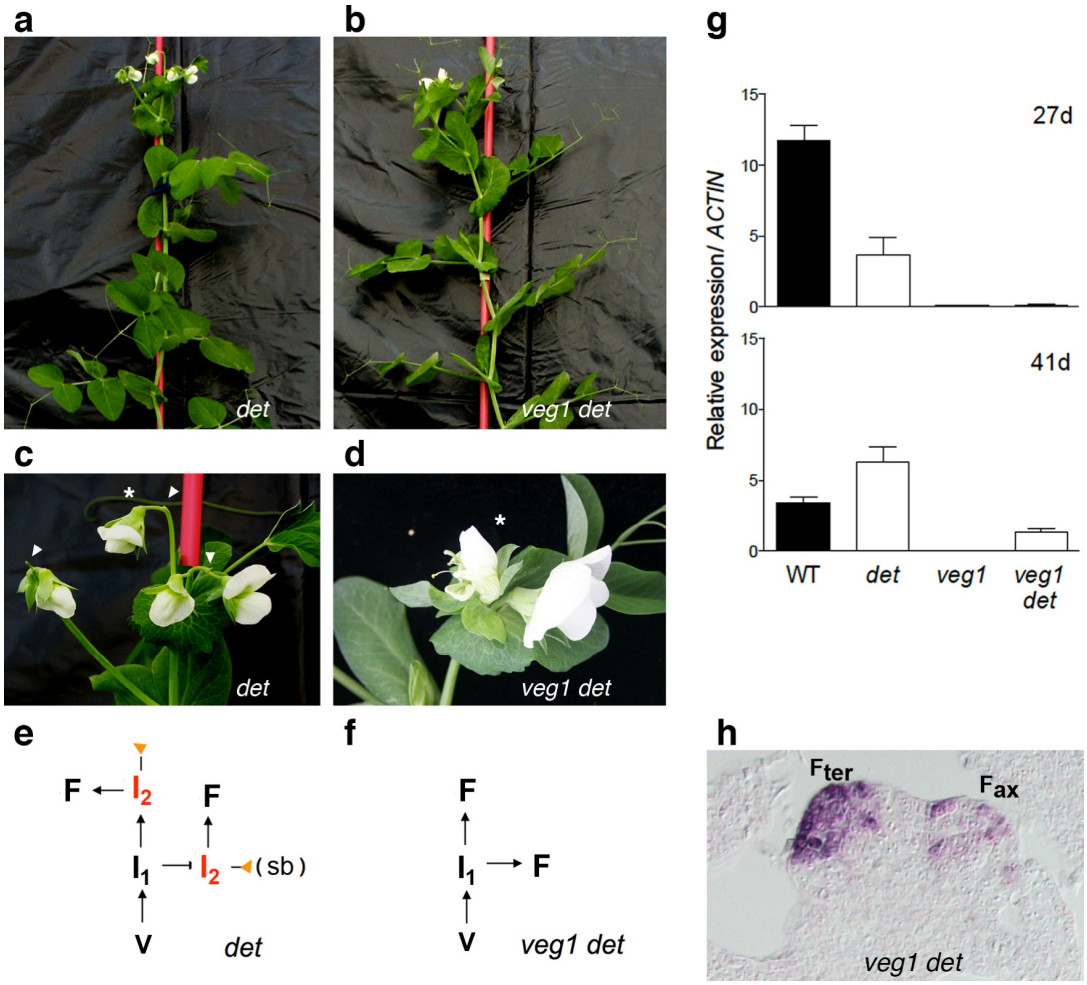
[6]Institute of Biological, Environmental & Rural Sciences Aberystwyth University, Gogerddan Campus, Aberystwyth SY23 3EB , UK

Supplementary Figures S1-S4

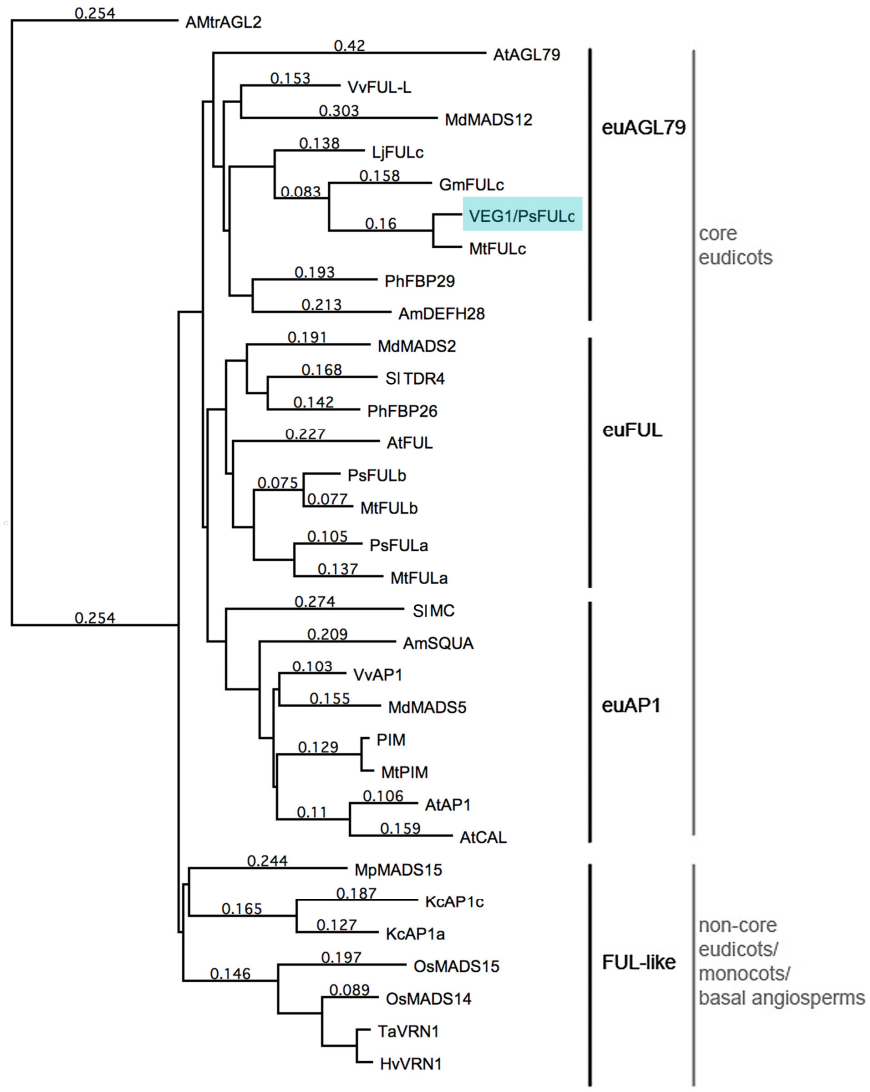
Supplementary Table S1



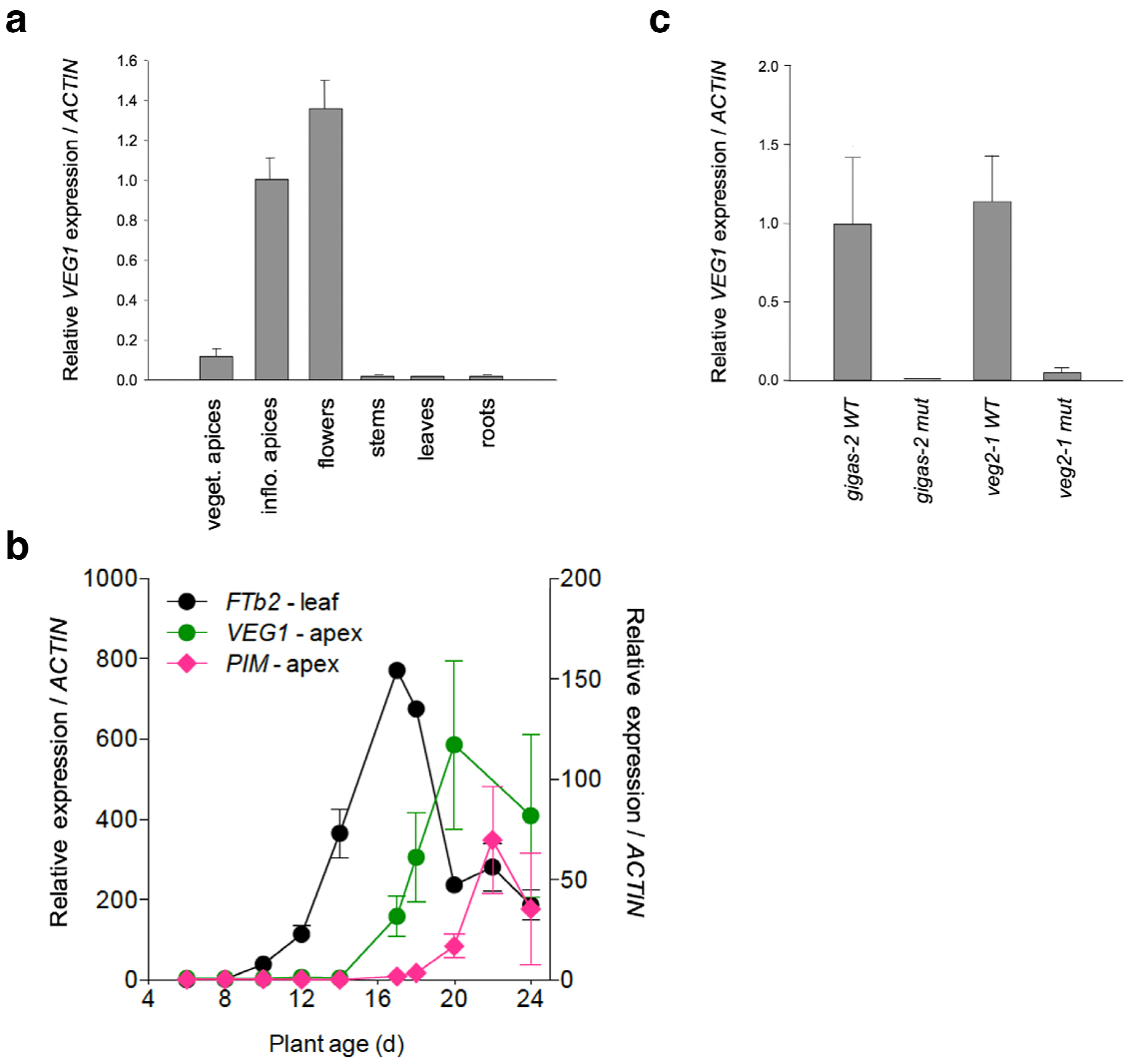
Supplementary Figure S1. In *veg1* mutant and in *PsFULc*VIGS plants branches grow out at equivalents positions where secondary inflorescences (I2) appear in wild-type plants. Top: diagrams representing the architecture of two sets of wild-type or *veg1* plants 10 weeks after germination, indicating the identity of the structure that had grown out in each node. Bottom: diagrams representing the architecture of two sets of control (plants inoculated with a pCAPE2-GUS construct) or *PsFULc*VIGS plants 8 weeks after inoculation. Empty nodes represent that the bud subtended by the corresponding leaf was smaller than 1 cm.



Supplementary Figure S2. The *veg1 det* double mutant produce flowers directly from the primary inflorescence (I1) and shows expression of the floral meristem identity gene *PIM*. (a and b) Upper part of *det* single and *veg1 det* double mutant plants after flowering. **(c)** Close-up of the inflorescence apex of the *det* plant in **(a)**. The primary inflorescence (I1) has produced two lateral secondary inflorescences (I2s) and ends with the formation of a terminal I2 (asterisk). The arrowheads mark the stubs of the I2s. **(d)** Close-up of the inflorescence apex of the *veg1 det* plant in **(b)**. The primary inflorescence has produced a flower from the axil of a leaf and a terminal flower (asterisk). The pedicel of the flowers emerge directly from the I1, not from I2s, as can be noticed by their short length and the absence of stubs. **(e and f)** Schematic representation of meristem identity in the inflorescences of *det* single and *veg1 det* double mutant plants. V: vegetative meristem; F: floral meritem; orange triangles: stubs. **(g)** Relative expression of *PIM* in dissected shoot apices of wild-type, *det* single, *veg1* single, and *veg1 det* double mutant plants. Plants were harvested at two timepoints: at 27 days after sowing, when wild-type and *det* plants had initiated flowering, and at 41 days after sowing, when the *veg1 det* double mutant had initiated flowering. The *veg1* mutant remained vegetative throughout the experiment. Relative transcript levels were determined by RT-qPCR. Values, normalized to the transcript level of the *ACTIN* gene, represent mean ± SE for n = 2 or 3 biological replicates, each consisting of pooled material from two plants. **(h)** *In situ* hybridization of *PIM* mRNA in the inflorescence apex of a *veg1 det* double mutant plant. Expression of *PIM* is observed in the floral meristems formed at terminal position (Fter) and at the axil of a leaf at the node below (Fax).



Supplementary Figure S3. Phylogenetic tree from the predicted amino acid sequences from the AP1/SQUA/FUL family. PsFULC/VEG1 belongs to the euAGL79 clade. AmtrAGL2 (AAX15917), AtAGL79 (AEE77628), VvFUL-L (AAT07448), MdMADS12 (Q7X9I6), LjFULc (GO026211), GmFULc (FG990175), VEG1/PsFULc (JN974184), MtFULc (ABE87778), PhFBP29 (AAK21258), AmDEFH28 (AAK72467), MdMADS2 (AAC83170), SlTDR4 (AAM33098), PhFBP26 (AAF19164), AtFUL (Q38876), PsFULb (JN974186), MtFULb (TC82227), PsFULa (AAX69065), MtFULa (TC84496), SlMC (AAM15774), AmSQUA (CAA45228), VvAP1 (AAT07447), MdMADS5 (ABG85297), PIM (AAL66379), MtPIM (AAZ67068), AtAP1 (AEE34887), AtCAL (AAG50679), MpMADS15 (BAB70749), KcAP1c (AAQ16201), KcAP1a (AAQ16199), OsMADS15 (Q6Q9I2), OsMADS14 (Q7Y023), TaVRN1 (ACI24357), HvVERN1 (ACU33960). Species names are: Amtr, *Amborella thrichopoda*; At, *Arabidopsis thaliana*; Vv, *Vitis vinífera*; Md, *Malus domestica*; Ps, *Pisum sativum*; Mt, *Medicago truncatula*; Ph, *Petunia hybrida*; Am, *Antirrhinum majus*; Sl, *Solanum lycopersicum*; Mp, *Magnolia praecocissima*; Kc, *Crocus sativus*; Os, *Oryza sativa*; Ta, *Triticum aestivum*; Hv, *Hordeum vulgare*.



Supplementary Figure S4. Expression of *VEG1* in the wild type and in other mutants with a *veg1*-like phenotype. (a) Relative *VEG1* transcript levels were determined in different tissues of the wild-type plant. **(b)** Expression of *VEG1* and other genes related to the floral transition during development. Developmental induction of *VEG1* (left axis), *FTb2* (marker for commitment to flowering; right axis) and *PIM* (marker for floral initiation; right axis) in wild type. Relative transcript levels were determined in dissected shoot apices (apex; *VEG1, PIM*) or the uppermost fully expanded leaf (leaf; *FTb2*). **(c)** *VEG1* is not expressed in the *gi-2* and *veg2-1* mutants. Relative *VEG1* transcript levels were determined in dissected shoot apices of *gi-2* and *veg2-1* mutants and their corresponding wild-type parental plants grown under long-day conditions for 28 days, when their corresponding wild types had gone through the floral transition. Relative transcript levels were determined by RT-qPCR. Values, normalized to the transcript level of the *ACTIN* gene, represent mean ± SE for n = 2 or 3 biological replicates, each consisting of pooled material from two plants.

Supplementary Table S1. List of primers used in this study

Gene	Accession	Purpose	Primers	
PsFULc genomic	JN974185	TILLING nested amplification 1	FULc-TILN1F	ATATGGAACAGCATGGAAGATATTTTGG
			FULc-TILN1R	GTTGATAGGAACTGTTTGAGCTTCACCTG
		TILLING nested amplification 2	FULc-TILN2F	ATGAGAGACAAAATCATACAGAACTTAC
			FULc-TILN2R	TCCAATGACCCTCTTGCTTGTATAGCACC
		veg1 genotyping	FULc-2F	CGATGCCTTGAAACCATAGG
			FULc-2R	AATTCCAATGACCCTCTTGC
		Mapping (CAPS, HinfI)	FULc-Map1F	CGATGCCTTGAAACCATAGG
			FULc-Map1R	AATTCCAATGACCCTCTTGC
PsFULc cDNA	JN974184	VIGS 193 bp fragment	FULc-V193F	AGTATTCTTCTGCACCAAGCATG
			FULc-V193R	AGTATTCTTCTGCACCAAGCATG
		VIGS 416 bp fragment	FULc-V416F	ACAGCAAGCTAGCAAAGACAAAG
			FULc-V416R	AGTTTGAACAGAATATGAAATTCTGC
		psfulc-3 dCAPs	FULc-dCF	TTGAATACATGAAGCTAACTGCTAAAGAT
			FULc-dCR	TTTCTTGTTCGGATGCGCTTTAGCGAC
		Southern probe	FULc-SPF	TCATACAGAACTTACTGGAGCTGC
			FULc-SPR	TCATACAGAACTTACTGGAGCTGC
		ISH probe	FULc-ISHF	TGGAGCTACCAATGAAACACAG
			FULc-ISHR	ACCTGCTTCTTCAAATTCCAATG
		RT-qPCR	FULc-qRTF	CACAGAGATTGGTTCCTTCTCTAGC
			FULc-qRTR	CTCTTGCTTGTATAGCACCACTGAG
ACT		RT-qPCR	ACT-F	GTGTCTGGATTGGAGGATCAATC
			ACT-R	GGCCACGCTCATCATATTCA
PIM	AJ291298	RT-qPCR	PIM-qRTF PIM-qRTR	TGCAGCTGAGCAGCAGGTA
				TAGTAAGTAATTTGGATTGACTCCATG
DET	AY340579	ISH probe	DET-ISHF	TTCTATTCAAACAAAGAGCGAGA
			DET-ISHR	ACAACCTCTCTTATTTCTTGAAATG
		RT-qPCR	DET-qRTF	CTTGTGTTGCAGAAAGGGAATC
			DET-qRTR	CAATGGACAGTAACTAACAACACACAG
		det-2 genotyping	TFL1a-1F	CGTTGGTAGAGTCATAGG
			TFL1a-REV03	CTTCTTGCAGCGGTTTCTCT
FTb1	HQ538822	Mapping (CAPS, BccI)	FTb1-Map1F	CTCTATTTCAACTGTCAGCGAC
			FTb1-Map1R	TGCACAATTGTTAGCTTGTTCG
FTb2	HQ538825	RT-qPCR	FTb2-F7	CGACTACCGGGACAGCATTT
			FTb2-R7	CGACTACCGGGACAGCATTT
SEP1	AY884290	Mapping (CAPS, BbvI)	PM6-Map1F	CATCTCTGAAGCATGTTAGG
			PM6-Map1R	TTGTTGAGCTTGACTTGTGG
COLa	AY830921	Mapping (size)	COLa-Map1F	GCTGGATTCAGTTACAATGG
			COLa-Map1R	CCGTTTCCTAGCAACCAAGC