New insights into pea compound inflorescence development: role of *FTc* and *VEGETATIVE1* as regulatory factors

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“If I don’t know something, I do my research”

Louis Pasteur

SUMMARY

Inflorescence architecture determines position and number of flowers (and fruits) in the plant. This affects plant shape, contributing to morphological diversity, and also influences seed yield. Therefore, understanding the genetics behind inflorescence development is relevant not only to plant developmental biology but also to agriculture, to design new breeding strategies.

Most legumes have compound inflorescences, in which the flowers do not form on the main stem but from secondary inflorescences (I2) at the flanks of the main primary inflorescence (I1). This is in contrast to plants with simple inflorescences, such as Arabidopsis, where the flowers directly form at the I1. Pea (*Pisum sativum*) belongs to the Fabaceae family and the galegoid clade of legumes and has a compound inflorescence.

It is well known that *VEGETATIVE1/ FULc* (*VEG1*) encodes a transcription factor that specifies the identity of the I2 meristem in legumes, but it is still unknown how and through which genes VEG1 controls I2 development and the genetic pathways in which it is involved. In this work, we aimed to identify regulatory targets of VEG1. For that, we compared the transcriptomes of inflorescence apices from wild type and pea mutants with defects in inflorescence development: *proliferating inflorescence meristems* (*pim* - with multiple I2 meristems), *veg1* and *vegetative2* (*veg2*), none of which produce neither I2 meristems nor flowers). Using this approach, we have isolated I2-expressed meristem genes and identified some possible targets of VEG1, among them some genes that seem promising tools to improve yield in legumes.

FLOWERING LOCUS T (*FT*) is a key regulator of the photoperiod inductive pathway that controls flowering time in Arabidopsis. In legumes, the *FT* clade has diversified into three subclades: *FTa*, *FTb* and *FTc*. Pea *FTc* is distant phylogenetically from the other *FTs* and has an unusual expression pattern, being expressed only at the inflorescence apex. In this work we have characterized pea *ftc* mutants and used them to analyze the genetic interactions of *FTc* with *DETERMINATE* and *LATE FLOWERING*, pea homologues of *TERMINAL FLOWER 1* of Arabidopsis. This analysis has revealed a function of *FTc* in the control of flowering and, interestingly, of I2 meristem development, this second function being possibly mediated through *FTc* regulation of *VEG1* expression.

RESUM

L'arquitectura de la inflorescència determina la posició i el nombre de flors (i fruits) en la planta. Això afecta a la forma de la planta, la qual cosa contribueix a la diversitat morfològica i influeix en la producció de llavors. Per tant, comprendre com funciona la genètica que està darrere del desenvolupament de la inflorescència és rellevant no sols per a saber més sobre la biologia del desenvolupament de les plantes, sinó també per a l'agricultura, amb la finalitat de dissenyar noves estratègies per a la millora de plantes.

La majoria de les lleguminoses tenen inflorescències compostes, en les quals les flors no es formen en la tija principal sinó a partir d'inflorescències secundàries (I2) en els flancs de la inflorescència primària o principal (I1). Això contrasta amb les plantes amb inflorescències simples, com Arabidopsis, on les flors es formen directament a partir de l'I1. El pésol (Pisum sativum) pertany a la família de les Fabaceae i al clade galegoide de les lleguminoses i té una inflorescència composta.

És ben sabut que *VEGETATIVE1/ FULc* (*VEG1*) codifica un factor de transcripció que especifica la identitat del meristemo I2 en lleguminoses, però encara es desconeix com i a través de quins gens VEG1 controla el desenvolupament de l'I2 i les vies genètiques en les quals està involucrat. En aquest treball, el nostre objectiu va ser identificar les dianes reguladores de VEG1. Per a això, comparem els transcriptomas provinents d'àpexs d'inflorescència del control silvestre (wild-type) amb els de mutants de pésol amb defectes en el desenvolupament de la inflorescència: *proliferating inflorescence meristems* (*pim* - amb múltiples meristemos I2), v*eg1* i *vegetative2* (*veg2* - cap dels quals produeix ni meristemos I2 ni flors). Usant aquest enfocament, hem aïllat alguns gens que s'expressen en l'I2 i identificat algunes possibles dianes de VEG1, entre elles alguns gens que semblen prometedors per a ser usats a manera d'eines genètiques per a la millora del rendiment en la producció en lleguminoses.

FLOWERING LOCUS T (*FT*) és un regulador clau en la xarxa genètica del fotoperíode que controla el temps de floració en Arabidopsis. En lleguminoses, el clade *FT* s'ha diversificat en tres subclades: *FTa*, *FTb* i *FTc*. En pésol, el gen *FTc* es troba distant filogenéticamente dels altres gens FT i té un patró d'expressió inusual, ja que només s'expressa en l'àpex de la inflorescència. En aquest treball hem caracteritzat mutants *ftc* de pésol i els hem utilitzats per a analitzar les interaccions genètiques de *FTc* amb *DETERMINATE* i *LATE FLOWERING*, que són els homòlegs en pésol de *TERMINAL FLOWER 1* d' Arabidopsis. Aquest anàlisi ha revelat una funció de FTc en el control de la floració i, curiosament, també en el desenvolupament del meristemo I2, estant aquesta segona funció possiblement mediada per la regulació de *FTc* en l'expressió de *VEG1*.

RESUMEN

La arquitectura de la inflorescencia determina la posición y el número de flores (y frutos) en la planta. Esto afecta a la forma de la planta, lo que contribuye a la diversidad morfológica e influye en la producción de semillas. Por lo tanto, comprender cómo funciona la genética que está detrás del desarrollo de la inflorescencia es relevante no solo para saber más sobre la biología del desarrollo de las plantas, sino también para la agricultura, con el fin de diseñar nuevas estrategias para la mejora de plantas.

La mayoría de las leguminosas tienen inflorescencias compuestas, en las que las flores no se forman en el tallo principal sino a partir de inflorescencias secundarias (I2) en los flancos de la inflorescencia primaria o principal (I1). Esto contrasta con las plantas con inflorescencias simples, como Arabidopsis, donde las flores se forman directamente a partir del I1. El guisante (Pisum sativum) pertenece a la familia de las Fabaceae y al clado galegoide de las leguminosas y tiene una inflorescencia compuesta.

Es bien sabido que *VEGETATIVE1/ FULc* (*VEG1*) codifica un factor de transcripción que especifica la identidad del meristemo I2 en leguminosas, pero aún se desconoce cómo y a través de qué genes VEG1 controla el desarrollo del I2 y las vías genéticas en las que está involucrado. En este trabajo, nuestro objetivo fue identificar las dianas regulatorias de VEG1. Para ello, comparamos los transcriptomas provenientes de ápices de inflorescencia del control silvestre (wild-type) con los de mutantes de guisante con defectos en el desarrollo de la inflorescencia: *proliferating inflorescence meristems* (*pim* - con múltiples meristemos I2), *veg1* y *vegetative2* (veg2 - ninguno de los cuales produce ni meristemos I2 ni flores). Usando este enfoque, hemos aislado algunos genes que se expresan en el I2 e identificado algunas posibles dianas de VEG1, entre ellas algunos genes que parecen prometedores para ser usados a modo de herramientas genéticas para la mejora del rendimiento en la producción en leguminosas.

FLOWERING LOCUS T (*FT*) es un regulador clave en la red genética del fotoperíodo que controla el tiempo de floración en Arabidopsis. En leguminosas, el clado *FT* se ha diversificado en tres subclados: *FTa*, *FTb* y *FTc*. En guisante,el gen *FTc* se encuentra distante filogenéticamente de los otros genes *FT* y tiene un patrón de expresión inusual, ya que solo se expresa en el ápice de la inflorescencia. En este trabajo hemos caracterizado mutantes *ftc* de guisante y los hemos utilizado para analizar las interacciones genéticas de *FTc* con *DETERMINATE* y *LATE FLOWERING*, que son los homólogos en guisante de *TERMINAL FLOWER 1* de Arabidopsis. Este análisis ha revelado una función de *FTc* en el control de la floración y, curiosamente, también en el desarrollo del meristemo I2, estando esta segunda función posiblemente mediada por la regulación de *FTc* en la expresión de *VEG1*.

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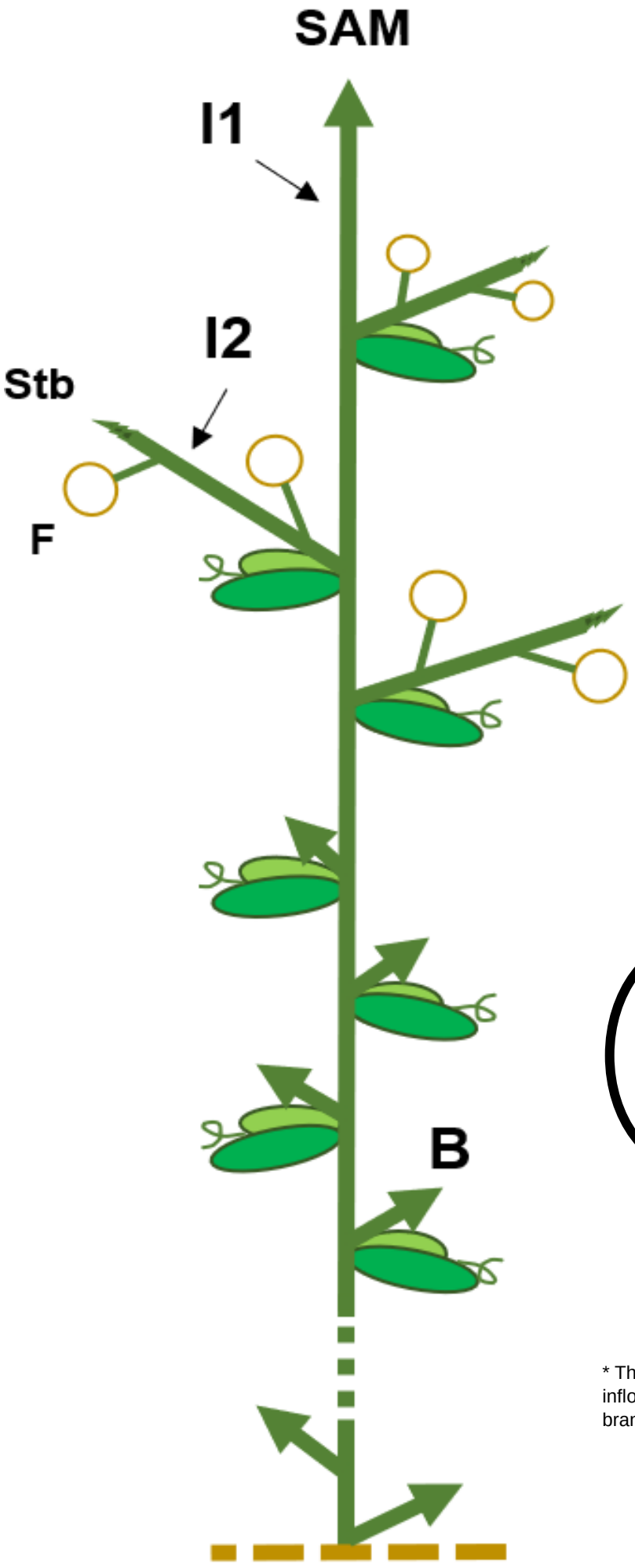
“The art and science of asking questions is the source of all knowledge”

Thomas Berger



General Introduction

* This diagram represents a wild-type pea plant. I1, primary inflorescence; I2, secondary inflorescence; F, flower; B, branch; Stb, stub.

The importance of crop research in the 21st century

Relationship between population growth and world productive capacity

Human population is growing steadily, and currently we are near 8,000 million of people in the world, although it seems that the growth rate is decreasing with respect to previous years. Nevertheless, it is expected that we will reach 9,000 million in 2050 and more than 10.000 million by the end of this century (Hickey et al., 2019). Currently, most of the population has the necessary resources to enjoy a balanced diet, although, according to data published by the Food and Agriculture Organization of the United Nations (FAO) more than 800 million suffer from malnutrition (Boliko et al. 2019), a number that has been increasing over the last 4 years, and that is similar to the data for the previous decade.

Both the population growth and the probable increase of food shortages linked to globalisation practices challenge the capacity of people to properly feed the world. It is thus important to respond with appropriate countermeasures, increasing the amount of food available, but also with the generation of a sustainable production system. In this context, it is a priority to promote the use of crops rich in proteins such as cereals and legumes (Tilman and Clark, 2015; Poore and Nemeck, 2018; Shepon et al., 2018; Eshed and Lippman, 2019).

On the other hand, humanity must confront another problem that has been notably accentuated in recent decades, which is the increasingly well-known global climate change. One of the clearest evidences of this phenomenon is the increase in the average temperature of the planet, which has been estimated to be 0.85 ºC since 1880, if we consider the first known reliable measurements (Intergovernmental Panel on Climate Change; 2014). As a result, extreme climate phenomena occur with greater frequency, including changes in precipitation patterns and extreme temperature swings, among others (Van Houtan et al., 2021). Furthermore, an increase in the proportion of non-arable land –at least by normal means- is produced, which greatly conditions the global food supply, and different public organisations, such as FAO, have warned that food supply is susceptible to worsen significantly if the appropriate measures are not taken (http://www.fao.org/3/i5188e/i5188e.pdf). Investing in plant science research is critical for future advancements in agricultural practices, and for this, biotechnology programs constitute the most advanced tool to address these problems (Hickey et al., 2019). In this regard, applied biotechnology provides with a number of solutions and possible approaches to tackle current and future challenges: global control of weeds and insect pests, improvement of crop tolerance to drought and increased salinity in soils, boosting nitrogen

efficiency usage, strengthening of crop resistance to diseases and increasing fruit production with improved nutritional properties, among others (Bailey-Serres, 2019).

The impact of legumes on world agriculture

Legumes are an essential source of nutrients in the human diet. If we consider the current set of crops that are being used for human consumption, legumes are the second most widespread crop after cereals, and they are of paramount importance for animal feed or forage (Waghorn and Clark, 2004; González-Bernal and Rubiales, 2016; Villalba et al., 2019). Cereals surpass legumes in productive capacity by weight, but growing sequential and recurrently cereal and legumes in crop rotation systems brings notable advantages in total efficiency for both, as legumes strongly improve the access to nitrogen in the soil (Jensen et al., 2020; Rodríguez et al., 2021). Legumes can incorporate atmospheric nitrogen to the soil thanks to their symbiotic association with certain soil nitrogen-fixing bacteria, called rhizobia (Soltis et al., 1999; Sprent, 2008). As a result of this symbiosis, characteristic structures called nodules are produced in the roots of the plant, where the nitrogen fixation process takes place (Lindström et al., 2020). Nodules, minimises the emission of N2O from the production and application of synthetic N fertilizers, N2O being the most important Greenhouse gas from arable agriculture (Canfield et al., 2010).

On the other hand, in terms of nutritional quality, grain legumes are rich in protein (in some cases up to three times as high as cereals), fibre and carbohydrates. Moreover, they have low contents of lipids (except for soybeans, peanuts, and lupins). These nutritional properties make them healthy foods and highly recommended for human consumption (https://fdc.nal.usda.gov), preventing multiple diseases like nutritional deficiencies, obesity, diabetes, and hypertension among others (Beltrán and Cañas, 2018).

Legume research in the genomics era

Plant genetics research has benefited greatly in the last decades by the increasing availability of sequenced genomes and annotated transcriptomes. The first legume genomes to be sequenced were those of *Lotus japonicus*, soybean and *Medicago truncatula*, and since then many other have been added to the list, including the recent publication of the pea genome (Sato et al., 2008; Tang et al., 2014; Yang et al., 2018; Kreplak et al., 2019).

In addition, reverse genetic tools such as public collection of mutants in soybean, Lotus, Medicago or pea (Soybean: soybase.org; Medicago: medicago-mutant.dasnr.okstate.edu; Lotus: lotus.au.dk; Pea: urgv.evry.inra.fr/UTILLd) and the increasing use of genome editing

by means of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPr)/ CRISPr associated protein 9 (Cas9) technology (Bhowmik et al., 2021; Rao and Wang, 2021) have allowed numerous advances, adding to the previous alternatives an additional and powerful tool for the study of genes and to generate new varieties with improved characteristics.

On the other hand, obtaining plants that overexpress a gene of interest is also a useful technique, especially for the study of genes that carry out their function redundantly with others, and that do not cause phenotypic effects when individually mutated (single mutants; Zhang, 2003). An alternative for the analysis of redundant genes is the generation of transgenic plants that simultaneously silence homologous genes by RNA interference (RNAi). RNAi-based methods have been widely used for functional characterization of individual genes, especially before the generalization of genome editing techniques (Younis et al., 2014), but they also allow to downregulate multiple targets to uncover redundancy effects, thus hastening the research workflow. However, this type of analysis implies obtaining stable transgenic lines, therefore limiting its use for only those species that are transformable (Chabaud et al., 1996). For those that are recalcitrant to genetic transformation, as it is the case with some legumes such as pea, an alternative for silencing gene expression is the virus-induced gene silencing (VIGS), a transient post-transcriptional gene silencing (PTGS) method that allows the generation of transient loss-of-function mutant for phenotype evaluation (Constantin et al., 2004).

Model organisms for plant science research

The model plant for which more information has been available for a long time, and currently continues to be one of the most important model species, is *Arabidopsis thaliana*, which belongs to the Brassicaceae family. Arabidopsis has a short-life cycle, which varies between 5 and 8 weeks depending on growth conditions, and it has a small genome (135 megabases) that hardly harbours repetitive sequences in addition to being diploid (Cheng et al., 2017). Moreover, Arabidopsis can tolerate a high degree of homozygosity and is self-fertile (Woodward and Bartel, 2018). All these attributes, along with the ease with which it is transformed by the *Agrobacterium tumefaciens* system (Clough and Bent, 1998), have allowed researchers to gain more in-depth knowledge into this species. Moreover, part of the biological knowledge that has been and continues to be obtained, serves as the basis for significant advancements in the research of other plants, both model plants and also species of great agricultural interest such as cereals, legumes and solanales.

Consequently, a huge amount of information on the genetics, physiology, metabolism and ecology of Arabidopsis is currently available, covering every different research area including flowering and plant development studies. Many of the data from Arabidopsis can be extrapolated to phylogenetically close species, which share a high proportion of conserved genes. However, other species that are phylogenetically distant require comparative studies to assess the degree of conservation of similar processes or to understand divergent mechanisms of development and evolutionary novelties underlying biodiversity (Chang et al., 2016).

Legumes, members of the Fabaceae clade, are relatively distant from Arabidopsis in terms of phylogenetic relationship, since they both belong to the Rosid group of Eudicotiledoneus plants, but are classified into different orders (Simpson, 2010). Legumes possess a number of distinctive characteristics not shared with Arabidopsis, such as the ability to symbiotically fix nitrogen, and developmental characters like compound leaf, distinct inflorescence types or architectures, papilionoid flowers and the typical legume fruit type, among many others (Simpson, 2010; Gepts et al., 2005).

Consequently, all these characters and developmental processes cannot be entirely understood by extrapolating the current knowledge from Arabidopsis, so new model plants must be sought. *Medicago truncatula* is one of the most studied plants among legumes, which has its genome sequenced and its own database (Young et al., 2011), with relatively high amount of information if we compare to that of other species of the same clade (https://www.noble.org/blog/top-5-medicago-resources/). Other frequently used model legume plants are *Lotus japonicus* and *Gycine max* (Chang et al., 2016).

Pea (*Pisum sativum*) is also a model plant for legumes. Mendel already used pea for his genetic studies (Mendel, 1866; Miko, 2008; van Dijk and Ellis, 2016; van Dijk et al., 2018) and the information that is available from all the different studies of many scientists that have worked with this species is especially rich and elaborate as regards to plant architecture, the time of flowering and nitrogen fixation (Engvild, 1987; Voisin et al., 2004; Flores-Félix et al., 2020; Enrico et al., 2020). Besides, the transcriptome has been available for a few years, which has made it possible to carry out studies based on molecular genetic approaches, such as expression analysis and functional studies with VIGS (Tayeh et al., 2015). Its genome has also been recently sequenced (Kreplak et al., 2019). Furthermore, it is a plant with a relatively short life cycle, self-fertile and easily out-crossed, which favours genetic studies. All these characteristics, together with the fact that it is an important crop, and as such it has numerous beneficial properties, has prompted the realization of numerous studies in recent years, among which the present PhD thesis is included.

Plant architecture

The meristem

In nature we can find a great variety of plants, with different shapes, sizes and architectures. Understanding the genetic bases of this diversity and associating each feature with specific gene functions and regulatory elements is one of the greatest challenges for researchers working on plant development.

All the organs that form the aerial part of most vascular plants develop from the Shoot Apical Meristem (SAM). The SAM can be divided into three functional zones (Tucker et al, 2007): the central zone (CZ), the peripheral zone (PZ), and the rib zone (RZ). The CZ contains a niche of pluripotent stem cells with low division rates that provide initial cells for the rest of the meristematic zones. At the flanks of the SAM, in the PZ, the pluripotent cells that come from the central zone divide rapidly to generate the founder cells for lateral organ formation, such as leaves and flowers. The RZ is located below the CZ and also contains pluripotent cells that divide to support stem growth and vascular differentiation. Right below the CZ a fourth domain is found, the so-called Organizing Centre (OC), whose main function is to maintain the pluripotency of the stem cells and control cell division rates in the CZ (Gaillochet et al., 2015).

Genetic pathways for meristem maintenance

WUSCHEL (*WUS*) and *SHOOTMERISTEMLESS* (*STM*) are two essential genes for SAM maintenance and they are both involved in the regulation of SAM activity. *WUS* is the founding member of the WOX family (*WUSCHEL*-like homeobox) of homeobox transcription factors and one of its functions consists in keeping the population of pluripotent cells of the CZ stable (Laux et al., 1996; Mayer et al., 1998). *WUS* is transcribed in the OC but the translated product moves to the CZ where it activates the expression of *CLAVATA3* (*CLV3*). The CLV3 peptide is then secreted and, through binding to membrane LRRRKs (Leucine Rich Repeat Receptor Kinase; Brand et al., 2000; Ogawa et al., 2008), like CLAVATA1 (*CLV1*), CLAVATA2 (CLV2) and CORYNE (CRN) (Clark et al., 1997; Jeong et al., 1999; Muller et al., 2008), triggers a signalling cascade that feeds back negatively on *WUS* to restrict its expression to the OC. In this way, the activity of the meristem is maintained by means of an activation-repression loop between *WUS*-*CLV3* that functionally delimits the zones that shape the SAM. This mechanism is conserved in Arabidopsis, rice, corn, tomato and in many other species (Pautler et al, 2013).

Like *WUS*, *STM* is a homeobox gene, which is also implicated in SAM maintenance (Long et al., 1996). *STM* encodes a transcription factor, belonging to the KNOX family (Knotted1-like homeobox) and it is expressed in most parts of the meristem (Lenhard et al., 2002). One of its functions is to promote the pluripotent nature of meristematic cells, which allows maintaining an adequate number of undifferentiated cells in the peripheral and rib areas of the SAM (Long et al., 1996).

The *WUS-CLV* loop along with *STM* maintain SAM function and activity. However, for organogenesis to initiate in an orderly manner, it is necessary the participation of other regulatory elements, including several plant hormones such as auxins, gibberellins and cytokinins (Raspor, et al., 2021). Auxins have a great impact on development and organogenesis. Before an organ primordium is initiated, an increase in auxin levels occurs in the corresponding area. PINFORMED 1 is an auxin carrier protein that localizes polarly in the plant cell membrane and controls auxin flux direction. In the case of *Arabidopsis thaliana*, *PINFORMED1* (*PIN1*) acts as a positioning marker for the development of organ primordia, in a process mediated by members of the *PLETHORA* (*PLT*) family of transcription factors (Prasad et al., 2011; Reinhardt et al., 2003). In addition to auxins, cytokinin (CK) and gibberellins (GAs) also play a role in the maintenance of meristems. CKs promote cell division and play a role in meristem maintenance, activating *WUS* and *STM* (Kurakawa et al., 2007; Gordon et al., 2009), while GAs promotes cell differentiation and cell elongation (Shani et al., 2006).

Organogenesis has a great impact on the architecture of the plant as it determines the arrangement of the leaves on the stem, also known as phyllotaxis, and therefore the position of the axillary meristems (AM) that develop in the axils of the leaves (Reinhardt et al., 2003). Those AMs will in turn give rise to axillary buds or branches at a later stage of development, thus shaping the aerial structure of the adult plant.

The axillary meristems

Cell proliferation at the SAM promotes upward or vertical growth of the plant, however there are two other types of meristems that control lateral growth. On one hand, the vascular cambium allows secondary growth (growth in thickness of the main stem) (Elo et al., 2009). On the other hand, AMs promote growth through the formation of buds that can give rise to lateral branches, which develops in a similar way as the main stem, modifying the architecture of the plant (Wang et al., 2018).

During the vegetative phase of the plant, the AMs appear at the junction of the stem with the adaxial zone of the leaves. After their formation, they transform into buds that remain initially dormant. If the right conditions are met, these buds start growing and develop into branches, either of vegetative nature or inflorescences (Müller et al., 2011). A number of hormones and other regulatory components have been shown to contribute to the control of AM activity, including polar auxin transport (PAT), strigolactones (SL) and abscisic acid (ABA). All these signals inhibit the growth of dormant buds (Müller et al., 2011). On the contrary, cytokinins (CKs) and the presence of sugars promote their activation (Wang et al., 2018; Xue et al., 2020).

The AMs behave differently depending on the plant species, the environmental conditions and the moment they become active. In the case of Arabidopsis, AMs usually remain dormant until floral transition, due to apical dominance from the main inflorescence. Once the floral transition takes place, the dormant buds develop into branches (Wang et al., 2018). On the contrary, in tomato, a species with sympodial growth, growth of the main shoot is determinate and it ceases after an initial growth phase (Lippman et al., 2008). Then, development of AM located in the axil of the youngest leaf is activated forming a new inflorescence. In the case of grasses, although they can vary depending on species, the SAM usually remains at the base of the plant producing leaves, which bear AM in their axils (Li et al., 2003). Those AMs are activated and form new shoots or branches depending on the species. Finally, in legumes the AMs can develop forming buds in most of the nodes as well as in the cotyledons depending of the species. In the case of *Lotus japonicus*, the AM are activated and keep proliferating, constantly producing branches during plant development (de G Alvarez et al., 2006). In other species of legumes such as pea, the axillary buds develop at most nodes, but they normally remain dormant until the optimal conditions are met, usually until flowering (Beveridge et al., 2003). Therefore, the architecture of a plant species varies greatly depending on the type of plant and its growth habit.

The inflorescences

Much of the morphological diversity of the aerial part of the plant or architecture depends on the structures from which the flowers develop, called inflorescences (Weberling, 1989). Understanding the genetic pathways that control the architecture of inflorescences is important to reveal the structural mechanisms that allow their development. Moreover, the inflorescence architecture does condition the production of flowers and fruits, having a potentially great impact on crop yield.

Depending on the fate of the main inflorescence meristem, inflorescences can be classified as determinate or indeterminate. Determined inflorescences are those in which the growth of the SAM ends with the formation of a terminal flower. In contrast, if the SAM continues to produce flowers until senescence occurs, the plant is said to possess indeterminate growth or indeterminate inflorescences (Weberling, 1992; Benlloch et al., 2007).

According to their complexity, inflorescences can be classified as simple or compound. Plants with a simple inflorescence develop flowers directly from the SAM, as is the case of *Arabidopsis thaliana*. On the other hand, in plants with compound inflorescences, flowers do not develop directly from the main apex but from secondary, tertiary or higher order inflorescences or axes; legumes and cereals have this type of compound inflorescence (Weberling, 1989; Weberling, 1992; Kellogg et al., 2007; Teo et al., 2013).

The meristem identity genes and inflorescence architecture

One of the key aspects that controls inflorescence development is the identity or nature of the meristems that form at the inflorescence apex. The identity of such meristems determines the position from which the branches and flowers will develop (Benlloch et al., 2015), therefore shaping the architecture of the plants. Here, I summarize what is known about the genetic networks controlling meristem identity in Arabidopsis, a model for simple, indeterminate inflorescences, and legumes, the focus of this thesis.

Arabidopsis thaliana

In Arabidopsis, when the floral transition occurs, the vegetative SAM transforms into an inflorescence meristem, producing floral meristems on its flanks that later develop into flowers. Among the meristem identity genes that are responsible for inflorescence and flower specification we find *TERMINAL FLOWER* 1 (*TFL1*), *LEAFY* (*LFY*), and *APETALA 1* (*AP1*) (Mandel et al., 1992; Weigel et al., 1992; Shannon and Meeks-Wagner, 1993; Bradley et al., 1997; Liljegren et al., 1999). *TFL1* specifies inflorescence meristem identity, contributing to the maintenance of the SAM in an undifferentiated state, while *LFY* and *AP1* specify floral meristem identity and promote cell differentiation and floral organ development. Hence, *TFL1* and *AP1/LFY* behave antagonistically in the control of meristem activity (Ratcliffe et al., 1999) in order to establish a correct inflorescence development (Gustafson-Brown et al., 1994; Wagner et al., 1999; Kaufmann et al., 2010).

LFY is a gene that codes for a transcription factor and it is expressed in floral meristems from very early stages, already in the groups of cells that appear at the flanks of the inflorescence SAM that will later develop into floral meristems (floral *anlagen*) (Weigel et al.,

1992; Blázquez et al., 1997). In addition, it is worth mentioning that *LFY* is also expressed in young leaves, similar to its legume homologues (Hofer et al., 1997; Blázquez et al., 1997). In the floral meristems, LFY activates *AP1* and both contribute to the robust specification of floral meristems (Wagner et al., 1999). *lfy* mutants display a partial loss of floral identity, with the first flowers replaced by branch-like structures with mixed vegetative and floral characters (Schultz and Haughn, 1991; Maizel et al., 2005). Later in development, some of these lateral structures acquire some floral identity, mainly due to the activation of *AP1* (Huala and Sussex, 1992) in a *LFY*-independent manner.

AP1 is a floral meristem identity gene and encodes a MADS-box transcription factor that is expressed in floral meristems (Mandel et al., 1992; Weigel and Meyerowitz, 1993*).* In the *ap1* mutants, the sepals are replaced by bracts and the petals are usually lost. New axillary meristems form, subtended by the bract-like sepals, and give rise to new floral structures that reiterate this pattern. The resulting structures are similar to branched flowers, what has been interpreted as a partial loss of floral identity (Irish and Sussex, 1990; Bowman et al., 1993). *AP1* fulfils its function in floral meristem specification redundantly with *CAULIFLOWER* (*CAL*), a closely related gene that also belongs to the MADS-box family of transcription factors (Kempin et al., 1995). In the double mutant *ap1 cal* a complete loss of floral identity is observed, and inflorescence meristems produce lateral meristems that, instead of acquiring floral identity, behave again as inflorescence meristems that form new meristems in spiral arrangement, reiterating this pattern almost indefinitely and thus producing curd-like structures composed of proliferating meristems that resemble cauliflowers (Mandel and Yanofsky, 1995; William et al., 2004).

TFL1 is a gene that codes for a protein from the family of phosphatidyl ethanolamine binding proteins (PEBP). It is expressed in a group of cells of the SAM, at low levels during the vegetative phase and at a higher level after floral transition (Bradley et al., 1997). *TFL1* contributes to the regulation of flowering time and specifies the identity of the inflorescence meristem. *TFL1* acts by repressing the floral transition, thus, *tfl1* loss-of function mutants show an early flowering phenotype. Furthermore, *TFL1* represses *AP1* and *LFY*, preventing both genes from being expressed in the SAM after floral transition and thus maintaining the identity of the inflorescence meristem. In *tfl1* mutants (Ohshima et al., 1997; Schultz and Haughn, 1993; Liljegren et al., 1999), *AP1* and *LFY* are expressed ectopically in the SAM and the plant gets determined with the formation of a terminal flower (Shannon and Meeks-Wagner, 1991; Weigel et al., 1992; Gustafson-Brown et al., 1994), consequently ceasing the indeterminate growth of the inflorescence (Alvarez et al., 1992). Conversely, *LFY* and *AP1/CAL* are also involved in the control of *TFL1* expression. Both *AP1* and *LFY,* when expressed constitutively, repress *TFL1* expression in the SAM and, conversely, *TFL1*

expression domain expands to the whole cauliflower-like structures of *ap1 cal* double mutants (Liljegren et al., 1999; Ratcliffe et al., 1999; Weigel et al., 1992).

Pisum sativum

Within legumes, pea (*Pisum sativum*) is one of the species in which the genetic network that orchestrates inflorescence architecture is best known. That network shows great similarity with that involved in floral and inflorescence meristem identity in Arabidopsis, but includes new elements related to the higher level of complexity of the compound inflorescence. Among the genes controlling flower and inflorescence development in pea we find the homologues of *LFY*, *AP1* and *TFL1*. *UNIFOLIATA* (*UNI*) and *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*) are the homologues of *LFY* and *AP1* respectively. As their counterparts, both are key genes that control the identity of the floral meristems (Hofer et al., 1997; Berbel et al., 2001; Taylor et al., 2002; Benlloch et al., 2015).

PIM is a MADS transcription factor closely related to AP1, whose expression is located initially in the floral meristems and later in the sepals and petals of the developing flowers (Berbel et al., 2001, 2012; Taylor et al., 2002). In *pim*, the floral meristems produced by the I2 in the first reproductive nodes are replaced by proliferating structures with I2-like identity, after several rounds of division some meristems produced by the *pim* I2s end up acquiring floral identity and give rise to abnormal flowers displaying defects in the sepals and petals (Berbel et al., 2001, 2012; Taylor et al., 2002). *PIM* function is conserved in other legume species, such as *Medicago truncatula* and *Lotus japonicus* (Benlloch et al., 2006; Dong et al., 2005)

UNI, the homologue of *LFY* in pea, works together with *PIM* to specify floral meristem identity. *UNI* is expressed in very young floral meristem primordia and also in the I2, although in the *uni* mutants there are no visible defects in these structures (Wang et al., 2008; Dong et al., 2005). Furthermore, *UNI* has a major role in the development of compound leaves in pea, since in the *uni* mutants the leaves have a reduced number of leaflets (Gourlay et al., 2000). As in *lfy*, the flowers that are produced in *uni* are abnormal and neither petals nor stamens are produced. Furthermore, the common primordia, ephemeral meristems that form between sepal and carpel primordia in early stages of floral development and that later produce petals and stamens, are replaced by new floral meristems that reiterate this pattern, thus producing indeterminate floral structures (Hofer et al., 1997). The function of UNI appears also to be conserved in legumes like *Medicago truncatula* and *Lotus japonicus* (Dong et al., 2005; Cheng et al., 2018)

The identity of the primary inflorescence (I1) meristem in pea is mainly maintained by *DETERMINATE* (*DET*) / *PsTFL1a*, which is one of the three *TFL1* homologues in pea along,

with *LATE FLOWERING* (*LF*) / *PsTFL1c* (see below) and *PsTFL1*b, for which no function has been described yet (Foucher et al., 2003). On the other hand, the I2 meristem, which does not have a functional equivalent in the simple inflorescence of Arabidopsis, is specified by a different gene product, belonging to the MADS-box family: *VEGETATIVE1* (*VEG1)* / *PsFUL* (Berbel et al., 2012).

DET and *LF* are two homologues of *TFL1* in pea. *DET* is expressed in the I1 after the floral transition and contributes to the maintenance and specification of I1 meristem identity (Berbel et al., 2012). In *det* mutants, the inflorescence is determined, forming one terminal I2 (with its respective flowers), but not directly in a terminal flower, as observed in Arabidopsis *tfl1* plants. Furthermore, *det* mutants do not display an early flowering phenotype, thereby showing that *DET* does not share with Arabidopsis *TFL1* the function of control of flowering time (Bradley et al., 1997; Foucher et al., 2003). On the other hand, *LF* is expressed more broadly, being detected very strongly in the axillary meristems of the vegetative nodes (Berbel, unpublished results) *lf* mutants shows early flowering, in a similar way to Arabidopsis *tfl1*, but *lf* inflorescences are not determined, indicating that *LF* does not share *TFL1* function in the maintenance of inflorescence meristem identity (Foucher et al., 2003). Therefore, *DET* and *LF* combined functions are somehow equivalent to the role of the *TFL1* gene in the regulation of flowering time and inflorescence architecture in Arabidopsis (Ratcliffe et al., 1998; Foucher et al., 2003).

Various studies that have been carried out on other legume species such as soybean (*Glycine max*), broad bean (*Vicia faba*), runner bean (Phaseolus vulgaris), mask bean (Vigna unguiculata) and stick bean (*Cajanus cajan*) (Avila et al., 2007; Liu et al., 2010; Repinski et al., 2012; Dhanasekar and Reddy, 2014; Saxena et al., 2017) show that the function of the *DET* homologues in legumes is generally conserved.

VEG1 or *PsFULc* codes for a MADS-box transcription factor. Its counterpart in Arabidopsis is *AGL79*, which belongs to the *AP1* / *SQUAMOSA* (*SQUA*) / *FRUITFULL* (*FUL*) gene clade (Berbel et al., 2012). Unlike Arabidopsis *AGL79*, for which there is no known function related to inflorescence development, *VEG1* is responsible for the specification of the identity of I2 meristems in pea (Berbel et al., 2012). Consistent with its function, *VEG1* is expressed after the floral transition at the inflorescence apex, specifically in the I2 meristems, just before the development of the floral meristem; no expression is detected in the rest of the I1 meristem nor in floral meristems (Berbel et al., 2012). *veg1* mutants do not produce flowers, and the secondary inflorescences are replaced by branches similar to primary inflorescences that reiterate this pattern forming new primary inflorescences (Gottschalk, 1979; Reid and Murfet, 1984; Berbel et al., 2012). The conversion of I2 into I1 in the *veg1* mutant can be explained

by the ectopic expression of *DET* found in the axillary meristems that should give rise to I2s, where *VEG1* would be expressed in the WT (Berbel et al., 2012). Interestingly, despite the absence of floral characters in *veg1* mutants, the transition from the vegetative phase to the reproductive phase appears to occur, as marked by the timely upregulation of *DET* in the SAM and the release of dormancy of the axillary meristems that arise from the I1, typical of I2 inflorescences (Reid and Murfet, 1984; Berbel et al., 2012). The recently characterized *MtFULc* gene (Zhang et al., 2021), for which has been described a very similar function of that of *VEG1,* indicates that the I2 development pathway appears to be highly conserved among grain legumes. Moreover, *dt1* and *dt2* soybean mutants (Tian et al., 2010; Ping et al., 2014) that have been associated with *DET* and *VEG1* genes respectively (Ping et al., 2014; Benlloch et al., 2015), display inflorescence-related phenotypes consistent with their predicted conserved roles. In addition, Dt2 (VEG1 homolog) repression upon *Dt1* (*DET* homolog) promoter (Liu et al., 2016) also resembles the proposed VEG1 repression of DET in pea (Berbel et al., 2012). Altogether, this suggests that VEG1 role in the control of the secondary inflorescence development is overall conserved in papilionoid legumes.

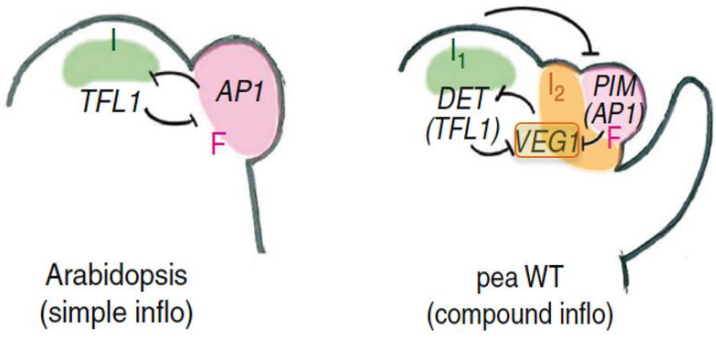


Figure 1. Model of the genetic networks controlling meristem identity specification in the simple inflorescence of Arabidopsis and in the compound inflorescence of pea. I: inflorescence meristem; I1: primary inflorescence meristem; I2: secondary inflorescence meristem; F: flower. Blocked arrows represent repression. The figure has been adapted from Berbel et at., 2012.

As in Arabidopsis, the basic network of meristem identity genes that define pea compound inflorescence architecture relies on antagonistic regulatory relationships. Thus, as mentioned before, *VEG1* negatively regulates *DET* expression, precluding its expansion to the I2 meristem. Conversely, *VEG1* is expressed in the SAM of *det* mutants, consistently with the observed I1-to-I2 conversion phenotype of *det* plants. In addition, it has also been shown that in *pim* mutants, *VEG1* expression is detected ectopically in the presumptive floral meristems, again in agreement with the partial FM-to-I2 conversion observed in *pim* mutants. Finally, in *det veg1* mutants, the primary inflorescence differentiates directly into a

terminal flower; accordingly, *PIM* expression invades the I1 SAM, indicating a likely additional negative regulation of *PIM* by *DET* (Berbel et al, 2012). Altogether, these data have led to propose a genetic model of mutually exclusive expression domains and negative regulatory interactions that resembles the Arabidopsis network but incorporates VEG1 as a new function essential for the development of secondary inflorescences (Figure 1; Berbel et al., 2012).

Flowering time

Genetic pathways in the induction of flowering in Arabidopsis

Most plants are sessile organisms, which means that they cannot move searching for the optimal conditions that would ensure reproductive success. Accordingly, a tightly regulated flowering time control is of vital importance for plants. They need to be able to sense environmental changes in their surrounding and respond to them, adapting their growth and development. The genetic network controlling floral transition has been extensively studied in Arabidopsis (Bäurle and Dean, 2006; Kinoshita and Richter, 2020; Quiroz et al., 2021). In Arabidopsis, the most determining exogenous or external signals in the control of flowering time are photoperiod (or day length) and temperature, among other factors such as the quality of the light or the presence of abiotic or biotic stress factors. In addition to these, other endogenous factors contribute to the regulation of flowering time, such as hormonal levels and the age of the plant. All these environmental and endogenous signals are integrated through a gene regulatory network that includes the so-called floral pathway integrators (Simpson and Dean, 2002; Moon et al., 2005; Blázquez et al., 2006; Srikanth and Schmid, 2011). The perception and transduction of these signals leads to floral transition taking place in the optimal conditions.

Photoperiod pathway

Depending on the kind of photoperiod conditions that plants need to undergo the floral transition, we can distinguish between long-day plants or short-day plants. Long-day plants flower only in long days, or their flowering is accelerated by long days. Opposite to that, short-day plants flower only in short days, or their flowering is accelerated by short days (Weller and Kendrik, 2008; Johansson et al., 2015; Song et al., 2015; Freytes et al., 2021). Moreover, there are Day-neutral plants, which are insensitive to day length and can flower under any photoperiodic condition. Arabidopsis, as a long-day plant, flowers in conditions

with more proportion of hours of light. However, Arabidopsis is also capable of flowering in non-photoinductive short-day conditions although it would take much longer to start the flowering transition process given the case (Rédei, 1962).

In the photoperiod pathway, CONSTANS (CO) protein plays a key role, and its activation depends on the perception of light and the circadian clock control mechanisms. *CO* is expressed in the vasculature of the leaves and the stem as a response to the control exerted by the circadian clock, displaying a low level of expression at the beginning of the day that progressively increases towards dawn (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). Additionally, CO protein is stabilized by light, thus is more abundant in long day conditions (Valverde et al., 2004; Imaizumi et al., 2005; Sawa et al., 2007).

CO positively regulates the expression of *FLOWERING LOCUS T* (*FT*) in the leaf vasculature, whose protein is transported to the SAM and forms a complex with the bZIP transcription factor FLOWERING LOCUS D (FD), activating genes that initiate the floral transition (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Takada and Goto, 2003; An et al., 2004; Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007). *TWIN SISTER OF FT* (*TSF*) also participates in this process acting redundantly with *FT* (Yamaguchi et al., 2005). The genes that initiate flowering after being activated by FT include *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *FRUITFULL* (*FUL*), and *AP1* (Wigge et al., 2005; Collani et al., 2019).

The vernalization pathway and the response to temperature

Many plants flower after winter has passed and for this, there are molecular mechanisms that allow the perception of long periods of time in cold temperature conditions, or vernalization (Amasino, 2004; Luo and He, 2020). In Arabidopsis, two main players involved in the vernalization response are *FLOWERING LOCUS C* (*FLC*), another member of the MADS box family, and *FRIGIDA* (*FRI*), which encodes a scaffold protein that forms part of different complexes that interact with the chromatin and positively regulates *FLC* expression (Choi et al., 2011). *FLC* protein is a strong floral repressor that negatively regulates different floral promoting factors, such as *FT*, members or the *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL*) family or *SOC1* (Michaels and Amasino, 1999; Johanson et al., 2000; Madrid et al., 2021). After cold exposure, *FLC* expression decreases, through a mechanism involving epigenetic changes on its promoter, allowing the upregulation of floral promoter genes and releasing the block for floral transition (Hyun et al., 2019; Madrid et al., 2021). In addition to *FLC*, other genes have been also shown to contribute to the vernalization

response, such as other MADS factors of the *FLC* family, like *FLOWERING LOCUS M* (*FLM*), *MADS AFFECTING FLOWERING 1* (*MAF1*) and *2* (*MAF2*) (Scortecci et al., 2001; Ratcliffe et al., 2003; Alexandre and Hennig, 2008).

In addition to the effect that vernalization, the exposure to long cold periods, has on flowering induction, ambient temperature also strongly influences the floral transition. It is well known that low temperatures generally cause a delay in flowering, while higher temperatures are usually associated with early flowering (Balasubramanian et al., 2006). The ambient temperature or thermosensory pathway carries out its control of flowering mainly through SHORT VEGETATIVE PHASE (SVP), a MADS box factor that acts as a flowering repressor, forming dimers with FLC to negatively regulate *FT* and *SOC1* levels (Li et al., 2008). *SVP* expression, which is not significantly affected by vernalization or photoperiod, is upregulated at low temperatures, while reduced in warmer conditions, thus modulating floral initiation through the regulation of *FT*, *SOC1* and other additional floral promoters (Hartmann et al., 2000; Lee et al., 2007; Li et al., 2008).

The response to endogenous cues: the autonomous and age pathways

In the same way that the plant is capable of perceiving exogenous stimuli, it also responds to endogenous cues, which together with the former allow modulating the time in which the floral transition finally occurs. This is the case of the autonomous and age pathways (Blázquez and Weigel, 2000; Cheng et al., 2017; Zheng et al., 2019).

The autonomous pathway of flowering acts by promoting the floral transition independently of environmental conditions to allow reproduction even in non-favourable scenarios. This pathway is mainly based on the regulation of the floral repressor *FLC*. To date, many mutants related to this pathway have been isolated and studied, and the corresponding gene products have been shown to mediate in different ways in the final repression of *FLC*. Many of them are related to RNA metabolism, like *FLOWERING CONTROL LOCUS A* (*FCA*), *FLOWERING LOCUS K HOMOLOGY DOMAIN* (*FLK*), *FPA*, *FY* and *LUMINIDEPENDENS* (*LD*) (Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003; Lim et al., 2004; Manzano et al., 2009). Others, such as *RELATIVE OF EARLY FLOWERING 6* (*REF6*), *FLOWERING LOCUS D* (*FLD*) and *FVE* encode proteins that are part of chromatin remodelling complexes that ultimately cause *FLC* repression (He et al., 2003; Ausín et al., 2004; Kim et al., 2004; Noh et al., 2004; Hennig et al., 2005). Finally, other factors play an important role in RNA-mediated gene silencing, providing a third pathway for the regulation of *FLC* (Baurle et al., 2007; Baurle and Dean, 2006; Veley and Michaels, 2008).

The age pathway integrates endogenous signals related to the acquisition of competence to flower, that is the transition from the juvenile phase to the adult phase of Arabidopsis development (Poethig, 2003). This transition is controlled by the balance that exists between miRNA156 and miRNA172 and the genes regulated by them, which includes some members of the *SQUAMOSA PROMOTER-BINDING LIKE* (*SPLs*) and of the *APETALA2 (AP2)* gene families. miRNA156 levels are high during the early stages of the life of the plant and progressively decrease with age, which causes the accumulation of its *SPL* targets (Wu and Poethig, 2006). *SPLs*, in turn, activate miRNA172 expression, which concomitantly reduce the activity of floral repressors of the *AP2* family that are miR172 targets, which then release the repression on *FT* and *SOC1* (Zheng et al., 2019).

In addition to the aforementioned pathways, plant hormones, especially gibberellins, also play an important role in flowering control. This function is key in non-photoinductive conditions. Moreover, the signalling cascade mediated by gibberellins allows for the floral transition to take place through the activation of the expression of *SOC1* and *FT*, thus as well as *AGL24* and *LFY* (Blazquez and Weigel, 2000; Moon et al., 2005; Hisamatsu and King, 2008; Liu et al., 2008; Izawa, 2021).

Flowering integrators

The different pathways involved in the floral transition converge in some genes that are called integrator genes. As easily deduced from the previous sections, *SOC1* and *FT* are major floral integrators, receptors of inputs from the different pathways and whose activation is closely related to the beginning of the floral transition (Blázquez and Weigel, 2000; Moon et al., 2005). Their induction can occur in a direct way (as is the case of the photoperiod, endogenous or gibberellin pathways) or by a repression of genes that repress their expression, finally resulting in their activation (it is the case of the routes of vernalization, temperature, autonomous and endogenous) (Blázquez and Weigel, 2000; Moon et al., 2005). However, there are other genes that can be considered as integrators of flowering since they respond to several of these flowering promoting routes, such as *LFY*, *AGL24* and *FUL* (Kardailsky et al., 1999; Blázquez and Weigel, 2000; Lee et al., 2000; Bemer et al., 2017).

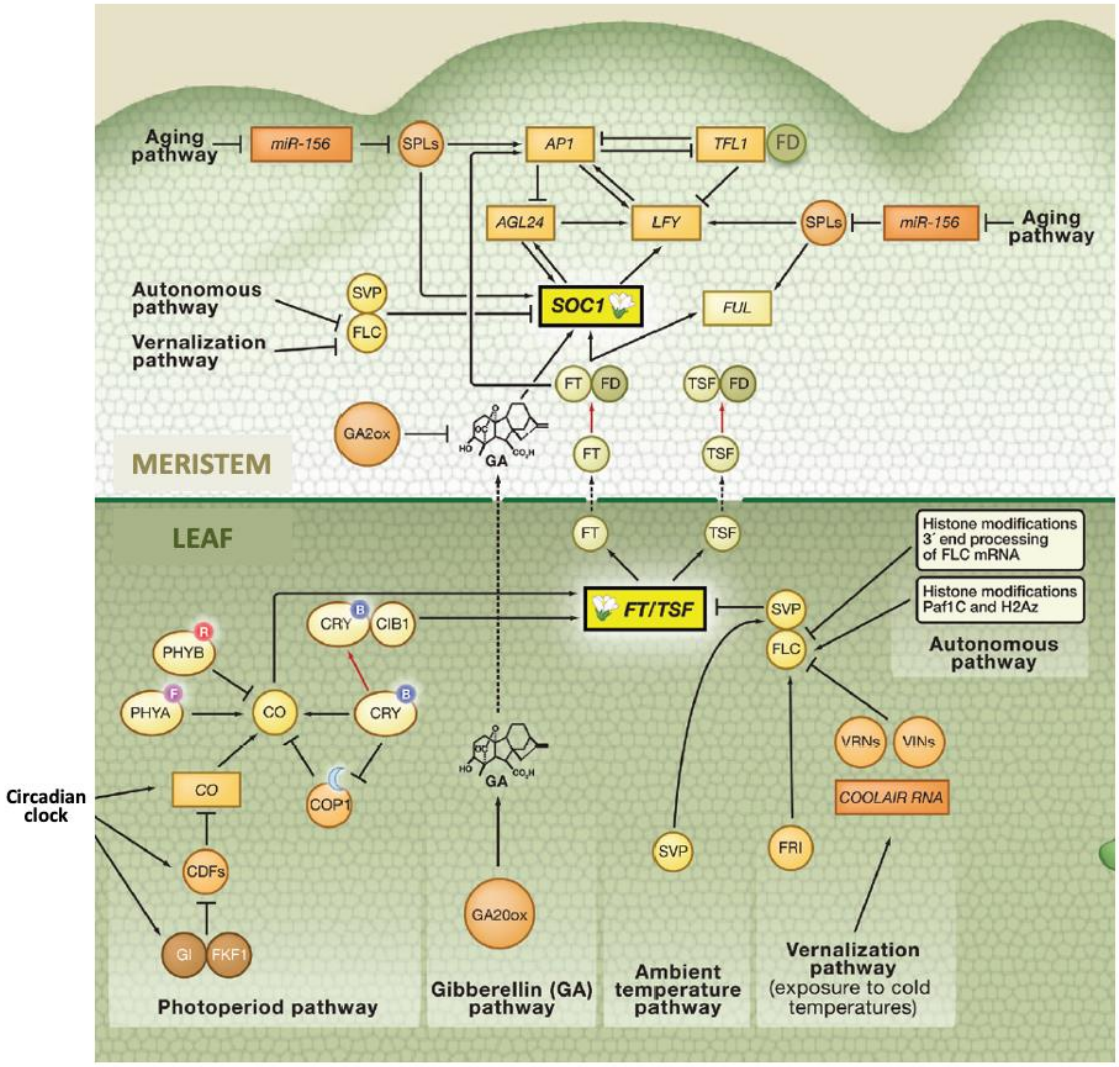


Figure 2. Model of the genetic pathways controlling flowering in Arabidopsis. Dashed lines from FT and TSF represent their movement from the leave to the meristem. Arrows represent induction and blocked arrows represent repression. The figure has been adapted from Fornara et al., 2010.

Floral initiation

Upon floral transition, activation of the floral meristem identity genes takes place, among them the MADS-box genes *AP1* and *CAL* (Mandel et al., 1992; Bowman et al., 1993; Kempin et al., 1995; Kaufmann et al., 2010) and *LFY* (Blázquez et al., 1997; Kaufmann et al., 2010). Their expression triggers the formation of floral meristems at the flanks of the inflorescence meristem. LFY and AP1 also activate the expression of the floral organ identity genes, whose expression specifies the identity of each of the organs of the flower (sepals, petal, stamens and carpels) (Prunet and Jack, 2014).

Genetic regulation of flowering time in legumes

The most economically important legume crops are found within the papillionoid clade, which can be classified in two subclades: the galegoid and the phaseoloid (Cronk et al., 2006). The species belonging to the galegoid clade (such as peas, lentils, chickpeas, etc.) are usually native of temperate regions and are long-day plants that respond to vernalization. On the other hand, phaseoloid legume species, such as soybeans and beans, are short-day plants generally found at lower latitudes (Bond et al., 1985).

In recent years there have been significant advances in the understanding of pathways that control flowering in legumes (Weller and Ortega, 2015; Weller and Macknight, 2018; Lin et al., 2021). These advances have been positively influenced by the recent availability of genetic and molecular tools and the availability of transcriptomes and genomes of various species, which made possible to advance in this area with expression analysis and reverse genetics experiments (Weller and Ortega, 2015). Known genes involved in flowering in legumes, for which more information is now available, are mainly those involved in the photoperiod pathway (including *FT*) and to a lesser extent those responsible for the vernalization response.

Photoperiod pathway in legumes

In Arabidopsis, the central core of this pathway is the CO-FT regulon, which, as described before, integrates day-length and circadian clock inputs. *FT* homologs in legumes (as for many other species) have been shown to act as key mediators of the photoperiodic response, behaving as the classic mobile flowering signal or florigen in a widely conserved manner. However, although *CO* homologs and CO-like genes have been shown to participate in the regulation of floral transition in different plant species, including legumes, the molecular mechanisms that finely regulate the daily oscillation of CO protein levels, as well as its role as a major integrator of photoperiodic input do not appear to be so conserved (Ballerini et al., 2011; Simon et al. 2015; Serrano-Bueno et al., 2017). This appears to be the case in legumes, where different studies show a putative role of CO homologs in *FT*-like gene regulation, but with a variable degree of functional importance that does not appear as prominent in pea, Medicago and other galegoid legumes (Wong et al., 2014; Weller and Ortega, 2015; González et al., 2021).

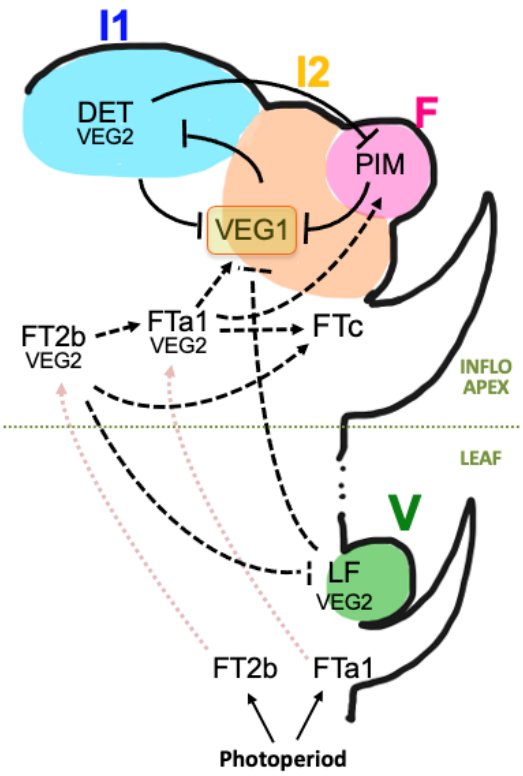


Figure 3. Genetic model for the role of *FT* genes in flowering and inflorescence development in pea. Dashed lines represent likely genetic interactions that require further experimental support. Arrows represent induction and blocked arrows represent repression. Red dashed lines from FTa1 and FTb2 represent their movement from the leave to the meristem. The figure has been adapted from Hecht. et al, 2011 and Sussmilch et al., 2015.

The *FT* gene family has been well studied in species such as pea, Medicago, Lotus and soybean (Kong et al., 2010; Laurie et al., 2011; Yamashino et al., 2013; Nan et al., 2014; Zhai et al.., 2014). In most species, at least 5 different genes homologous to the Arabidopsis *FT* have been found, which are divided into three subclades: *FT*a, *FT*b and *FT*c. *FT*c is considered the most divergent of the three subclades since it has several residue substitutions at conserved positions within the *FT* subclade (Hecht et al., 2011; Weller and Ortega, 2015). In pea, *FTa* and *FTb* genes are expressed in the leaves and are likely part of the mobile florigen, while *FTc* is expressed only in the inflorescence apex, which may suggest a role as integrator of signals from the other *FTs* genes (Hecht et al., 2011). All pea *FTs* can promote flowering when expressed in Arabidopsis, but among all of them, *FTb2* is considered the most likely candidate to initiate the flowering process, since it is the first one to be expressed in the leaf upon photoperiodic inductive conditions (Hecht et al., 2011). *FTa1*/*GIGAS* is also able to generate a mobile signal from the leaves but *gi* mutants mostly have defects in inflorescence specification, although are still responsive to daylength, suggesting that *FTa*/*GI* may have a different developmental role to *FTb* (Beveridge and Murfet, 1996; Hecht et al., 2011).

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OBJECTIVES

OBJECTIVES

Recent and significant advances in the development of genetic and genomic tools in legumes facilitated the analysis of functioning of different genetic networks in various legumes of great agronomic importance, including chickpea, lentils, and pea. Our work begins with the hypothesis that it is possible to optimize the production of legumes and its stability by modifying the genes of the network that controls the architecture of their inflorescences, obtaining and using alleles that have an impact it in the desired direction. On the other hand, the genetic network of inflorescence development is conserved in grain legumes, which makes it possible to transfer the knowledge and tools obtained in one species to other legumes of interest.

VEGETATIVE1 codes for a transcription factor that is a key regulator of development of the compound inflorescence of pea. *VEG1* specifies the identity of the secondary inflorescence meristem (I2) and is essential for the formation of the compound inflorescence. The increasing availability of genomic resources for pea research has prompted us to study in more depth the genetic and molecular basis of the development of the compound inflorescence in pea. Around the central role of VEG1 in the genetic network of the compound inflorescence, many questions arise: through which genes does VEG1 act to direct the development of the I2? How does VEG1 regulate these genes? Also, which genes are regulating VEG1? Are those genes conserved with Arabidopsis or have they gained new functions?

To answer these questions, we propose two specific objectives:

1.- To isolate inflorescence genes with VEG1-dependent expression. We hypothesise that a new strategy, based on comparing transcriptomes of pea mutants with opposite defects in inflorescence development (*veg1*, *pim* and *veg2*), will allow to identify VEG1 targets and will shed light on how VEG1 controls I2 development.

2.- To identify regulators of the expression of the inflorescence network genes. We hypothesise that characterizing the function of *FTc*, an unusual pea *FT* gene specifically expressed in the inflorescence apex, will shed light on how the genetic network of the inflorescence and its development is regulated in pea.

CHAPTER 1

Identification and functional characterization of putative targets of VEGETATIVE1/FULc, which directs formation of secondary inflorescence meristems in pea

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* In Capter 1, Marcos Serra-Picó performed all the experimental work. In the bioinformatic analysis he had the help from Dr Valèrie Hecht and Dr Reyes Benlloch. Finally, he also actively contributed to the design of the research and to the writing of the manuscript

Identification and characterization of putative targets of VEGETATIVE1/FULc, a key regulator of development of the compound inflorescence in pea and related legumes

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ABSTRACT

Inflorescence architecture contributes to essential plant traits. It determines a major component of plant shape, contributing to morphological diversity, also determines position and number of the flowers and fruits produced by the plant, influencing seed yield. Most legumes have compound inflorescences, where flowers are produced in secondary inflorescences (I2), formed at the flanks of the main primary inflorescence (I1), in contrast to simple inflorescences of plants like Arabidopsis, in which flowers are directly formed on the I1. The pea *VEGETATIVE1/ FULc* (*VEG1*) gene, and its homologues in other legumes, specify the formation of the I2 meristem, a function apparently restricted to legumes. To understand the control of I2 development it is important to identify the genes working downstream of *VEG1*. In this study, we adopted a novel strategy to identify genes expressed in the I2 meristem, as potential regulatory targets of VEG1. To identify pea I2-meristem genes we compared the transcriptomes of inflorescence apices from wild-type and mutants affected in I2 development, *proliferating inflorescence meristems* (*pim* - with more I2 meristems), *veg1* and *vegetative2* (both without I2 meristems). Analysis of the differentially expressed genes using Arabidopsis genome databases combined with RT-qPCR expression analysis in pea, allowed the selection of genes expressed in the pea inflorescence apex. *In situ* hybridization of four of these genes showed that all four genes are expressed in the I2 meristem, proving that our approach to identify I2-meristem genes was successful. Finally, analysis by VIGS in pea identified one gene, *PsDAO1*, whose silencing lead to small plants and another gene, *PsHUP54*, whose silencing leads to plants with very large stubs, meaning that this gene controls activity of the I2 meristem. PsHUP54-*VIGS* plants also are large and, more importantly, produce large pods with almost double the number of seeds of the control. Our study shows a new useful strategy to isolate I2-meristem genes and identifies a novel gene, *PsHUP54*, which seems a promising tool to improve yield in pea and in another legumes.

INTRODUCTION

The aerial organs of most flowering plants derive from the shoot apical meristem (SAM). In annual angiosperms, the SAM goes through two developmental phases. During the vegetative phase, the SAM produces vegetative organs, leaves and branches and, after transition to the reproductive phase, the vegetative SAM is transformed into an inflorescence meristem that produces floral meristems that develop into flowers (Benlloch et al., 2007; Prusinkiewicz et al., 2007; Teo et al., 2014). Much of the huge diversity of plant forms depends on the wide variety in the architecture of the inflorescences (Weberling, 1992; Benlloch et at., 2007). Inflorescence architecture is important not only for its contribution to plant diversity but also because it conditions the production of flowers and fruits, having a great impact on crop yield (Park et al., 2014).

Legumes are the second most important crops, after cereals, with a world production of around 340 million tons per year (González-Bernal and Rubiales, 2016). Cereals surpass legumes in productive capacity, nevertheless, combining both crops bring notable advantages in total efficiency, as legumes strongly improve the access to nitrogen in the soil (Jensen et al., 2020; Rodríguez et al., 2021). Legumes are an essential source of nutrients in the human diet, and they are also of paramount importance for animal feed or forage. They are rich in protein (up to twice and even three times as high as cereals), fiber, unsaturated fatty acids, and carbohydrates (Iqbal et al., 2006; Cañas and Beltrán; 2018). Their nutritional properties make of legumes a very healthy food highly recommended for human consumption. In addition, they compensate for some nutritional deficiencies in cereals, such as lysine and other valuable amino acids (Iqbal et al., 2006; Beltrán and Cañas; 2018).

In legumes, the most common inflorescence type is the compound inflorescence (Weberling, 1989). In contrast to simple inflorescences, such as that from Arabidopsis, where flowers are directly formed by the SAM at the primary inflorescence stem (Benlloch et al., 2007), in compound inflorescences the flowers are formed at secondary or higher order axes (Weberling, 1992; Benlloch et al., 2015). Thus, in legumes the primary inflorescence (I1) meristem laterally forms secondary inflorescence (I2) meristems that produce the floral meristems (Fig. 1). After producing a number of flowers, the I2 meristem terminates in formation a residual organ or stub (Fig. 1; Benlloch et al., 2015).

Within legumes, pea (*Pisum sativum*) is the species in which the gene network controlling the identity of meristems in the inflorescence was first elucidated and possibly the one where it is best known (Berbel et al., 2012; Benlloch et al., 2015). Specification of inflorescence

and floral meristem identity is governed by three types of genes. Pea I1 meristem identity is specified by *DETERMINATE*/*PsTFL1a* (*DET*), a homologue of the Arabidopsis *TERMINAL FLOWER 1* (*TFL1*) gene (Bradley et al., 1997; Foucher et al., 2003). As in the mutants of the Arabidopsis *TFL1* gene, development of the primary inflorescence (I1) meristem of pea *det* mutants is determinate, in contrast to wild-type pea, where development of the I1 meristem is indeterminate (Singer et al, 1999; Benlloch et al., 2015). *DET* function appears strongly conserved in legumes and determinate mutants due to mutation in *DET* homologues have been described in different legumes (Tian et al., 2010; Repinsky et al., 2012; Dhanasekar and Reddy, 2015; Cheng et al. 2018). Pea floral meristem identity is mainly specified by *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*), homologue to the Arabidopsis *APETALA1* (*AP1*) gene (Taylor et al., 2002). *AP1* and *PIM* encode MADS domain transcription factors required for the formation of the floral meristem (Mandel et al., 1992; Berbel et al., 2001; Taylor et al., 2002). In the *pim* mutant, the initiation of floral meristems from I2 meristems is impaired, and as a result, the I2 meristems proliferate dramatically in an undifferentiated state and only eventually form some floral meristems (Taylor et al., 2002). Due to this proliferation, the inflorescence apices of *pim* mutants have more I2 meristems than the wild-type (Figure 1B).

Finally, specification of I2 identity in pea depends on *VEGETATIVE 1/PsFULc* (*VEG1/PsFULc*), another MADS domain transcription factor gene from the same clade as Arabidopsis *AP1* and *FRUITFUL,* which is specifically expressed in the I2 meristem (Berbel et al., 2012). In *veg1* mutant plants, I2s are not formed and instead they are replaced by vegetative I1 (vegetative) branches (Berbel et al., 2012). Mutants in the *VEGETATIVE2/PsFD* gene (*VEG2/PsFD*) exhibit an inflorescence phenotype that strongly resembles that of *veg1*, with defects in the formation of I2s, which in plants of the *veg2-1* null mutant allele are not produced but instead are replaced by vegetative branches (Sussmilch et al., 2015). *VEG2* codes a bZIP transcription factor, homologue to Arabidopsis *FD*, which is required to upregulate *VEG1* upon floral transition (Abe et al., 2005; Sussmilch et al., 2015). This genetic network controlling identity of the meristems in the inflorescence is conserved also in other legumes. For example, in *Medicago truncatula* the whole network has been shown to work in the same way, with the *M. truncatula* homologues of the pea inflorescence genes, *MtFULc* (*VEG1/PsFULc*), *MtAP1* (*PIM*) and *MtTFL1* (*DET/PsTFL1a*), playing the same role in the inflorescence meristem identity genetic network than the pea genes (Cheng et al., 2018). Moreover, in soybean, *Dt1*, a homologue of pea *DET/PsTFL1a*, controls determination of the I1 stem and *Det2*, an orthologue of *VEG1/PsFULc*, has also been isolated and characterized (Tian et al. 2010, Ping et al. 2014).

The formation of the I2 meristem, specified by *VEG1*, is a key step in the development of the legume compound inflorescence, and functional homologues of *VEG1* have been described only in legumes, which possibly reflects the fact that the function of this gene is most likely unique to the compound inflorescence of legumes. The activity of I2 meristem determines the number of flowers that it produces. The number of flowers in the I2 is characteristic of each legume species and variety, and influences the number of pods and, therefore, yield (French, 1990; Rubio et al., 2004).

Very little is known about how *VEG1* controls the formation of the I2s. The goal of this study is to identify genes expressed in the I2 meristem that might play a role in its development as regulatory targets of VEG1. With that aim we have taken advantage of some of the many molecular genetic tools now available in pea: mutant lines in inflorescence meristem genes, as biological material (Benlloch et al., 2015), transcriptome and genome sequences, to analyse transcriptome of these mutants (Alves-Carvallo et al., 2015; Kreplak et al., 2019), and virus induced gene silencing (VIGS), as method to study the function of the selected candidate genes (Constantin *et al*., 2004). In this study we have compared the transcriptome in developing inflorescence tissue of the wild-type, and the *veg1*, *pim* and *veg2* mutants. That has led us to the identification of five genes expressed in meristems of the inflorescence apex. Finally, silencing of these genes by VIGS showed that two of them control plant development and that one of these genes, *PsHUP54*, contributes to controlling the activity of the I2 meristem, and its silencing increases plant size and seed production.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type (NGB5839 and Boneville) and mutant pea lines (*veg1/psfulc-3*, *pim-2* and *veg2-1*) have been previously described (Murfet and Reid 1993; Taylor et al., 2002; Berbel et al., 2012). The original mutants *psfulc-3*, *pim-2* and *veg2-1* were introgressed in the dwarf NGB5839 line. Plants were grown in a greenhouse at 21 ºC day 16 ºC night and under long-day (LD) photoperiod (16h light / 8h darkness). When needed to maintain LD photoperiod conditions, natural light was supplemented with lighting (400W Phillips HDK/400 HPI (R)(N)). Plants were irrigated periodically using Hoagland Nº1 solution supplemented with oligoelements.

Transcriptome analysis

For RNA-seq experiments, inflorescence apex samples (three biological replicates) from pea wild-type, NGB5839 line, and *veg1*, *pim* and *veg2* mutant plants were collected at floral transition, when the primary stem plant had formed 10 nodes (approximately 4 weeks after germination). Each biological replicate consisted of 3-4 inflorescence apices. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with DNaseI (Turbo DNA-free kit INVITROGEN; Ref-AM1907) following the manufacturer's instructions. The quality of the RNA was checked on an Agilent 2100 Bioanalyzer instrument using the RNA6000 nano kit. Strand-specific RNA libraries were constructed using the TruSeq stranded mRNA kit (Illumina). Libraries were sequenced in a HiSeq2500 platform (Illumina) to produce 50-nucleotide single-end reads. Library construction and sequencing was performed at the genomics core facility at Centre for Genomic Regulation, Barcelona, Spain. Approximately 20 million reads were generated from each sample.

For RNA-seq analyses, ribosomal RNA sequences were filtered out using SortMeRNA (Kopylova et al., 2012). Sequences of adapters were trimmed from the remaining reads using Trimmomatic (Bolger et al., 2014). The trimmed sequences were then aligned against the Pea transcriptome (PsUniLowCopy database, Ps Cameor database) using STAR (Dobin et al., 2013) and reads were counted with HTSeqCount (Anders et al., 2015). DESeq2 with default parameters was used to perform differential expression analysis (Love et al., 2014). Identification of genes with opposite expression patterns was performed by constructing Venn Diagrams with the online tool Venny (Oliveros, 2007-2015). Genes with opposite expression pattern in *veg1* and *pim* samples were visualized in a heatmap created with the tool ClustVis (Metsalu et al., 2015).

RNA-seq data was validated by checking gene expression by RT-qPCR of selected genes in the wild-type and mutants. For that, wild-type, *veg1* and *pim* plants were grown for approximately 4 weeks, to node 10, as described above. Samples from inflorescence apex were collected and RNA extraction and cDNA synthesis were done as described at the RT-qPCR section.

Gene ontology (GO) term analysis

The analysis of the enrichment in gene ontology terms corresponding to *VEG1*, *PIM* and *VEG2* differential expressed genes was performed using the online tool AGRI-GO http://bioinfo.cau.edu.cn/agriGO/ (Du et al., 2010; Tian et al., 2017). For each pea transcript we identified the best Arabidopsis homologue by using Basic Local Alignment Search Tool (BLAST). Then, we applied the Singular Enrichment Analysis (SEA) for the identification of

corresponding GO terms that were statistically overrepresented for each DEGs list (P value < 0.05) (Supplementary table 3).

Characterization of gene expression levels by RT–qPCR

Total RNA was extracted with the SV Total RNA isolation system (Promega) according to manufacturer instructions. RNA concentration of the samples was determined by spectrophotometer analysis using a NanoDrop 8000 (Thermo Scientific). Reverse transcription (RT) was conducted in a final volume of 20µl and using 1µg of total RNA as a template (MMLV high-performance reverse transcriptase, Epicenter), according to manufacturer instructions. RT-negative controls were performed to monitor sample contamination with genomic DNA. For each time point and/or tissue, three biological replicates were analysed, and results are presented as the average +/- standard deviation. Statistical significance was tested by one-way ANOVA test, followed by Dunnett's multiple comparison test (one to four asterisks indicating P value <0.05, <0.01, <0.001 or <0.0001 respectively). Relative transcript levels were calculated following the Delta-Delta CT method (Livak and Schmittgen, 2001), using pea actin (PEAc14; accession U76193) as reference gene. The primers used for PCR and RTqPCR are detailed in Supplementary Table 5.

Histological sections and *in situ* hybridization

The histological study of floral transition and the *in-situ* hybridization experiments (Figures 2 and 6) were done with 8µm-thick longitudinal sections of inflorescence apices embedded in paraffin. RNA *in situ* hybridization experiments were performed according to a previously described protocol (Ferrándiz et al, 2000). For each gene, digoxigenin-labelled probes were generated using as a template a fragment of the coding sequence corresponding gene: PsCam039164 (350-bp fragment; positions 677-1026), PsCam043276 (312-bp fragment; 39–350), PsCam043354 (350-bp fragment; positions 807-1156), PsCam050808 (350-bp fragment; positions 744-1093) and PsCam057706 (350-bp fragment; positions 1-350). Nucleotide positions are indicated using as reference the ATG codon. Each of the fragments was amplified by PCR using inflorescence apex cDNA as a template and cloned into the pGEM-T Easy vector (Promega). RNA anti-sense probes were generated with T7 RNA polymerase; sense probes were used as a control in each case, and they were generated with SP6 RNA polymerase.

Virus Induced Gene Silencing (VIGS)

Gene fragments for VIGS constructs to downregulate expression of *PsCam039164*, *PsCam043354*, *PsCam050808* and *PsCam057706* genes were amplified from pea cDNA. The VIGS system combines two different plasmids: pCAPE1 and pCAPE2-PDS. These vectors contain respectively the RNA1 and RNA2 of the Pea early-browning virus (PEBV), under the control of the CaMV 35S promoter and the NOS terminator, in the binary vector pCAMBIA-1300 (Constantin *et al*., 2004). To generate the VIGS constructs, the vector pCAPE2-PDS was used, where PDS is flanked by several restriction enzymes that makes it possible to replace the PDS fragment with the cDNA fragment of the gene to be silenced (Constantin *et al*., 2004). In all cases NcoI and EcoRI restriction sites we used to subclone the gene fragments, with the exception of the *PsCam050808* gene, where NcoI and PstI were used. Gene fragments were PCR amplified as described in the previous section, using primers carrying the aforementioned restriction sites (Supplementary Table 5). The plasmid pCAPE2-Con, containing 400bp of the GUS coding sequence, was used as VIGS control (Constantin *et al*., 2008).

Inoculation of plants was carried out as previously described in Constantin *et al*., 2004 with minor modifications as follows. For each experiment, 30 plants of the pea Boneville cv., about 3-weeks old, when they have produced 5 leaves, were infiltrated with two *Agrobacterium tumefaciens* strains carrying the pCAPE1 and the pCAPE2 plasmids. Infiltrated plants were decapitated 5 days after inoculation and in each plant, a single, basal, axillary shoot was kept and allowed to form a new primary shoot. Those newly formed shoots were characterized as described below.

Plant phenotypic characterization

For phenotypic characterization of VIGS plants we scored a set of phenotypical traits: number and nodal position of secondary inflorescences, length of the main shoot internodes, number and complexity of leaves, length and structure of the secondary inflorescences (I2s) length of the stub, and length of the floral pedicels. For stub length, those ones that were that small that were barely visible and not measurable were considered to be 1mm long, the rest of stubs were measured accordingly.

To determine when to collect the shoot apices from the pea plants, we scored the number of nodes in the primary stem to the node with the first folded leaf. For analysis of flowering, we considered the “flowering node” the first node with an I2-structure that produced a flower.

For statistical analysis of parameters of VIGS plants, all the data containing multiple variables were analyzed by one-way ANOVA with post hoc HSD Tukey's test taking as significant difference depending on Bonferroni and Holm multiple comparisons. Statistical significance calculations for variables with data from two groups were performed with two-tailed Student's t test. Differences in expression were considered significant (*) when $P < 0.05$ and highly significant (**) when $P< 0,01$.

Multiple sequence alignment

Putative *PsHUB54* homologue genes in other species were identified by using the protein-protein Basic Local Alignment Search Tool (BLAST) Blastp at https://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1990). Sequences from *Arabidopsis thaliana* HUP54 (AT4G27450), *Glycine max*_XP_003543254.1, *Medicago truncatula* XP_013464732.1 and *Cicer arietinum* XP_004487686.1 proteins were aligned using the MEGAX and applying the ClustalW algorithm.

RESULTS

Determining timing of floral transition in the pea line NGB5839

In order to elucidate the molecular mechanisms involved in the development of the secondary inflorescences (I2) of pea, we aimed to investigate the action of the VEG1 transcription factor, which specifies the identity of the I2 meristems (Berbel et al., 2012), by identifying its target genes. For this, we took a genetic approach in which we compared the transcriptomes of inflorescence apices of wild-type pea and mutants in which the formation of I2 meristems is affected: *veg1*, *pim* and *veg2*. In wild-type pea plants, after the floral transition, the flowers arise from secondary inflorescences that develop from the primary inflorescence (I1; Figure 1; Benlloch et al., 2015). In the *veg1* mutant, plants fail to produce these I2s, which are replaced by I1s (Figure 1). Conversely, in *pim* mutant plants, the I2 meristems proliferate before producing some flowers, so that more I2 meristems are formed (Figure 1; Taylor et al., 2002). Finally, *veg2* mutant plants show a phenotype similar to that of *veg1*, with no I2s, although the molecular basis for the phenotype is different to that in *veg1*, reflecting a defect in *VEG1* induction rather than direct impairment of its function (Figure 1; Sussmilch et al, 2015).

To select the most suitable time to compare the transcriptomes of the pea inflorescence mutants, we determined the timing of floral transition, when the I2 meristems are initiated.

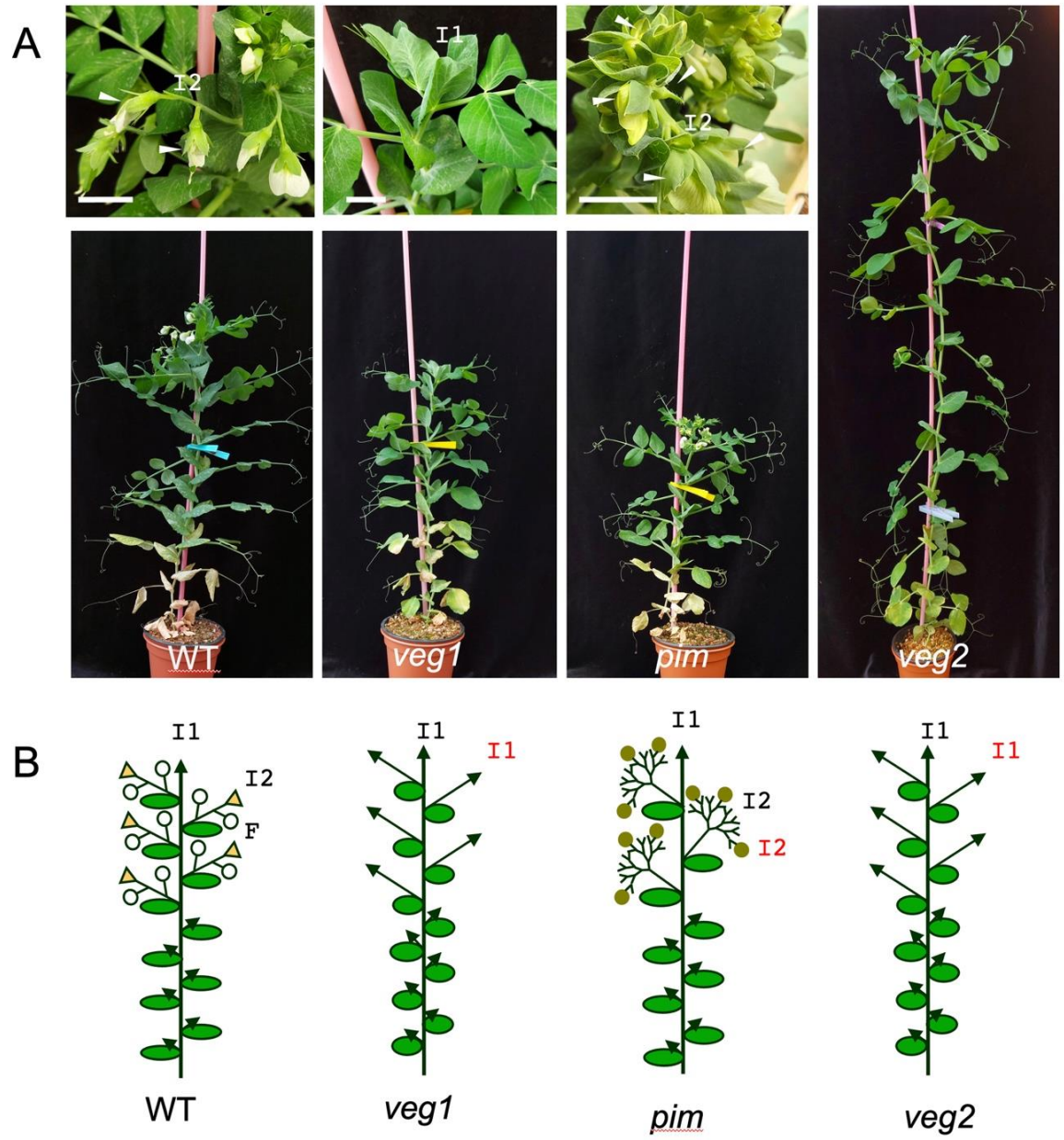

Figure 1. Inflorescence architecture of wild-type pea and *veg1*, *pim* and *veg2* mutants in NGB5839 background. (A) Images of wild-type (WT), *veg1*, *pim* and *veg2* plants (lower panels). In the close-ups of the inflorescences of those plants (upper panels) I1 and I2s are marked. Flowers are marked with arrowheads. The WT usually has two flowers per I2 while in *pim* more flowers are produced. The *veg1* and *veg2* mutants neither produce I2s nor flowers. **(B)** Diagrams showing the inflorescence architecture of these genotypes. F, flower; white circles represent WT flowers; brown circles represent abnormal *pim* flowers; yellow triangles represent the stubs; scale bars: 2cm.

With that aim, we characterized, both at the morphological and molecular level, the development of the inflorescence of the reference line NGB5839, the wild-type genetic background of the inflorescence mutants used in this work (Hecht et al., 2007). First, we analyzed a series of shoot apex samples at different developmental stages (including apices from plants where the primary shoot had formed 6, 8, 10 or 12 nodes). Shoot apices were dissected and histological sections were prepared to determine the first node at which I2 and floral meristems could be observed. These observations revealed that I2 or floral meristems could be readily observed in plants having formed 10 nodes but that were not apparent in plants having formed only 8 nodes (Figure 2A).These results indicated that these plants underwent floral transition between node 8 and node 10. Secondly, in the same type of samples, we used RT-qPCR to examine the expression of *VEG1* and *PIM*, I2 and floral meristem marker genes, respectively. Consistent with our previous observations, both genes displayed an expression increase in samples having

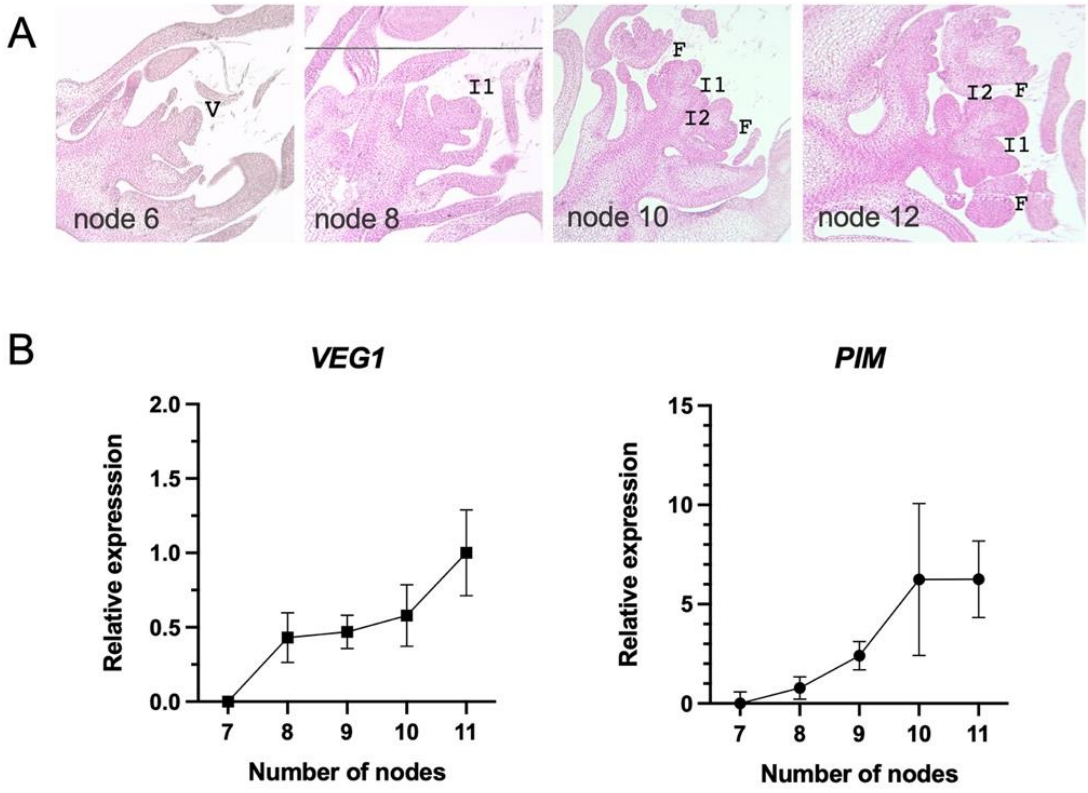

Figure 2. Floral transition in the NGB5839 pea line. (A) Histological sections of shoot apices of NGB5839 pea plants that had produced 6, 8, 10 or 12 nodes. I2 and floral meristems are observed from 10-node plants. **(B)** Relative expression levels of *VEG1* (left) and *PIM* (right), determined by RT-qPCR, of shoot-apex samples collected from plants that had produced 7, 8, 9,10 or 11 nodes. Error bars correspond to standard deviation. V, shoot vegetative meristem; I1, primary inflorescence meristem; I2, secondary inflorescence meristem; F, floral meristem.

formed 8 and 10 nodes (Figure 2B). From these results, we decided to compare the transcriptome of apices of wild-type and mutant plants having formed 10 nodes. In these plants, I2 meristems were visible and *VEG1* expression was clearly detected, hence being an appropriate time to detect the expression of VEG1 regulatory targets.

Transcriptome analysis of inflorescence apices of pea *veg1*, *pim* and *veg2* mutants

In order to identify genes whose expression is associated to I2 meristems, inflorescence apex samples from wild-type, *veg1*, *pim* and *veg2* plants were used to perform a transcriptome analysis by RNA-seq, comparing the transcriptome of each mutant to that of the wild-type line (WT). Comparison of the inflorescence apex transcriptome of *veg1* with that of the WT identified 2792 differentially expressed genes (DEGs). Among those, 1584 were upregulated and 1208 were downregulated in *veg1* (Figure 3A; Supplementary Table 1). A similar comparison of WT and the *pim* mutant identified 2148 DEGS (Figure 3A; Supplementary Table 1). Since *veg1* and *pim* have opposite phenotypes in terms of I2 meristem development, with *veg1* developing no I2 meristems and *pim* displaying a proliferation of I2 meristems, we identified which of the WT/*veg1* and WT/*pim* DEGs showed an opposite expression pattern. In this way, we found that 42 genes were upregulated in WT/*veg1* and downregulated in WT/*pim* and 43 genes that were downregulated in WT/*veg1* and upregulated in WT/*pim*, giving a total of 85 genes with an opposite expression pattern between WT/*veg1* and WT/*pim* (Figure 3A and D; Supplementary Table 2).

To validate the results of the RNAseq, we randomly choose twelve genes among those 85 with opposite expression pattern and a LFC (log2 fold change) ≥ 1 for at least one of the transcriptomes (WT/*veg1* or WT/*pim*) and analyzed the expression of those genes by RT-qPCR in WT, *veg1* and *pim* mutant apices (Supplementary Figure 1). We could confirm the results of the transcriptome analysis in 10 out of these 12 genes displaying a clear opposite expression pattern in *veg1* and *pim* mutant background compared to the wild-type. Overall, these results indicate that our approach consisting of comparing transcriptomes of apex samples in these mutants was an effective method to identify genes with an opposite expression pattern and possibly involved in VEG1-mediated control of I2 meristem development.

Finally, we characterized the transcriptome changes in the samples of the *veg2* mutant in comparison to WT. This comparison identified 4059 DEGs, among which 2163 were up regulated and 1923 were downregulated in *veg2*. Both *veg1* and *veg2* mutants display a similar I2 phenotype (lacking I2 meristems), but the molecular bases for those phenotypes are different. Since we were interested in characterizing how VEG1 specifies the I2 meristem

identity and controls its activity, we identified those genes with an opposite expression pattern in WT/*veg1* and WT/*pim* but that showed no expression change in *veg2* (Figure 3B and C).

In order to identify the possible function of DEGs in each transcriptomic comparison (WT/*veg1*, WT/*pim* and WT/*veg2*) we identified the putative homologues for each pea transcript by blasting their sequences against the Arabidopsis and *Medicago truncatula* databases. In the case of Arabidopsis, we identified close homologues for 74% of the pea transcripts, while in the case of Medicago, we could find the corresponding homologues for 77% of the pea transcripts. Because the information on genes was much more complete in

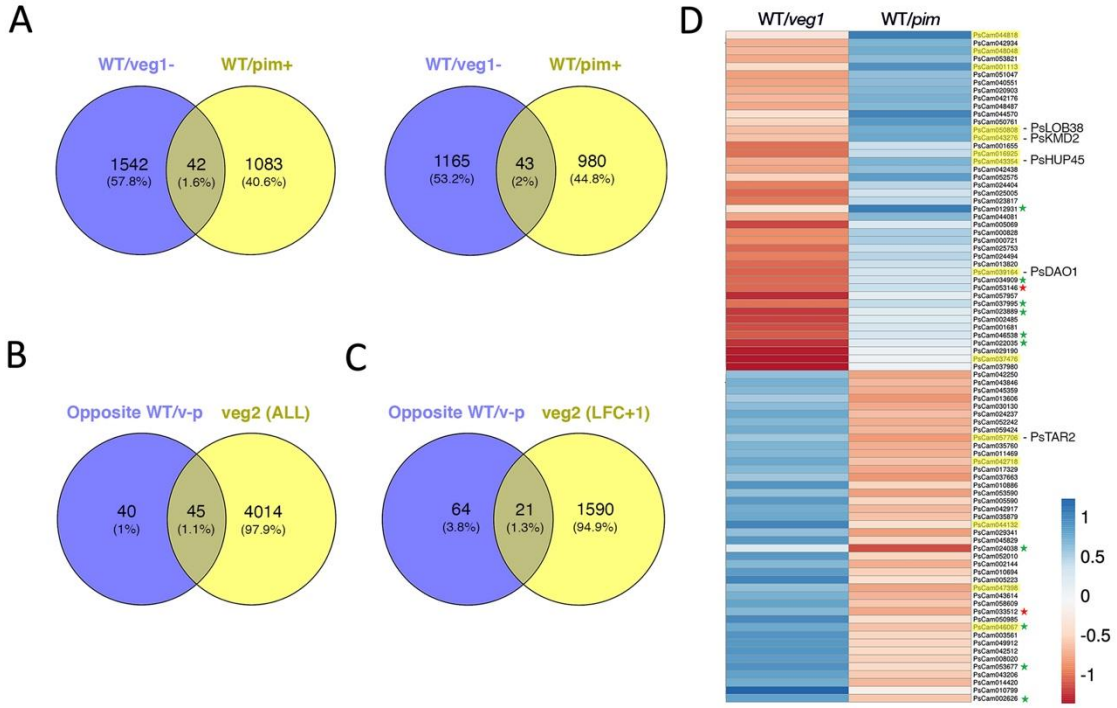

Figure 3. Transcriptome analysis of *veg1*, *pim* and *veg2* apex samples compared to wild-type. (A) Venn diagram displaying genes with an opposite expression pattern in WT/*veg1* and WT/*pim* comparisons: 42 genes are down-regulated in *veg1* (WT/veg1-) and up-regulated in *pim* (WT/*pim*+) (left panel); 43 genes are up-regulated in *veg1* (WT/*veg1*+) and down-regulated in *pim* (WT/*pim*-) (right panel). **(B)** Venn diagram identifying differentially expressed genes in WT/*veg2* among those with an opposite expression patter in *veg1* and *pim* mutant backgrounds (opposite WT/v-p). **(C)** Venn diagram identifying differentially expressed genes in WT/*veg2* with a Log of Fold Change >1 among those with an opposite expression patter in *veg1* and *pim* mutant backgrounds (opposite WT/v-p). **(D)** Heatmap displaying fold change expression of the 85 genes with an opposite expression pattern in WT/*veg1* and WT/*pim* (red shows down-regulation and blue indicates up-regulation). Genes highlighted in yellow were selected for further characterization. Validation of the RNAseq data was performed for twelve genes (marked with a star). We confirmed the opposite expression pattern in ten out of twelve of those genes (green star). For two of the genes, the expression profile could not be confirmed (red star) (**Supplementary Figure 1**).

this species, we used the Arabidopsis homologue genes to perform a gene ontology analysis (GO) with up- and down-regulated genes and identified biological processes that were overrepresented in each of our datasets. GO term enrichment analysis with Arabidopsis homologues of WT/*veg1* up-regulated pea transcripts returned enriched processes tightly related to reproduction, flower and meristem development, hormone transport and regulation of transcription (Figure 4A; Supplementary Table 3), indicating that, as intended, we have identified genes that are involved in reproductive development (as expected from a mutant such as *veg1*, showing impairment of I2 meristem initiation). Other biological processes that are enriched in this analysis include those referring to RNA metabolic processes (including non-coding RNA) and chromatin modification. Those terms, although less related to meristem development, point out to alternative mechanisms that could be contributing to the control of I2 meristem specification or activity. Analysis of GO term enrichment with Arabidopsis homologues of WT/*veg1* down-regulated pea transcripts returned processes related to metabolism of different compounds, including lipids, amino acids, phenylpropanoids, or related to hormone response (more specifically response to abscisic acid) (Figure 4B; Supplementary Table 3).

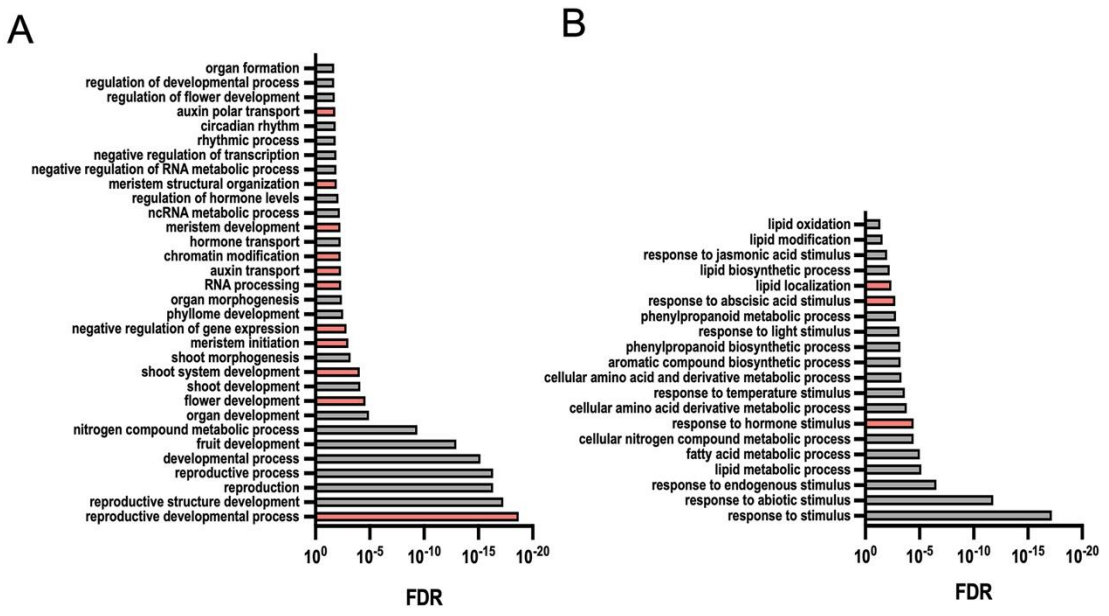

Figure 4. Gene ontology term (biological processes) enrichment among differentially expressed genes in WT/*veg1* transcriptome. (A) Selected GO terms returned from the analysis of genes up-regulated in *veg1*. **(B)** Selected GO terms returned from the analysis of genes down-regulated in *veg1*. Terms were selected according to their relation to previously described VEG1 function and/or potential VEG1 mechanisms of action. All depicted terms were overrepresented (False Discovery Rate (FDR) < 0.05). A complete list of all enriched GO terms for each analysis is detailed in Supplementary Table 3.

We performed similar GO term enrichment analysis with DEGs identified in WT/*pim* and WT/*veg2* comparisons (Supplementary Table 3; Supplementary Figure 2). In both cases, enriched GO terms found with the up-regulated genes returned processes that are unequivocally related to reproduction, meristem initiation and development and response to different environmental signals. In the case of WT/*pim* among those terms we found meristem and flower development, maintenance of meristem identity, floral organ development or regulation of meristem growth. Negative regulation of developmental processes is another GO term, indicating that we have identified genes related to the control of meristem specification and activity. A similar analysis with down-regulated genes showed that terms such as response to hormones (abscisic acid, ethylene, salicylic acid and gibberellins), signal transduction and transcription are significantly overrepresented in this dataset, in agreement with the loss of function of a transcription factor such as PIM, controlling floral meristem specification (Supplementary Figure 2A).

Finally, GO term enrichment with the WT/*veg2* dataset also identified significantly overrepresented processes pointing out to an important environmental and hormonal regulation of meristem development (Supplementary Table 3; Supplementary Figure 2). On one hand, among the enriched terms identified with WT/*veg2* up-regulated genes, we found overrepresentation of terms related to meristem development (meristem initiation, meristem maintenance and meristem growth), to reproductive development (flower and fruit development), environmental signals (temperature) or hormone response. On the other hand, using the WT/*veg2* down-regulated dataset we find an overrepresentation of terms related to different biosynthetic processes (nitrogen compounds, protein and amines, organic acids) (Supplementary Figure 2B). This points to processes that control the metabolic status of the apices, as it was also observed in the enriched terms using the WT/*veg1* down-regulated dataset. Interestingly, WT/*veg2* down-regulated terms pointed out at ribosome biogenesis and ribonucleoproteins complexes as very strongly enriched, which could indicate a unknow role of these processes in meristem regulation.

In order to narrow down our selection of genes putatively involved in the control of VEG1-mediated I2 meristem development, we applied several additional criteria to the list of 85 genes displaying opposite expression patterns between WT/*veg1* and WT/*pim*. Those criteria included *in-silico* analysis of gene expression pattern in different pea plant organs (Pea gene expression atlas; Alves-Carvallo et al., 2015), function and expression pattern of the Arabidopsis homologue genes contribution of these Arabidopsis homologues to relevant biological processes overrepresented in the GO analysis of WT/*veg1* DEGs, and whether the gene was up- or down-regulated specifically in *veg1* (i.e., not differentially expressed in

WT/*veg2*). With these criteria, we selected 14 genes for further functional characterization (Table 1). Some of the selected genes did not comply with all criteria but all of them showed opposite regulation in *veg1* and *pim* inflorescences and met at least one other criterium.

Table 1. Genes selected for RT-qPCR and further expression analysis.

Ps Cameor ID	LFC (WT/*veg1*)	LFC (WT/*pim*)	LFC (WT/*veg2*)	PsCam expression atlas[a]	Arabidopsi shomologue	Arabidopsis protein and function	Arabidopsis GO-associated terms
PsCam057706 (PsTAR2)	-0.3	0.4	-	yes (2)[b] LFPA	AT4G24670	Tryptophan aminotransferase involved in IAA biosynthesis	**indoleacetic acid biosynthetic**[b] **process; flower development;** maintenance of root meristem identity; **shoot system development**
PsCam050808 (PsLBD38)	0.5	-0.5	-	yes (3) LFPA	AT3G49940	Transcription factor involved in anthocyanin biosynthesis and nitrogen availability signals	**regulation of gene expression**
PsCam043354 (PsHUP54)	0.6	-0.6	0.6	yes (1) LFPA	AT4G27450	Cellular response to hypoxia	-
PsCam039164 (PsDAO1)	2.5	-0.7	3.4	no PA	AT1G14130	IAA oxidase contributing to IAA degradation	**auxin homeostasis**
PsCam043276 (PsKMD2)	0.5	-0.6	-	yes (3) LFPA	AT1G15670	F-box protein involved in targeting type B-ARR proteins for degradation	negative regulation of cytokinin
PsCam048048	0.3	-0.3	0.4	yes (3) LFPA	AT2G36490	Repressor of transcriptional gene silencing	nucleus; **chromatin silencing**
PsCam047398	-0.8	1.0	1.6	yes (3) PA	AT5G59310	Lipid transfer protein	**response to abscisic acid** [c]
PsCam046067	-1.0	0.8	-1.3	yes (2) LFPA	AT3G14160	2-oxoglutarate-dependent dioxygenase protein	nucleus; oxidative DNA demethylase activity
PsCam044818	0.2	-0.7	-	yes (2) LFPA	AT1G01040	RNA helicase involved in microRNA processing (dicer-like1)	nucleus; DNA binding; **flower development;** vegetative to reproductive phase transition of meristem; **RNA processing**
PsCam044132	-0.6	0.3	-0.4	yes (0) FPA	AT1G02205	-	-
PsCam042718	-0.3	0.3	-	yes (2) 7476LFPA	AT1G69040	ACT-domain protein involved in feedback regulation of amino acid metabolism	-
PsCam037476	10.7	-0.3	-	yes (3) LFPA	AT3G02300	Regulator of chromosome condensation	-
PsCam016925	0.6	-0.2	-	yes (0) LFPA	AT3G29075	Glycine-rich protein	-
PsCam001113	0.3	-0.6	-	yes (2) LFPA	AT2G19810	Oxidation-related zinc-finger 1 involved in oxidative stress	nucleus; **chromatin silencing**

[a] Expression according to the Pea gene expression atlas- 0: expression in shoot apices, NPKM value higher or close to 40; 1: NPKM value higher or close to 20; 2: NPKM value higher or close to 10; 3: NPKM value lower than 10. L: expression in leaves; F: expression in flowers; P: expression in pods; expression in other organs (different to leaves, flowers, pods or shoot apices).

[b] Green font represents go-terms associated to genes up-regulated in WT/*veg1.*

[c] Red font represents go-terms associated to genes down-regulated in WT/*veg1.*

The GO term enrichment analysis suggested that several hormones are likely to play an important role in meristem initiation and development during floral transition in pea. In particular, auxin homeostasis and response to abscisic acid were enriched terms in up- and down-regulated gene sets, respectively (Figure 4). Accordingly, we select three genes: PsCam039164, homologue of the *DIOXYGENASE FOR AUXIN OXIDATION 1* (*DAO1*) gene in Arabidopsis, involved in auxin degradation (Porco et al., 2016; Zhang et al., 2016), hereafter named *PsDAO1*; Pscam057706, corresponding to the pea *PsTAR2* gene, homologue of the Arabidopsis *TRYPTOPHAN AMINOTRANSFERASE RELATED 2* (*TAR2*) gene, both of them involved in auxin biosynthesis (Stepanova et al., 2008; Tivendale et al., 2012; MacAdam et al; 2017); and PsCam047398, homologue to a Arabidopsis lipid transfer protein strongly upregulated by abscisic acid (Gao et al., 2016). *PsTAR2* was of particular interest since expression of its Arabidopsis orthologue *TAR2* increases strongly at the shoot apical meristem upon floral induction and its expression at the SAM was restricted to the peripheral zone, where lateral organs are initiated (ePlant: https://bar.utoronto.ca/eplant/; Waese et al., 2017). Selection of *PsDAO1* as a candidate was supported as well by a very discrete expression pattern of its Arabidopsis homologue in the rib meristem at the SAM (ePlant). Finally, PsCam047398, besides being up-regulated by ABA, is related to lipid transport and lipid localization, biosynthesis, and modification, which came up as enriched GO-terms in our previous analysis, supporting the selection of this gene for further investigation. A fourth hormone-related selected gene was PsCam043276, a homologue of the *KISS ME DEADLY 2* (*KMD2*) gene of Arabidopsis, which, together with *KMD1*, is involved cytokinin signaling and has been shown to have an impact on shoot apical meristem size when overexpressed (Kim et al., 2013).

PsCam050808, hereafter named *PsLBD38*, is a homologue of *LATERAL ORGAN BOUNDARIES 38* (*LBD38*), an Arabidopsis transcription factor involved in defining lateral organ boundaries that is repressed at the SAM upon floral induction and that has been related to the control of flowering time in rice plants (ePlant; Albinsky et al., 2010). PsCam043354 (thereafter *PsHUP54*) is a homologue of the *HYPOXIA REPSONSE UNKNOWN PROTEIN 54* (*HUP54*) for which very little functional information is available. In Arabidopsis, *HUP54* is transiently upregulated at the SAM during floral transition. Its expression at the SAM is restricted to the rib meristem and the peripheral zones, being quite low at the central zone (ePlant). The *HUP54* gene has been shown to be regulated in floral buds by SHINE transcription factors and gibberellin (Shi et al., 2011). From all these data, *PsLBD38* and *PsHUB54* were selected for further characterization.

Finally, we selected a number of genes that could eventually reveal a role for different mechanisms in the regulation of I2 meristem specification and activity: PsCam048048, PsCam46067 and PsCam001113 were selected based on the function of their Arabidopsis homologue genes in chromatin silencing (GO term enriched in the VEG1 up-regulated dataset) and PsCam044818 based on the relation of its Arabidopsis homologue with RNA processing (RNA metabolic process including ncRNA) and to the vegetative to reproductive phase transition. The Arabidopsis homologues of these genes are expressed in different domains of the SAM and their expression level change during floral transition (ePlant). PsCam037476 was selected based on its strong up-regulation in the *veg1* mutant, together with the fact that its homologue in Arabidopsis, belonging to the Regulator of Chromosome Condensation (RCC) protein family, displays a strong expression at the SAM (ePlant). PsCam044132, PsCam042718 and PsCam016925 were included in the selected list based on the expression changes of their corresponding homologue genes in Arabidopsis, which in all cases were up-regulated in the SAM upon floral transition (ePlant) and the last two were in addition VEG1 specific (not significant changes were detected in WT/*veg2* transcriptome).

Expression analysis of genes differentially expressed in *veg1*

Since functional characterization of pea genes is still challenging, we decided to narrow down the list by performing an initial gene expression analysis of the 14 selected genes to see whether they are expressed in the inflorescence apex, as expected for genes involved in I2 meristem development, and whether this expression is inflorescence specific. We analyzed the expression of these genes in different organs of pea wild-type plants including roots, stem, leaves, vegetative apices, inflorescence apices and flowers. Expression of the 14 genes was detected in the inflorescence apex at various levels (Figure 5). However, only in the case of *PsDAO1* and PsCam048048 was higher expression in inflorescence apex statistically significant when compared to the vegetative apex. Expression of PsCam00113, PsCam037476, PsCam042718, PsCam044132, PsCam047398, *PsLBD38* and *PsTAR2* was similar in inflorescence apices than in other organs, indicating that these genes could have a role in I2 meristem, but they would probably have additional roles in the development of other plant organs. We could distinguish a third group of genes, including PsCam016925, *PsKMD2*, *PsHUP54*, PsCam044818 and PsCam046067, in which we were able to detect expression in the inflorescence apex but the level of expression was lower than in other organs of the plant. Low level of expression in the inflorescence apex of these genes did not discard them as candidates since a restricted expression in specific regions of the apex (as in the case of I2 meristem - specific expression only) can be masked in RT-qPCR assays.

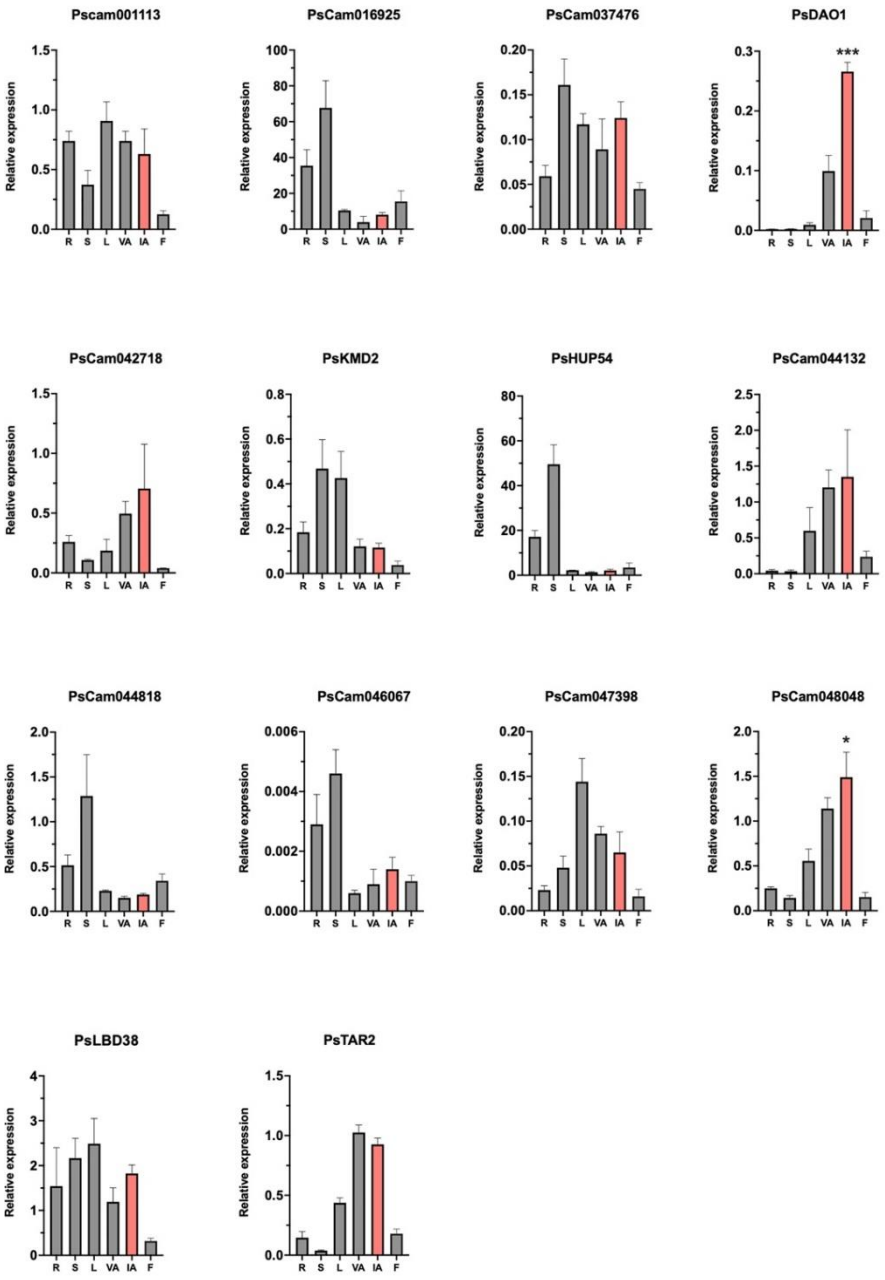

Figure 5. Expression analysis by RT-qPCR of candidates for VEG1 target genes in different pea plant organs. Relative mRNA levels were determined by RT-qPCR. For the analysis, different samples were collected from wild-type plants: roots (R), shoots (S), leaves (L), vegetative apices (VA), inflorescence apices (IA - highlighted in red), and flowers (F). Roots, shoots, leaves and vegetative apices were collected from 3-week-old plants, before the floral transition; the inflorescence apices (highlighted in red) were collected from approximately 5-week-old plants after the floral transition has occurred, and flowers were collected at anthesis. Error bars correspond to standard deviation.

Considering the expression and the information on the corresponding Arabidopsis homologues genes, we decided to characterize the expression pattern by *in situ* hybridization in pea inflorescence apices of five of these candidates: *PsDAO1*, *PsKMD2*, *PsHUP54*, *PsLBD38* and *PsTAR2* (Figure 6).

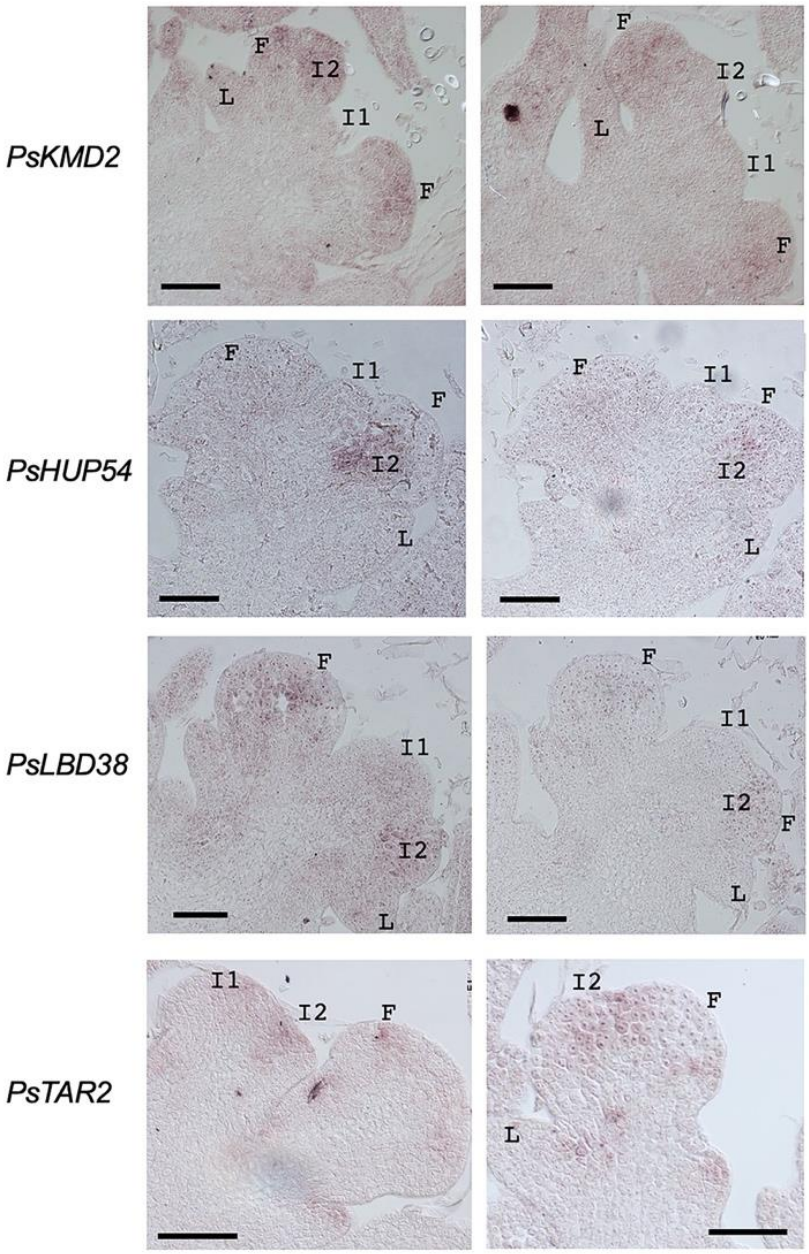

Figure 6. Expression analysis by *in situ* hybridization in pea inflorescence apices of selected candidates for VEG1 target genes. Sections of wild-type pea inflorescence apices were hybridized with antisense probes for the genes *PsKMD2*, *PsHUP54*, *PsLBD38* and *PsTAR2*. L, leaf primordium; I1, primary inflorescence meristem; I2, secondary inflorescence meristem; F, floral meristem/primordium. Scale bars: 100 µm.

The *in situ* hybridization experiments worked for four of the five genes, but not for *PsDAO1*. The probes of the four remaining genes showed hybridization in meristems of the inflorescence apices (Figure 6), while no signal was detected in this tissue for any of these genes with the negative control sense probes (Supplementary Figure 3). *PsKMD2* and *PsLBD38* showed hybridization in both I2 and floral meristems, indicating that expression in the inflorescence of these genes is not specific for the I2 meristem (Figure 6). In contrast, for *PsHUP54* and *PsTAR2,* the hybridization signal was apparently restricted to the I2

meristems, implying possible function of these genes in the specification of I2 identity (Figure 6).

Functional analysis by VIGS of selected genes points to *PsHUP54* / PsCam043354 as possible regulator of I2 activity

In order to analyze the function of selected candidates, we carried out VIGS experiments in pea to silence the expression of four genes: *PsHUP54*, *PsTAR2*, *PsLBD38*, and *PsDAO1*. *PsHUP54* and *PsTAR2* were selected for functional analysis because they showed an expression profile apparently restricted to the I2 meristem (Figure 6). *PsLBD38* was also selected because, although it showed expression in both I2 and floral meristems, RT-qPCR detected high level of expression in inflorescence apices (Figures 5, 6). Finally, we decided to also include *PsDAO1*, even though we could not detect its expression by *in situ* hybridization, due to its strong expression in inflorescence apices detected by RT-qPCR (Figure 5).

VIGS constructs for these four genes were generated using the pCAPE2-PDS vector (Constantin *et al*., 2004). As a control, plants agroinfiltrated with a *GUS*-VIGS construct, containing the *Escherichia coli UidA* gene (*GUS*; Jefferson et al., 1978). The effect on the wild-type plants of the VIGS constructs for the four different pea genes was studied by analyzing different parameters: length of the stem internode, leaf, secondary inflorescence, stub and of the floral pedicel, as well as the number of leaflets (Table 2). *PsTAR2*- and *PsLBD38*-VIGS plants did not show evident phenotypic differences and the only apparent defect observed was in the length of the floral pedicels, which was significantly shorter in *PsLBD38*-VIGS plants than in the *GUS*-VIGS control plants (Supplementary Figure 4; Supplementary Table 4).

In contrast, *PsDAO1*- and *PsHUP54*-VIGS plants consistently showed strong phenotypes as compared to *GUS*-VIGS control plants. In the case of *PsDAO1*-VIGS plants, 3 weeks after infiltration with the VIGS construct they displayed a conspicuous leaf necrosis not observed in plants infiltrated with the *GUS*-VIGS control construct (Supplementary Figure 5). Later on, once the *PsDAO1*-VIGS plants had grown for approximately 10 weeks, they were notably smaller than the *GUS*-VIGS plants (Figure 7A) and the internodes, leaves and floral pedicels were significantly shorter than those of the *GUS*-VIGS plants (Figure 7B).

In contrast, *PsHUP54*-VIGS plants were notably bigger than *GUS*-VIGS plants (Fig 7A) overall, with significantly larger leaves, stubs and floral (Figure 7B, D; Table 2). The phenotype was particularly striking in the case of the I2 stem and the stub, the residual organ

Table 2. Characterization of morphological alterations in *PSDAO1* and *PsHUP54*-VIGS plants

VIGS construct	Parameters								
	Internode length[b] (cm)	**Leaflet number[c]**	**Leaf length[c] (cm)**	**I2 length[d] (mm)**	**Stub length (mm)**	**Floral pedicel length (cm)**	**Pod size[e] (mm)**	**Seed weigth[f] (gr)**	**Seed no. /pod**
GUS	2.25 ± 0.48	3.62 ± 0.72	6.53 ± 1.62	9.75 ± 3.45	1.52 ± 0.67	5.74 ± 0.94	56.93 ± 8.03	3.66 ± 0.19	2.53 ± 1.25
PsDAO1	**0.74 ± 0.30** [a]	3.32 ± 0.98	**2.89 ± 1.41** [a]	3.14 ± 1.53	1.77 ± 0.82	**4.45 ± 1.15** [a]	n.d.	n.d.	n.d.
PsHUP54	2.83 ± 0.95	3.66 ± 0.78	**8.16 ± 2.66** [a]	**26.81 ± 17.10** [a]	**5.66 ± 5.78** [a]	**7.61 ± 2.01** [a]	n.d.	n.d.	n.d.
PsHUP54-Ph[g]	**3.15 ± 0.87** [a]	3.75 ± 0.75	**9.19 ± 2.04** [a]	**30.46 ± 16.73** [a]	**5.97 ± 6.07** [a]	**8.16 ± 1.84** [a]	**67.31 ± 8.65**[a]	3.55 ± 0.18	**4.33 ± 1.97** [a]

The values correspond to mean ± standard deviation.
[a] The data in bold correspond to values with statistically significant variation respect the values of control *GUS*-VIGS plants. For statistical analysis, one-way ANOVA test with Bonferroni and Holm inference test was used.
[b] Values correspond to the average length of the internodes of the stem before the first reproductive node.
[c] Leaflet number and leaf length correspond to the leaves at the first reproductive node and at the previous one.
[d] Values correspond to the length of the "stem" of the I2s of each plant.
[e] The values correspond to the pods at first three reproductive nodes. Seeds in those pods were used to estimate seed weight,
[f] Values for seed weight correspond to the average weight of five groups of 10 seeds from each plant.
n.d. = not determined
[g] Analyzed only in the *PsHUP54*-VIGS plants construct that showed moderate-strong phenotype, characterized for being very high plants > 15 cm (15 out of 19 plants in the experiment exhibited that moderate-strong phenotype)

formed by the I2 meristem once it stops producing floral meristems (Benlloch et al., 2015). The I2 stem and the stub showed close to three- four-fold increase in length, respectively, in the *PsHUP54*-VIGS plants relative to the control. In addition, in one of the *PsHUP54*-VIGS plants, the elongated I2 gave rise to an additional second flower. Notably, the *PsHUP54*-VIGS plants with a moderate-strong phenotype (big plants 15-40 cm high) produced pods that were significantly larger and contained more seeds than those from the control plants, with no decrease in seed weight (Figure 7C; Table 2). This phenotype, although relatively weak, is consistent with a role of *PsHUP54* in regulating the period of time in which the I2 meristem stays active.

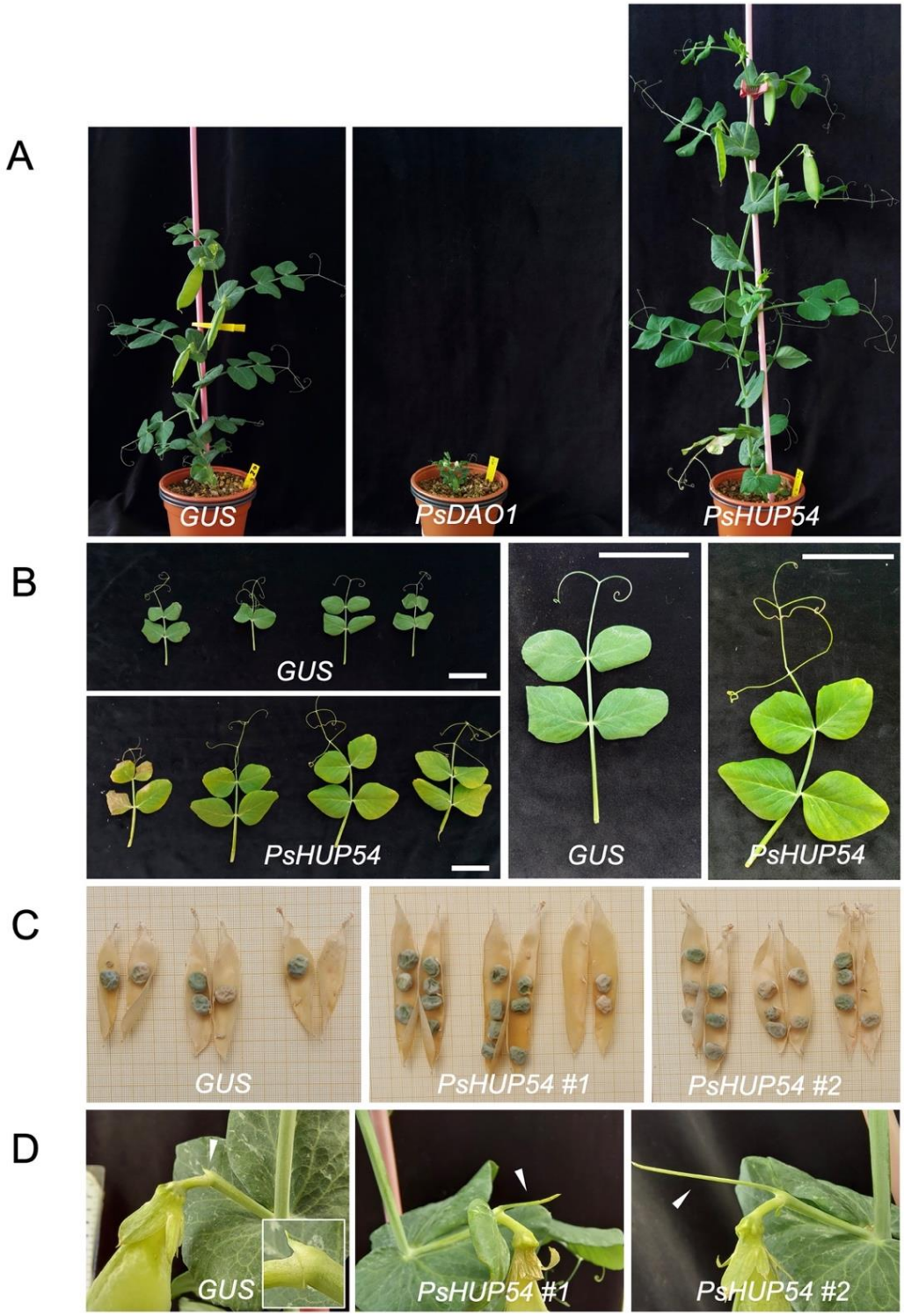

Figure 7. Phenotype of pea VIGS plants for genes PsDAO and PsHUP54. (A) Representative 10-week-old GUS-VIGS (control), PsDAO1-VIGS and PsHUP54-VIGS plants. (B) Leaf defects of PsHUP54-VIGS. Leaves were sampled from the fourth node of different plants. PsHUP54-VIGS leaves are bigger and slightly lighter than GUS-VIGS leaves (C) Seed number and and pod defects of PsHUP54-VIGS plants. The images show the three first pods of different plants. (D) Increased stub length in PsHUP54-VIGS plants. Stubs are marked with arrowheads. Detail of the stub in a control GUS-VIGS plant is shown in a close-up. Scale bars: 5 cm.

DISCUSSION

Recent development of genomic resources, such as high-quality transcriptome assemblies and full genome sequence (Kreplak et al., 2019), represent an important step forward for molecular genetic studies in pea, one of the most studied model plants among legume crops. These new resources have been instrumental for this study aimed at understanding the control of I2 development.

Our experimental approach has been based on the idea that it would be possible to identify genes expressed in the I2 meristem (therefore candidates to be regulated by VEG1) by comparing the transcriptomes of inflorescence apices from pea mutants with defects in I2 development. This experimental strategy has proved successful, and the comparison of inflorescence-apex transcriptomes from wild-type pea, the *pim* mutant (enriched in I2 meristems) and *veg1* and *veg2* mutants (both without I2 meristems) (Taylor et al., 2002; Berbel et al., 2012; Sussmilch et al., 2015) has allowed us to identify a number of genes with preferential expression in the I2 meristem.

In our study the selection of promising candidate genes from the list of genes differentially expressed (DEGs) between the different mutants has suffered from the still limited functional annotation of the pea genome, only relatively recently published (Kreplak et al., 2019). To overcome this limitation, we have used functional information on the homologues of the pea genes in *Medicago truncatula* and Arabidopsis. Since some Medicago databases are still not fully developed, ultimately our main source of information has been the Arabidopsis databases even though Arabidopsis is phylogenetically not so closely related to pea, and the available knowledge for Arabidopsis genes is not ideal to make predictions about their pea homologues. Nevertheless, the Arabidopsis-based information, combined with expression studies and functional analysis by VIGS in pea, has allowed us to identify several interesting genes likely involved in the development of the I2.

Several candidate genes with an interesting expression pattern were identified. Thus, eight out of twelve genes whose expression pattern was analyzed by RT-qPCR showed moderate to high expression level in the inflorescence apex. Among them, the *PsDAO1* gene showed much higher expression in the inflorescence than in the vegetative apex. Moreover, expression of *PsKMD2*, *PsLBD38* and *PsTAR2* was also detected in the I2 meristem by *in* situ hybridization.

PsTAR2 encodes a key enzyme involved in the initial steps of auxin synthesis, which belongs to a small gene family of at least two additional members (Tivendale et al., 2012).

Interestingly, *PsTAR2* specific expression in the I2 meristem of the inflorescence apex suggests that local auxin production in this domain could be important for the correct specification or development of the secondary inflorescence. However, when VIGS was used to study the function of *PsTAR2* no evident phenotypic defect was observed, maybe because redundancy with other close homologs precluded the effective reduction of TAA/TAR activity (Bala et al., 2017; Tivendale et al., 2012). Likewise, in *PsLBD38*-VIGS plants, only a subtle phenotype was observed, where the flower pedicels were significantly shorter than in the *GUS*-VIGS control plants. This phenotype, together with the *in situ* hybridization data, which showed that *PsLBD38* is expressed in the floral meristem, may suggest a possible role of *PsLBD38* in floral development in pea. The LBD family of plant-specific transcription factors is relatively large, with 43 members in Arabidopsis, that can be grouped in two classes. Functional studies have associated class I LBD genes from different species to general roles in lateral organ patterning and in auxin signal transduction (Xu et al., 2016), while for class II genes, to which *PsLBD38* belongs, functional information is still limited, although they appear to be involved in metabolic processes, such as anthocyanin synthesis in response to N availability (Rubin et al., 2009). Intriguingly, the potential function of *PsLBD38* in the control of floral pedicel length resembles more the described role of class I LBD genes in petiole development of leaves in legumes, expanding the evidence on the functional versatility of the family (Chen et al., 2012). Again, it is possible that redundancy masked the phenotypic effects of *PsLBD38* silencing, making it necessary to address this possibility in future studies.

In contrast, *PsDAO1*-VIGS plant exhibited a dramatic phenotype. *PsDAO1*-VIGS plants were very small, with short internodes, small leaves and short floral pedicels. *PsDAO1* is a homologue of the Arabidopsis *DAO1* (*DIOXIGENASE FOR AUXIN OXIDATION 1*) gene, which encodes an indole acetic acid (IAA) oxidase, the major contributor to IAA oxidation in Arabidopsis, whose activity is tightly coordinated with auxin biosynthesis and conjugation (Porco et al., 2016; Zhang et al., 2016). The VIGS-*PsDAO1* phenotype, could possibly reflect an alteration in these VIGS plants of auxin homeostasis, a hormone with a key role in the regulation of plant growth. The dramatic organ size reduction found in the pea VIGS-*PsDAO1* plants contrasts with the phenotype of Arabidopsis *dao1* mutants that, apparently depending on the growing conditions, show either a slight reduction in inflorescence stem and siliques (Porco et al., 2016) or moderate enlargement of rosette leaves and inflorescence stem (Zhang et al., 2016). However, it should be noted that in Arabidopsis two closely related genes, *AtDAO1* and *AtDAO2*, are found in tandem in the genome (At1G14130 and At1G14120, respectively) and the double mutant has not been generated yet, so the full consequences of the lack of IAA oxidation have not been uncovered so far.

Moreover, functional analyses of *DAO1* homologues in rice show a prominent role of these enzymes in reproductive development, where the mutants showed severe defects in anther dehiscence, pollen maturation and flower aperture (Zhao et al, 2013). The *PsDAO1*-VIGS plants from this work show a different effect of potentially reduced auxin catabolic processes. It is clear, then, that more studies are required to better understand the full spectrum of IAA oxidation roles in development, and how it is integrated in auxin signaling pathways.

Finally, despite a relatively low expression in the apex, after floral transition *PHUP54* expression becomes spatially restricted to the I2 meristem. The most prominent effect of silencing *PsHUP54* is a dramatic increase in plant growth, which affects most aerial organs, including shoot length, leaf size and pod length, with a subsequent increase of seed production, indicating that *PsHUP54* could function as a general repressor of growth. Regarding I2 activity, a conspicuous defect in *PsHUP54*-VIGS plants was that the stubs, the residual organs formed by the I2 meristems after producing the flowers (Benlloch et al., 2015), were usually much longer than in the control *GUS*-VIGS plants. Together with the specific expression of *PsHUP54* in the I2 meristem, this suggests that *PsHUP54* could be a target of VEG1 that promotes I2 meristem termination, so that in the *PsHUP54*-VIGS plants the I2 meristems stay active for longer. Accordingly, in the *PsHUP54*-VIGS plants, a higher number of flowers per node was observed in one plant, which supports this hypothesis.

The Arabidopsis *HUP54* gene belongs to a small gene family with homologs present in all plant groups, which contain conserved YGL and LDRD motifs (Cheng et al., 2017). Despite the high level of conservation, especially in angiosperms, where homologs with a percentage of identity higher than 60% can be found even in the most basal clades, limited functional information based in mutant phenotypes is available for these proteins, and their molecular function is still basically unknown. In Arabidopsis, *AtHUP54* appears to be involved in hypoxia tolerance (Mustroph et al, 2010) and plant cell wall remodeling as target of the SHINE transcription factors in a GA-dependent manner (Shi et al., 2011). The possible link of these putative functions with the control of plant growth in pea remains to be explored in detail.

It seems noticeable that the conspicuous phenotype of *PsHUP54*-VIGS plants, which were notably larger, featured longer pods with up to double number of seeds, with no concomitant decrease in seed weight, has not apparently been identified in mutant screenings in pea or other legumes. A possible explanation is that the *PsCam027351* gene, present in the pea genome, with a high level of similarity to *PsHUP54* (77% nucleotide identity), is functionally redundant to *PsHUP54*. Since the *PsHUP54*-VIGS construct might be silencing both genes, the phenotype of the *PsHUP54*-VIGS could be equivalent to that of a double *PsHUP54*

PsCam027351 mutant, something that will have to be considered if these genes are to be exploited in breeding programs. Notably, our work has revealed a novel role for a protein of unknown function in growth control, which looks to be a promising tool to improve yield in pea and possibly also in other legumes where highly conserved homologues of *PsHUP54* are present (Supplementary Figure 6).

In summary, this study presents a successful strategy to identify genes with expression in the I2 meristem of the pea inflorescence, likely controlling different aspects of inflorescence architecture in legumes. Although more detailed functional analyses should be carried out to elucidate the precise functions of these genes, our approach has already served as a proof of concept to validate the use of the new genomic tools available for pea and to identify at least one novel gene that is a potential target for breeding programs.

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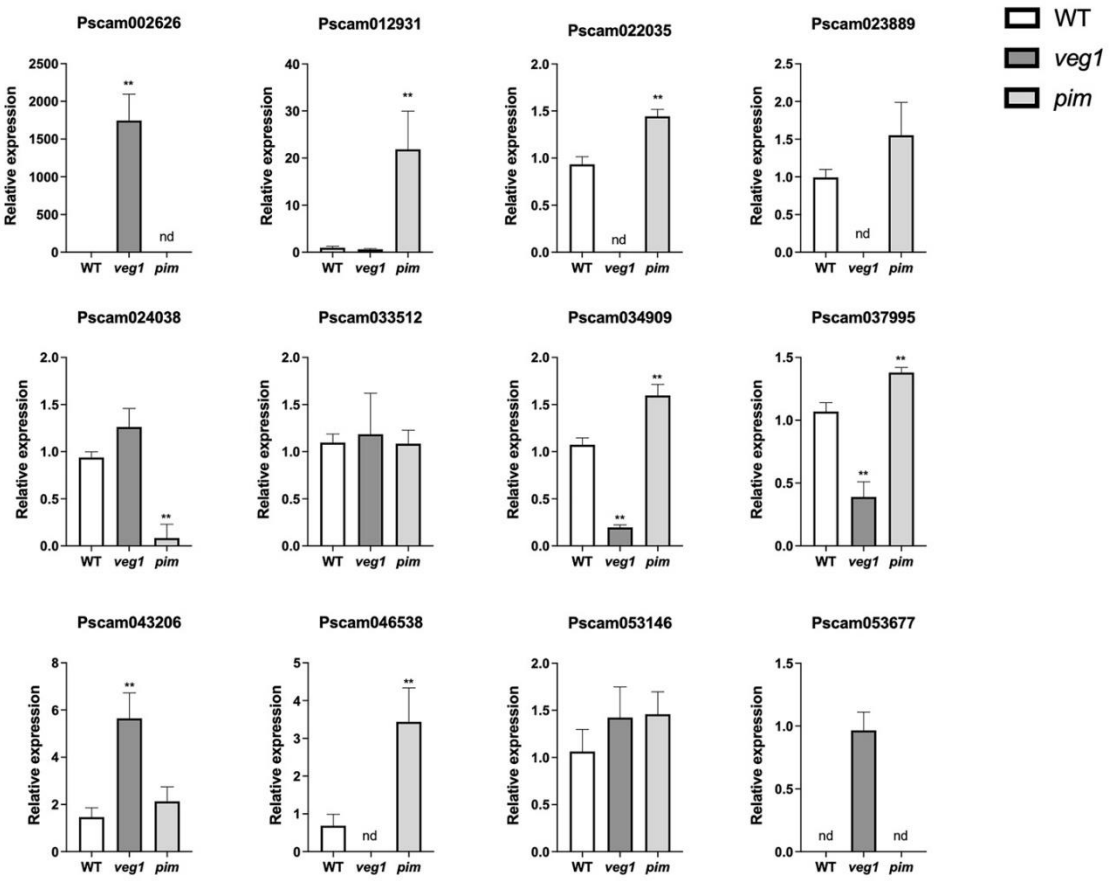
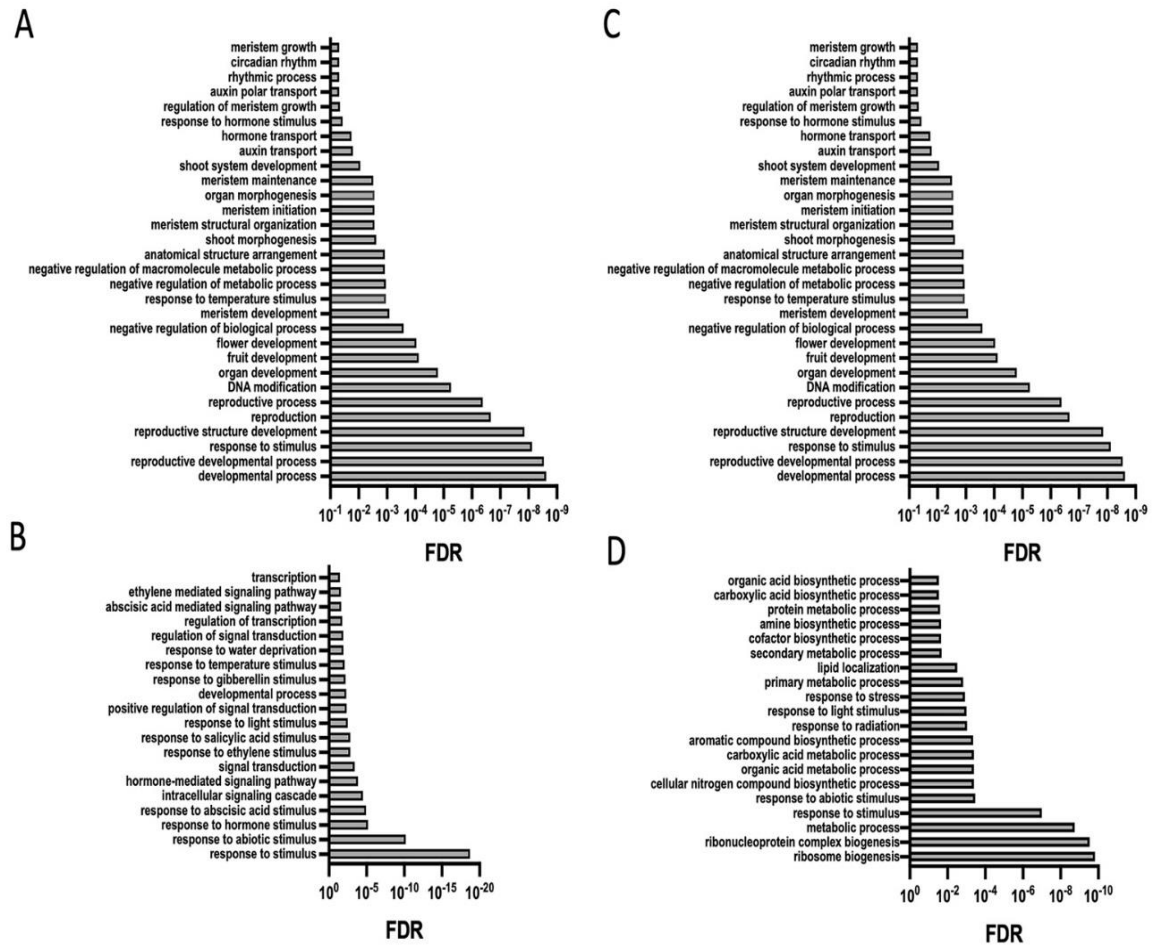
SUPPLEMENTARY FIGURE 1



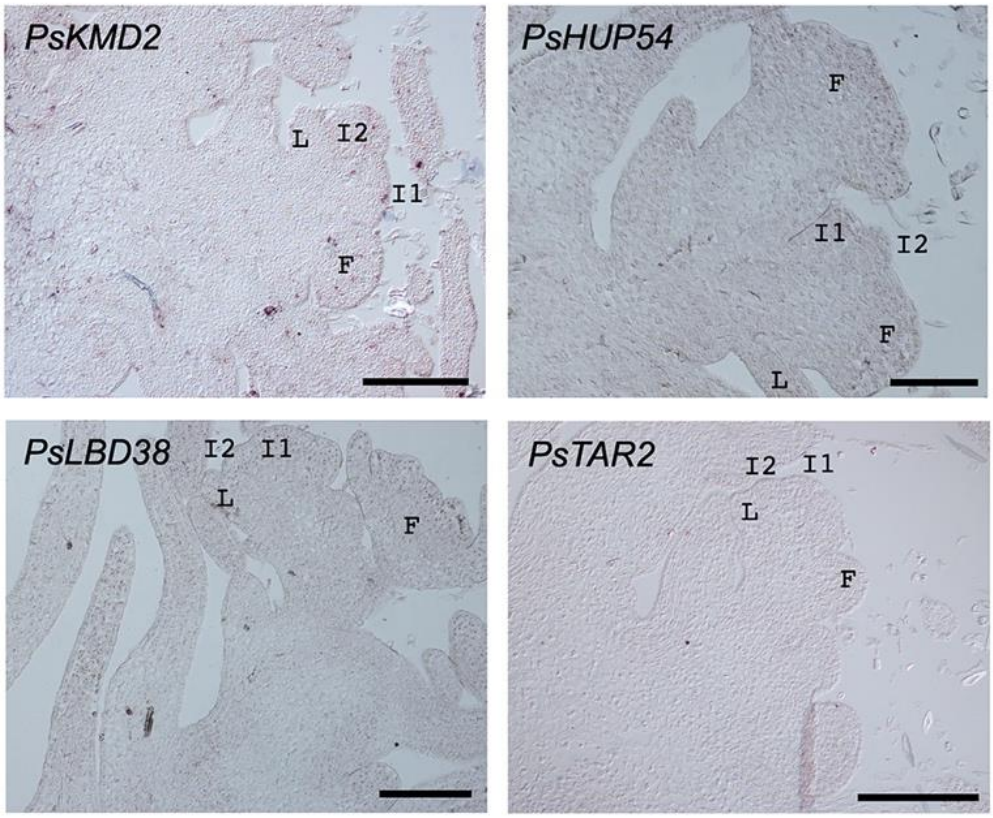
Supplementary Figure 1. Validation of RNAseq data by RT-qPCR analysis. RT-qPCR expression analysis, in wild-type, *veg1*, and *pim* inflorescence apices, of 12 genes randomly chosen among those showing opposite expression between WT/*veg1* and WT/*pim.*

SUPPLEMENTARY FIGURE 2



Supplementary Figure 2. Gene ontology term (biological processes) enrichment among differential expressed genes in WT/*pim and* WT/*veg2* transcriptomes. (**A)** Selected GO terms returned from the analysis of genes up-regulated in *pim*. **(B)** Selected GO terms returned from the analysis of genes down-regulated in *pim*. **(C)** Selected GO terms returned from the analysis of genes up-regulated in *veg2*. **(D)** Selected GO terms returned from the analysis of genes down-regulated in *veg2*. Terms were selected according to their relation to previously described PIM or VEG2 function and/or potential mechanisms of action. All depicted terms were overrepresented (False Discovery Rate (FDR) < 0.05). A complete list of all enriched GO terms for each analysis is detailed in Supplementary Table 3.

SUPPLEMENTARY FIGURE 3



Supplementary Figure 3. Negative controls (sections hybridized with sense probes) for *in situ* hybridization experiments in Figure 6. L, leaf primordium; I1, primary inflorescence meristem; I2, secondary inflorescence meristem; F, floral meristem/primordium Scale bars: 200 mm.

SUPPLEMENTARY FIGURE 4

Supplementary Figure 4. Phenotype of pea VIGS plants for genes *PsTAR2* and *PsLOB38* Representative six-week-old *GUS*-VIGS (control), *PsTAR2*-VIGS and *PsLOB38*-VIGS plants.

SUPPLEMENTARY FIGURE 5

Supplementary Figure 5. Morphological alteration of plants infiltrated with the VIGS-*PsDAO1* construct. Pea plants three weeks after infiltration with the *GUS*-VIGS or *PsDAO1*-VIGS constructs.

SUPPLEMENTARY FIGURE 6

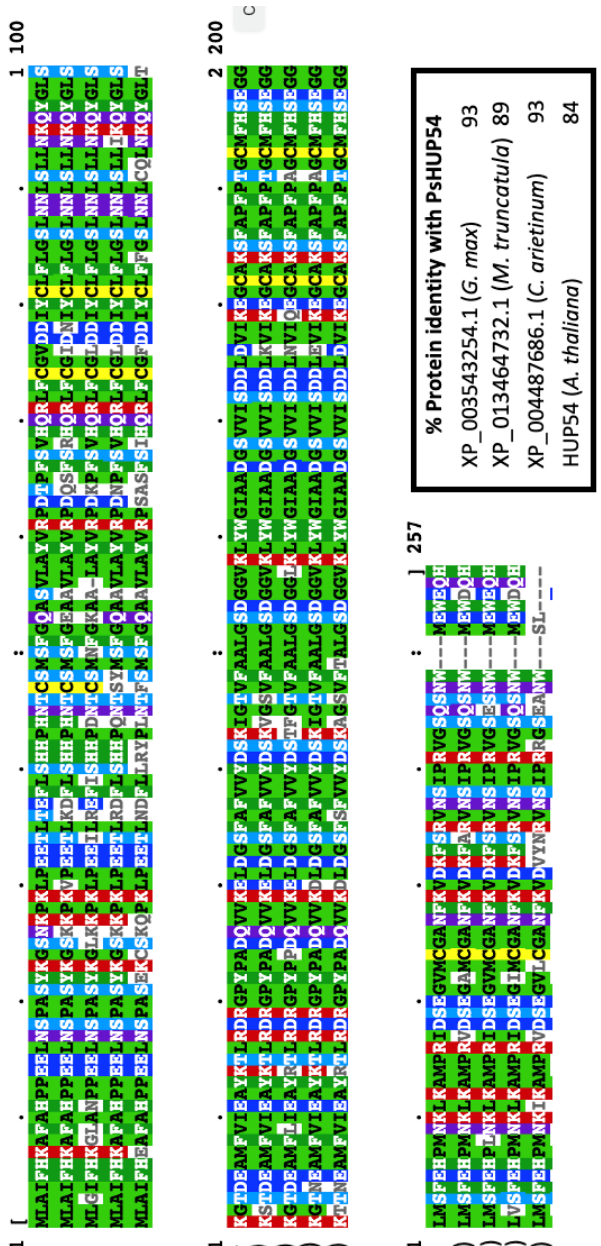


Figure 6. Sequence alignment of PsHUP54 with homologues from *Glycine max* (XP_003543254.1), *Medicago truncatula* (XP_013464732.1), *Cicer arietinum* (XP_004487686.1), *Arabidopsis thaliana* (HUP54, Q93V62) and *Nicotiana tabacum* (TSJT1, A0A1S4D0M8). The percentage of protein identity for each homologue is shown in the square.

SUPPLEMENTARY TABLES

Supplementary Tables 1-3 are available through the following link (stored in a Mendeley Data resource):

https://data.mendeley.com/datasets/krf3rhbv4n/draft?a=4d166568-0751-47bd-9131-6a12c7f2bf4e

- Supplementary Table 1. Differentially expressed genes for all three mutant transcriptomes versus wild type
- Supplementary Table 2. Genes with opposite expression in *veg1* and *pim*.
- Supplementary Table 3. Go term enrichment DEGs for all three transcriptomes

Supplementary Table 4. Characterization of morphological alterations in *PsLOB38*-VIGS and *PsTAR2*-VIGS plants

VIGS construct	Parameters			
	Internode length[b] (cm)	Leaflet number[c]	I2 length (cm)	Floral pedicel length (cm)
GUS	2.07 ± 0.38	3.30 ± 0.98	7.78 ± 5.50	4.83 ± 1.29
PsTAR2	1.75 ± 0.40	3.22 ± 0.81	5.47 ± 2.22	4.10 ± 1.02
PsLOB38	1.91 ± 0.33	3.68 ± 0.75	5.10 ± 2.47	**3.30 ± 2.51** [a.]

Values correspond to mean ± standard deviation.

[a] The data in bold correspond to values with statistically significant variation respect the values of control *GUS*-VIGS plants. For statistical analysis, one-way ANOVA test with Bonferroni and Holm inference test were used.

[b] Values correspond to the average of the internodes of the stem before the first reproductive node

[c] Leaflet number correspond to the leaves at the first reproductive node and at the previous one.

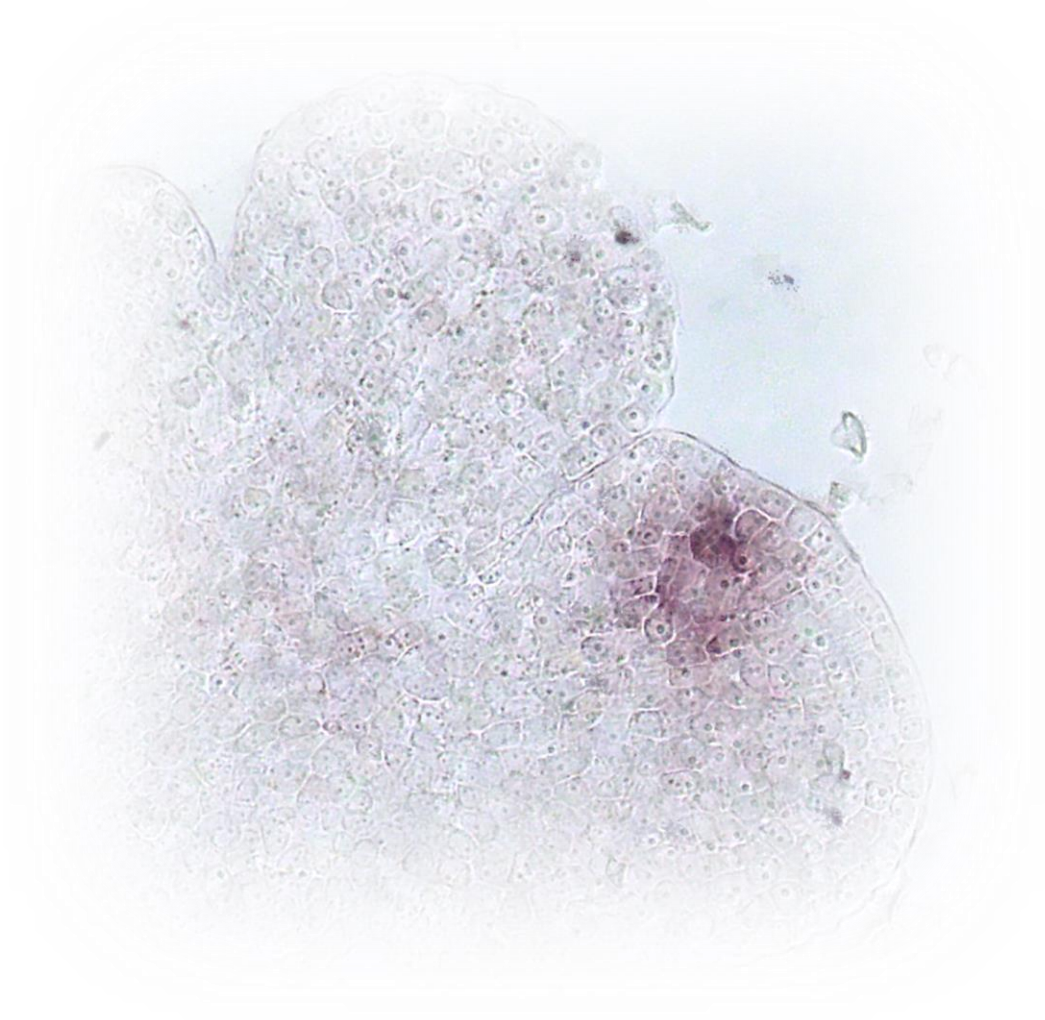
Supplementary Table 5. Primers used in this work

Primer name	Sequence
RT-qPCR primers for detecting floral transition (Fig 1)	
VEG1 F	CACTCAGTGGTGCTATACAAG
VEG R	GCATCCATGCAGGAATAAGA
PIM F	TGCAGCTGAGCAGCAGGTA
PIM R	TAGTAAGTAATTTGGATTGACTCCATG
RT-qPCR primers for reference control gene	
ACTIN11 (PEAc14) F	AGGTGCTGTACCAACCATCCA
ACTIN11 (PEAc14) F	CGTGAATTCCTGCTGCTTCC
RT-qPCR primers for RNAseq validation (Supplementary Fig 1)	
Pscam002626 F	AGTTAAATCCGCTGCTGCTG
Pscam002626 R	TGGGAATTTCGGCTTGGATG
PsCam012931 F	TTCGGCAGAAGATGAGGATGAG
PsCam012931 R	TGCCACTGCAACAAAAGCTG
PsCam022035 F	ATAACCACGGACATGCCTTG
PsCam022035 R	AATCATCGTCGCCTACCAAC
PsCam023889 F	AACAGGGCCATGGTCAATTG
PsCam023889 R	TTTCCAAACGCAAGCCAAGC
PsCam024038 F	AACGGAGATGAAGGCGTTTG
PsCam024038 R	ACCACACTCCAATCGTCAAC
PsCam033512 F	GGCGTGATTGCAAAATCGAG
PsCam033512 R	TTGCCACTTCAATGCGTTGC
PsCam034909 F	AACCTGGTTTGCACCTTCAC
PsCam034909 R	TGATTTTGGGTGCAGCACTG
Pscam037995 F	AGGTTTACCATGTCGGGTCTTC
Pscam037995 R	TCCATTCTACCAGGACGCAAC
PsCam043206 F	TGTGTGGCTGCATTTGCTTC
PsCam043206 R	ACATGGTGTGGTCCAAACTC
PsCam046538 F	AACCACTCATTCAGCTGTGC
PsCam046538 R	TCCCAAGTAGTTCAAGCTCAGG
PsCam053146 F	TGTGATTCCTGCCTTTGCAC
PsCam053146 F	GTGGAAGAGACCTTGTGCAAAG

PsCam053677 F	AATCTGGCCGTTGAATCTGC
PsCam053677 R	GGGCTTACACCAACAGACAAAC
RT-qPCR primers for expression of candidate genes (Fig 5)	
PsCam001113 F	AACTCAGCCATGCAAAGACG
PsCam001113 R	AGTTGTTCAGTCGTGTGAGC
PsCam016925 F	TTTTTCAAGGGGTGGTGGTG
PsCam016925 R	ATGTCGTAACCTCCACCGTATG
PsCam037476 F	TTTTGGGGAGCCTTCAAACC
PsCam037476 R	AAAGCAACCACGTGAACAGC
PsCam039164 F	TCGTGTGCAATGCAAGGAAG
PsCam039164 R	TTGGAGCCTCAACATTTCCC
PsCam042718 F	TGCAGCTGCTGTAATGCATG
PsCam042718 R	TCCGTGACAACAGTTTTGGC
PsCam043276 F	TTCCGAACGAACATGTGGAG
PsCam043276 R	TGTCCACCGGCAACAAAAAC
PsCam043354 F	TGCAGCTGATGGATCTGTTG
PsCam043354 R	GCAAAGGATTTAGCGCAACC
PsCam044132 F	GCCTAGTTGCTGCAATTGTG
PsCam044132 R	AACTTGCCTCGAAGAACCAC
PsCam044818 F	TTACATTTGCGGTGCGTGTG
PsCam044818 R	TCTTGACACTTGGCATTGGC
PsCam046067 R	ATTTGCACCCCCAAACCAAG
PsCam046067 R	TTCCCCTGTTCACTGCAAAC
PsCam047398 F	AATGGTGGTGCAGTTCCATG
PsCam047398 R	ACAACAACGCGGAGAAACAG
PsCam048048 F	AAGCCCGCACAATCAAAAGG
PsCam048048 R	TGACATTTCGGCAGCTTCAG
PsCam050808 F	ACGCAAGCGATTGAGTGAAG
PsCam050808 R	AACTGTTGGTCAACGCGTTC
PsCam057706 F	AGGTCGCCACATTGTTGTTG
PsCam057706 F	TGGCAGAAACAACGCTGATG
PCR primers for probes for *in situ* hybridization (Fig 6)	
PsCam039164 F	ATGAAAATGTTGGTGGTCTTGAAAT
PsCam039164 R	TTAAACCAAGCGTAATAACTCGAGA

PsCam043276 F	TTGGTGACTGGGTCCGAATACGAAG
PsCam043276 R	AAAGTTCACTGCGAAACCATACATG
PsCam043354 F	ATCCAATGAACAAGTTGAAAGCAAT
PsCam043354 R	AAACACTTGAATATATTGTGTTACT
PsCam050808 F	ACAGTTACCGGAGGGACGTTTG
PsCam050808 R	TCCTCACCGACTAACCTTCATCTTT
PsCam057706 F	ATATTCTGAAACCAAGTTGTTTTCT
PsCam057706 R	TTTTTGAATAATCCGATTATACCCT
Primers for VIGS constructs (Fig 7)	
PsDAO1-VIGS F	TCCATGGATGAAAATGTTGGTGGTCTTGAAAT [a] (NcoI)
PsDAO1-VIGS R	TGAATTCTTAAACCAAGCGTAATAACTCGAGA (EcoRI)
PsHUT54-VIGS F	TCCATGGATCCAATGAACAAGTTGAAAGCAAT (NcoI)
PsHUT54- VIGS R	TGAATTCAAACACTTGAATATATTGTGTTACT (EcoRI)
PsLOB38- VIGS F	TCCATGGACAGTTACCGGAGGGACGTTTG (NcoI)
PsLOB38- VIGS R	TCTGCAGTCCTCACCGACTAACCTTCATCTTT (EcoRI)
PsTAR2-VIGS F	TCCATGGATATTCTGAAACCAAGTTGTTTTCT (NcoI)
PsTAR2-VIGS R	TGAATTCTTTTTGAATAATCCGATTATACCCT (EcoRI)

[a]Restriction sites in the primers are underlined.



CHAPTER 2

PsFTc, a homologue of the florigen FLOWERING LOCUS T, regulates the development of the pea compound inflorescence

Marcos Serra-Picó, Valérie Hecht, Frances C Sussmilch, Reyes Benlloch, Francisco Madueño, James L Weller

*In Capter 2, isolation of *ftc* mutants, generation of the mutant combinations and a preliminary characterization of them was carried out by Dr Valèrie Hecht and Dr Frances Sussmilch. Marcos Serra-Picó has carried out the expression analysis of *FTc* by *in situ* hybridization and the detailed quantitative characterization of the *ftc* mutant combinations that is presented in this thesis. He has also actively contributed to the analysis of the data and to writing the paper.

PsFTc, a homologue of the florigen *FLOWERING LOCUS T*, regulates the development of the pea compound inflorescence.

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ABSTRACT

Legumes usually have compound inflorescences, where flowers are not directly formed from the main primary inflorescence (I1) stem, like in simple inflorescences such as that of Arabidopsis, but instead they are formed from secondary inflorescences (I2). In pea and other temperate legumes, flowering is induced by long-day photoperiods (LD). *FLOWERING LOCUS T* (*FT*) is as main promoter of flowering in Arabidopsis. LDs induce *FT* in the leaf and its protein moves to the shoot apical meristem where it activates genes responsible for floral initiation. In pea, the *FT* family has expanded to six members, one of which, *PsFTc*, is peculiar both in its protein sequence as well as in its expression pattern, which is not found in leaves, but only in the shoot apex after the floral transition. We have studied *PsFTc* expression domain, finding that it is specifically restricted to the I2 meristem, overlapping with *VEGETATIVE1(VEG1)/PsFULc*, a pea I2 meristem identity gene. We have isolated and characterized loss-of-function mutants in *PsFTc*. *psftc* mutations delay flowering, common for *ft* mutants. Interestingly, they also cause defects in development of I2s, which show novel abnormal structures. Genetic analysis showed that these *psftc* I2 defects are enhanced when combined with mutations in *DETERMINATE(DET)*/*PsTFL1* or *LATE FLOWERING*(*LF*)/*PsTFL1c*. The nature of the abnormal I2 structures and their frequency in the different mutant combinations suggest that *PsFTc* contributes to inflorescence development by inducing *VEG1* expression.

INTRODUCTION

Plants encompass a great morphological diversity and one of the features that most conditions this is their architecture, the number and disposition of their organs. The inflorescences, the reproductive structures that bear flowers and fruits, are a major component of plant architecture. In the past decades, many studies have been carried out trying to find out the mechanisms that condition the different forms that inflorescences adopt depending on their genotype, and nowadays, the genetic pathways that control inflorescence morphology are relatively well known in quite many species (Gauley and Boden, 2019; Zhu and Wagner, 2020). This is specially the case for Arabidopsis, which has a simple inflorescence, where flowers directly develop from the main apex (Weberling, 1992; Teo et al., 2014).

Important crop species such as those in the large families of legumes and cereals typically have compound inflorescences (Weberling, 1989; Kellogg, 2007). In plants with this type of inflorescence the flowers develop from secondary or higher order axes relative to the primary inflorescence stem (Weberling, 1992). This higher complexity potentially involves a concomitant increase of the number of genes involved in inflorescence development and/or their regulatory interactions (Hecht et al., 2011; Gauley et al., 2019) and, thus, further studies are required to fully understand the genetic networks that control the architecture in plants with complex inflorescence types. The ecological and economical importance of cereal and legume crops has fuelled the research to understand development of compound inflorescences, since plant architecture impacts on yield, and obtaining information of the genes involved in its regulation can be important to improve breeding programs (Wang and Li, 2008; Schilling et al., 2018; Basu and Parida, 2021). The aim of our work has been to expand our knowledge on the genetic interactions that direct the development of the compound inflorescence in pea.

The architecture of the inflorescence is controlled by meristem identity genes. In Arabidopsis, these genes mainly are *TERMINAL FLOWER 1* (*TFL1*), *LEAFY* (*LFY*) and *APETALA 1* (*AP1*) (Shannon and Meeks-Wagner, 1993; Liljegren et al., 1999; Blázquez et al., 2006). *TFL1*, which encodes a protein from the Phosphatidyl Ethanolamine Binding Protein family (PEBP), acts by maintaining the identity of the primary inflorescence meristem, so that in *tfl1* mutants the inflorescence meristem is transformed into a floral meristem, which ceases its indeterminate growth with the formation of a terminal flower (Shannon and Meeks-Wagner,1991; Bradley et al, 1997). On the other hand, the transcription factors AP1 and LFY specify the identity of the floral meristem and, thus, in the *ap1* and in *lfy* mutants, flowers are replaced by inflorescence-like ramified structures. *TFL1*

and *AP1/ LFY* are expressed in complementary domains in the shoot apex, with *TFL1* being expressed in the shoot apical inflorescence meristem (inflorescence SAM), and *AP1* and *LFY* in the flanks of the inflorescence meristem where the floral meristems are formed. This complementary expression pattern, which is maintained by mutual repression between *TFL1* and *AP1 / LFY*, makes sure that the primary inflorescence SAM grows indeterminately while generating flowers in its flanks, therefore giving rise to a simple inflorescence (Irish and Sussex, 1990; Schultz and Haughn, 1991; Weigel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al, 1994).

A common inflorescence type in legumes is compound, characterized by the presence of a main/primary inflorescence (I1), secondary inflorescences (I2) and flowers. The I2s arise laterally from the I1 stem and gives rise to the flowers. The key regulators of inflorescence meristem identity in legumes have been best studied in pea. In this species, the main genetic functions fulfilling these roles are *DETERMINATE* (*DET*) and *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*), homologues to *TFL1* and *AP1*, respectively, and *VEGETATIVE1* (*VEG1*).

PIM codes for a MADS transcription factor that is expressed in the floral meristem (Berbel et al., 2001; Taylor et al., 2002). In *pim* mutants, the floral meristems fail to acquire their correct identity producing a proliferation of I2-like meristems (Taylor et al., 2002).

DET, also known as*TFL1a,* is expressed in the I1 SAM after the floral transition and acts by maintaining I1 meristem identity (Foucher et al, 2003; Berbel et al., 2012). The only evident defect of *det* mutant plants is that they are unable to maintain I1 identity, and opposite to wild-type, which show indeterminate growth, *det* mutants show determinate growth with the I1 meristem terminating with the formation of a I2, while flowers develop normally without displaying any defect (Singer et al., 1990). In addition to *DET*, pea has another *TFL1* homologue, *LATE FLOWERING (LF)/TFL1c* which is expressed during vegetative growth and appears to carry out the flowering-time related function of *TFL1*, acting as a repressor of floral transition (Foucher et al, 2003). Therefore, *DET* and *LF* seem to jointly bring together the whole function of the Arabidopsis *TFL1* gene (Foucher et al, 2003).

VEG1/FULc codes for another MADS-box transcription factor responsible for maintaining the identity of I2 meristems in pea (Berbel et al., 2012). Consistent with its function, *VEG1* is expressed after the floral transition at the inflorescence apex, specifically in the I2 meristems. In *veg1* mutants, the plants do not produce flowers, and the I2 meristems appear to acquire a I1 identity, so the lateral structures that are produced by the primary I1 resemble vegetative branches that in turn produce laterally more vegetative branches (Gottschalk,

1979; Reid and Murfet, 1984; Berbel et al., 2012). This phenotype is consistent with the ectopic expression of *DET* in lateral meristems produced by the I1 in the *veg1* mutant, where *VEG1* is normally expressed in the wild-type. This explains the conversion of the I2 meristems into I1 meristems, which do not form flowers (Gottschalk, 1979; Reid and Murfet, 1984; Berbel *et al.*, 2012). Interestingly, despite this non-flowering phenotype, in *veg1* mutants the transition from the vegetative phase to the reproductive phase takes place, as marked by the onset of *DET* expression at the equivalent time and node as it would be upregulated in wild-type plants (Berbel et al., 2012).

PIM, *DET and VEG1* homologs, as well as their genetic interactions, have been further studied in other legume species, where they appear to be functionally conserved (Dong et al., 2005; Benlloch et al., 2006; Ping et al, 2014; Cheng et al, 2018).

The timing of flowering is regulated by a number of different environmental cues including daylength and temperature, as well as by endogenous and hormonal pathways. A wealth of knowledge about these complex routes has been acquired in the last decades, most extensively in Arabidopsis, but also in many other species (Kim et al., 2009; Amasino and Michaels, 2010; Andrés and Coupland, 2012; Song and Imaizumi, 2013; Brambilla and Fornara, 2013; Kinoshita and Richter, 2020; Cao et al., 2021). The Arabidopsis *FT* gene, which as TFL1 codes for a member of the PEBP family, is a key factor of the photoperiod inductive pathway regulating flowering time (Yanovsky and Kay, 2002; Wigge et al., 2005; Kardailsky et al., 1999). *FT* is expressed in the leaves and its protein moves to the SAM where it interacts with the FD bZIP transcription factor to activate the floral meristem identity genes (Abe et al., 2005; Wigge et al., 2005; Zhu et al., 2020). Thus, opposite to *TFL1*, *FT* promotes flowering. Some studies have demonstrated that the antagonistic roles of TFL1 and FT depend on a few amino acid residues on the fourth exon of both proteins, and that a single aminoacidic change is enough to switch the function of these proteins from an activator to a repressor of flowering and vice versa, which therefore emphasizes the similarity between them and the importance of the conserved residues in this region (Hanzawa et al., 2005; Ahn et al., 2006; Ho and Weigel 2014).

In legumes, the FT subclade of the FT/TFL PEPB family has diversified, and in diploid species it consists of six members in contrast to Arabidopsis, that only possesses two *FT* genes, *FT* and *TWIN SISTER OF FT (TSF)*. Previous phylogenetic analyses have shown that legume *FT*s fall into three different subclades: *FTa*, *FTb* and *FTc* (Hecht et al., 2011). The *FTa* clade includes three genes: *PsFTa1*, which is expressed at both apex and leaves; *PsFTa2,* which is expressed at the apex; and *PsFTa3,* recently identified when the pea genome sequence has been completed. The *FTb* clade includes two genes: *PsFTb1*, with

apparently no expression in leaves or apex, and *PsFTb2*, which is expressed specifically in leaves. Finally, the *FTc* subclade consists of a single gene, *PsFTc* (hereafter *FTc*) which is expressed solely at the apex. Among the *FT* genes, aside of being the only one that is exclusively expressed at the apex, *FTc* is also the most distant phylogenetically, with several important changes on its sequence. The most remarkable change in FTc is that His140 replaces Gln140, a highly conserved residue in FT proteins that unambiguously distinguishes FT from TFL1 proteins, where the equivalent, highly conserved, residue is Asp144 (Supplementary Figure 1; Ahn et al., 2006; Hecht et al., 2011).

Several functional interactions between FT/TFL1 family members and meristem identity genes have been previously established, as for example, the induction of *AP1* and *LFY* by FT/FD (Abe et al., 2005; Wigge et al., 2005; Blázquez et al., 2006; Jaeger and Wigge, 2007; Mathieu et al., 2007; Zhu et al., 2020), or the repression of floral genes by TFL1 (Hanano and Goto; 2011; Goretti et al., 2020; Zhu et al., 2020). However, in legumes, given the expansion of the FT/TFL1 family into different subclades, it is likely that these interactions have increased in complexity. Among the pea *FT* genes, *FTb2* meets the requirements for being the first mobile signal that orchestrates the onset of the floral transition together with *FTa1* (or *GIGAS*), although they may possess slightly different developmental roles (Hecht et al., 2011; Weller and Ortega, 2015). On the other hand, *FTc*, which is expressed specifically at the apex, has been suggested to integrate different FT-derived signals from leaves (Weller and Ortega, 2015), which points to it as a possible inducer of the meristem identity genes and the subsequent floral transition.

In this sense, the question of the role of *FTc* and its possible relationship with other meristem inflorescence genes remains unknown. In order to shed light in this subject, in this work we have isolated and characterized pea *ftc* mutants and used them to study the genetic interactions of *FTc* with the pea *TFL1* homologue genes *DET* and *LF*. Our results reveal a function of *FTc* in the control of flowering and, unexpectedly, in the control of inflorescence meristem identity, with this second function being possibly mediated through the regulation of *VEG1*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pea mutants (*det-3 and lf-22*) have been previously described (Foucher et al., 2003; Weller et al., 2009) and had been introgressed in the NGB5839 pea line (Hecht et al., 2007). Plants

were grown in a greenhouse at controlled conditions under long-day (LD) photoperiods (21.5°C- 16 h light / 16°C - 8 h darkness). When needed, natural light was supplemented with artificial lighting (400W Phillips HDK/400 HPI (R)(N)). Plants were irrigated periodically using Hoagland Nº1 solution supplemented with oligoelements (Hewitt, 1966). *ftc* alleles were isolated in an EMS-mutagenized TILLING population of Cameor cultivar (Dalmais et al., 2008) and introgressed by backcrossing seven times into the NGB5839 background.

Histological sections and *in situ* RNA hybridization

In situ hybridization (ISH) assays were performed on 8mm-thick longitudinal sections of wild-type pea (NGB5839) inflorescence apices embedded in paraffin. Detailed anatomical observations were carried out to determine the optimal sampling time in order to hybridize apices around the floral transition; in our conditions, plants that still had visible but unfolded leaves in the 10th node were chosen for apex fixation. *In situ* RNA hybridization was carried out according to previously described protocols (Ferrándiz et al., 2000) with the following modifications: contiguous individual sections were placed alternating between two slides and used to perform the hybridization assay with *FTc* and with *VEG1* probes in parallel. For each gene, digoxigenin-labelled probes were generated using as a template a fragment of the coding sequence of the corresponding gene. The *VEG1* probe was described in Berbel et al. (2012). For the *PsFTc* gene, the RNA probes were generated using as a template a 639-bp fragment corresponding to nucleotide positions 1-639 of the *PsFTc* transcript (PsCam040405). Nucleotide positions are indicated using as reference the ATG codon. Each fragment was amplified by PCR using inflorescence apex cDNA as a template and cloned into the pGEM-T Easy vector (Promega). RNA anti-sense probes were generated with SP6 RNA polymerase; RNA sense probes were used as a control in each case, and they were generated with T7 RNA polymerase. *FTc* ISH probes were hydrolysed for 46 min at 60 ºC in order to generate fragments of approximately 150 pb.

Phenotypic characterization

For the phenotypic characterisation of simple, double and triple mutant combinations (*ftc-2; ftc-2 lf-22; ftc-2 det-3; ftc-2 det-3 lf-22*) and the control plants (WT, *lf-22*, *det-3*, *det-3 lf-22*) we scored the occurrence of a set of phenotypical traits affecting I2 development. Frequency of occurrence of these traits was compared in each case with that of the wild-type NGB5839 control plants and/or corresponding single mutants. For flowering time analysis, we considered the “node of flowering initiation” as the first node bearing an I2-structure that produced a flower.

Statistical Analyses

For statistical analysis of the previously mentioned parameters in *ftc* mutant combinations and controls, all the data containing multiple comparisons between different genotypes were analysed by one-way ANOVA with posthoc HSD Tukey's test taking as significant difference depending on Bonferroni and Holm multiple comparison system.

RESULTS

ftc mutants show defects in flowering time and in the development of secondary inflorescences

To understand the function of the pea *FTc* gene, we followed a reverse genetics approach. Two pea mutant lines in the *FTc* gene were generated by TILLING in the pea Cameor genetic background: *ftc-1* and *ftc-2*. These mutations are found in the first exon of *PsFTc* and consist of Pro8Leu and Gln47STOP transitions in *ftc-1* and *ftc-2*, respectively (Figure. 1A). The Pro8 residue is conserved in all PsFTs and also most FT proteins outside fabales (Supplementary Figure 2) so it could be expected that an amino acid switch in this position, especially the substitution of a proline where the imino acid bond is topologically constrained, would impact on protein function. On the other hand, the premature STOP codon in *ftc-2*, which is located at position 47, most likely results in a complete loss-of-function of the gene product.

The original lines carrying the *ftc-1* and *ftc-2* alleles in the TILLING population were backcrossed to Cameor wildtype, and homozygous *ftc-1* and *ftc-2* plants were identified in the corresponding segregating F2 populations. A preliminary phenotypic characterization was carried out for these mutants, comparing with plants from the same segregating population that were homozygous for the FTc wild-type allele and also with Cameor. During the vegetative phase, the mutants did not show strong defects in comparison with the controls (wild-type Cameor and FTc plants in the F2 population). The floral transition, quantified as the node of floral initiation (NFI), was significantly delayed in *ftc-2*, but not in *ftc-1* plants (Figure 1B). Furthermore, in both the *ftc-1* and *ftc-2* mutants the length of the I2s ("I2 stem") was severely reduced compared to Cameor and, to a lesser extent, also to plants homozygous for the wildtype FTc allele in the corresponding F2 populations (Figure 1C), suggesting that other unknown mutations affecting this trait could be also present. In addition, some *ftc-2* plants also showed abnormal I2s and stipule-like structures at the base of flowers (Figure. 1D). Because Cameor carries a mutation in the *LF* gene (JL Weller, unpublished), which controls flowering time and might affect *FTc* regulation (Foucher, 2003),

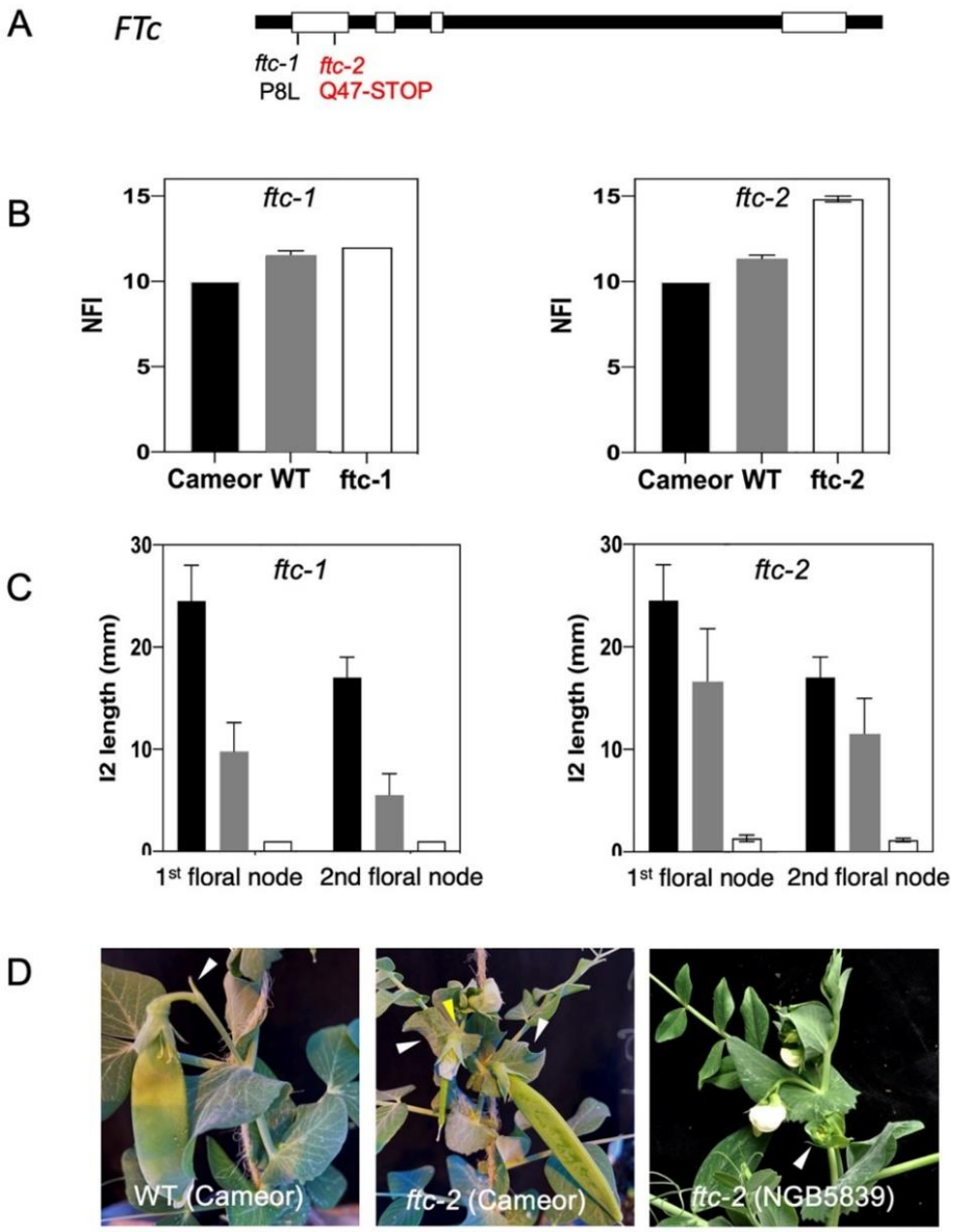

Figure 1. Isolation of *ftc* mutants and characterization of *ftc-1* and *ftc-2* (in Cameor and NGB5839 backgrounds). (A) Diagram of the *FTc* gene showing the mutations in *ftc-1* and *ftc-2***.** White boxes represent exons and black boxes introns. **(B)** Number of the node of flowering initiation (NFI) of *ftc-1* and *ftc-2* mutants. Black bars, grey bars and white bars represent wild-type Cameor plants, FTc wild-type plants from a population segregating for the corresponding *ftc* mutation, and homozygous *ftc-1* or *ftc-2* mutant plants in these populations, respectively. Error bars correspond to standard deviation. **(C)** Length of the I2 stem of *ftc-1* and *ftc-2* mutants. Bars represent the length of the I2 stem of the first and second flowering nodes. Black bars, grey bars and white bars represent wild-type Cameor plants, wild-type plants from a population segregating for the corresponding *ftc* mutation, and homozygous *ftc-1* or *ftc-2* mutant plants, respectively. Error bars correspond to standard deviation. **(D)** Inflorescence apices from wild-type Cameor, the original *ftc-2* mutant (Cameor background) and the *ftc-2* mutant introgressed in the NGB5839 background. In Cameor, arrowhead points to a normal stub in the I2. In *ftc-2* (Cameor), white arrowheads point to two short I2s, with stipule-like structures at the base of the flowers. In *ftc-2* (NGB5839), the arrowhead points to an abnormal I2 at the first flowering node.

we performed an additional characterization of the *ftc* mutants introgressed into the NGB5839 genetic background (Figure 1D). NGB5839 is an isogenic dwarf derivative of the spring cultivar Torsdag which carries a recessive allele at the *HIGH RESPONSE TO PHOTOPERIOD* (*HR*) locus that confers early flowering in SD, but that has functional alleles of genes in the TFL/FT family (Hecht, 2007). In this genetic background, a flowering delay of approximately 2.5 nodes was observed in *ftc-2* compared to the WT (Table 1), but abnormal I2 structures were seldom observed (Figure 1D).

These first insights into the *PsFTc* function indicate that strong mutations in *FTc* (*ftc-2*) cause a moderate delay in flowering and some defects in I2 development, although those appear to be more frequent in the Cameor background. As mentioned previously, Cameor carries a mutation in *LF*, which is one of the pea homologues of the Arabidopsis *TFL1* gene (Foucher et al., 2003). This data suggests a possible contribution of *FTc* to the regulation of inflorescence meristem identity genes and I2 development and a potential functional interaction with *LF*.

FTc is specifically expressed in the I2 meristem, overlapping with _VEG1_ expression

Previous studies established that *FTc* is expressed in the shoot apex after floral transition (Hecht et al., 2011), however the precise spatial expression pattern of *FTc* in the inflorescence apex remained unknown, so we performed RNA *in situ* hybridization (ISH) with *FTc* in inflorescence apices. As *VEG1* specifies the identity of I2 meristems, and is expressed specifically at this domain (Berbel et al., 2012), we decided to include in this experiment a parallel *in situ* hybridization with a *VEG1* probe in serial sections to aid in the interpretation of the results.

ISH assays showed that *FTc* is expressed specifically at I2 meristems in a domain partially overlapping with that of *VEG1* (Figure 2A, B). As previously described, expression of *VEG1* was detected uniformly in the I2 meristem (Berbel et al., 2012), while *FTc* is expressed mainly in the inner cell layers of the I2 meristem (Figure 2A).

The specific expression of *FTc* in the I2 meristem suggests a possible involvement of *FTc* in I2 meristem development.

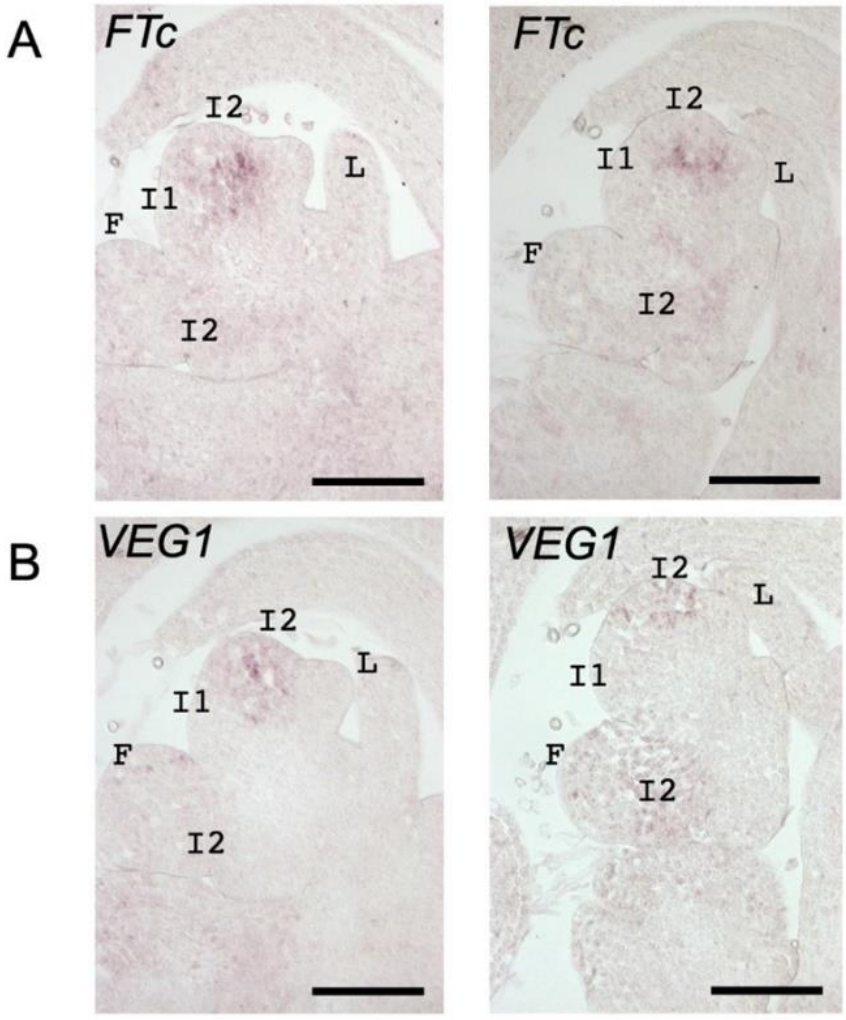

Figure 2. Analysis of *FTc* expression pattern in the inflorescence apex by *in situ* hibridization. Serial sections of shoot apices of wild-type pea plants (NGB5839). **(A)** Apex sections hybridized with a *FTc* probe. (B) Apex sections hybridized with a *VEG1* probe. I1, primary inflorescence meristem; I2, secondary inflorescence meristem; F, floral meristem; L, leaf primordium. Scale bars: 100 µm.

The I2 defects of *ftc* mutants are enhanced in the double *ftc lf* mutant

LF is also known as *TFL1c*, and homologue of the *TFL1* gene of Arabidopsis. Previous studies have shown that mutations in *LF* cause early flowering (Murfet, 1975; Foucher et al., 2003) but *LF* has not been related so far with possible defects in inflorescence development, and the putative null allele *lf-22* (Weller et al., 2009) alone does not display any phenotype of this kind (Figure 3A; Table 1). However, the inflorescence defects of the *ftc-2* mutants are attenuated in NGB5839 background, which carries a wild-type allele of the *LF* gene, in contrast to Cameor, which carries a weak *lf* mutant allele. This suggests a possible genetic interaction of *FTc* and *LF* with an effect in inflorescence development. To assess this possibility, we generated the double mutant *ftc-2 lf-22* in the NGB5839 background.

The double mutant *ftc-2 lf-22* displayed different defects in I2 development. Although most of the I2 structures showed a wild-type phenotype (Figure 3C), around 16% (Table 1) of the total I2s analysed in *ftc-2 lf-22* plants carried defects in the stub, which was transformed into abnormal flowers (Figure 3 G-H). We called these structures “stub-flowers” as the stub is replaced by abnormal flower-like structures. Apart from being abnormal, stub-flowers characteristically appeared at the end of the I2 stem, which did not produce any stub. No sign of the junction between the floral pedicel and the I2 could be observed, and the flowers appeared to differentiate terminally at the I2 stem (insets in Figure 3G-H). Stub-flowers were found in 70% of the plants analysed (Table 1). Moreover, some the I2s (4,9% of total I2s; Table 1) that came out in the first flowering node had 3 flowers instead of the 1 or 2 of the wild-type, and did not show stub-related effects, so we called these newfound structures “three-flower-I2” (Figure 3D). Three-flower-I2s usually had stipule-like organs at the base of the third flower, and the rudimentary stub (reduced to a ring around the base of the floral pedicel) could be observed only when the stipule-like structure was removed (Figure 3E). Three-flower-I2 structures were present in 30% of the plants analysed (Table 1). None of the wild-type plants or *lf-22* single mutant plants showed any stub-flower, neither three-flower-I2 (Table 1).

In addition to these features *ftc-2 lf-22* plants also showed branch-like structures at unusual positions and timing. Usually, secondary branches develop from axillary meristems of leaves at the lower nodes of the plant when the plant has already developed pods but, in some *ftc-2 lf-22* plants a branch eventually formed at the preceding position of the first flowering node and at about the time when the I2s were formed (Figure 3F). In addition, some of these branch-like structures resembled I2 since in some cases they contained only flowers with hardly any leaf tissue. Accordingly, we called these structures as "branch-I2". Branch-I2s were found in around 28% of the plants analysed and represented 5.5% of the total I2s scored (Table 1). None of the WT or *lf-22* plants analysed showed "branch-I2” structures (Table 1).

Altogether, these findings suggest that *FTc* along with *LF* might be involved in specifying the development of secondary inflorescences of pea.

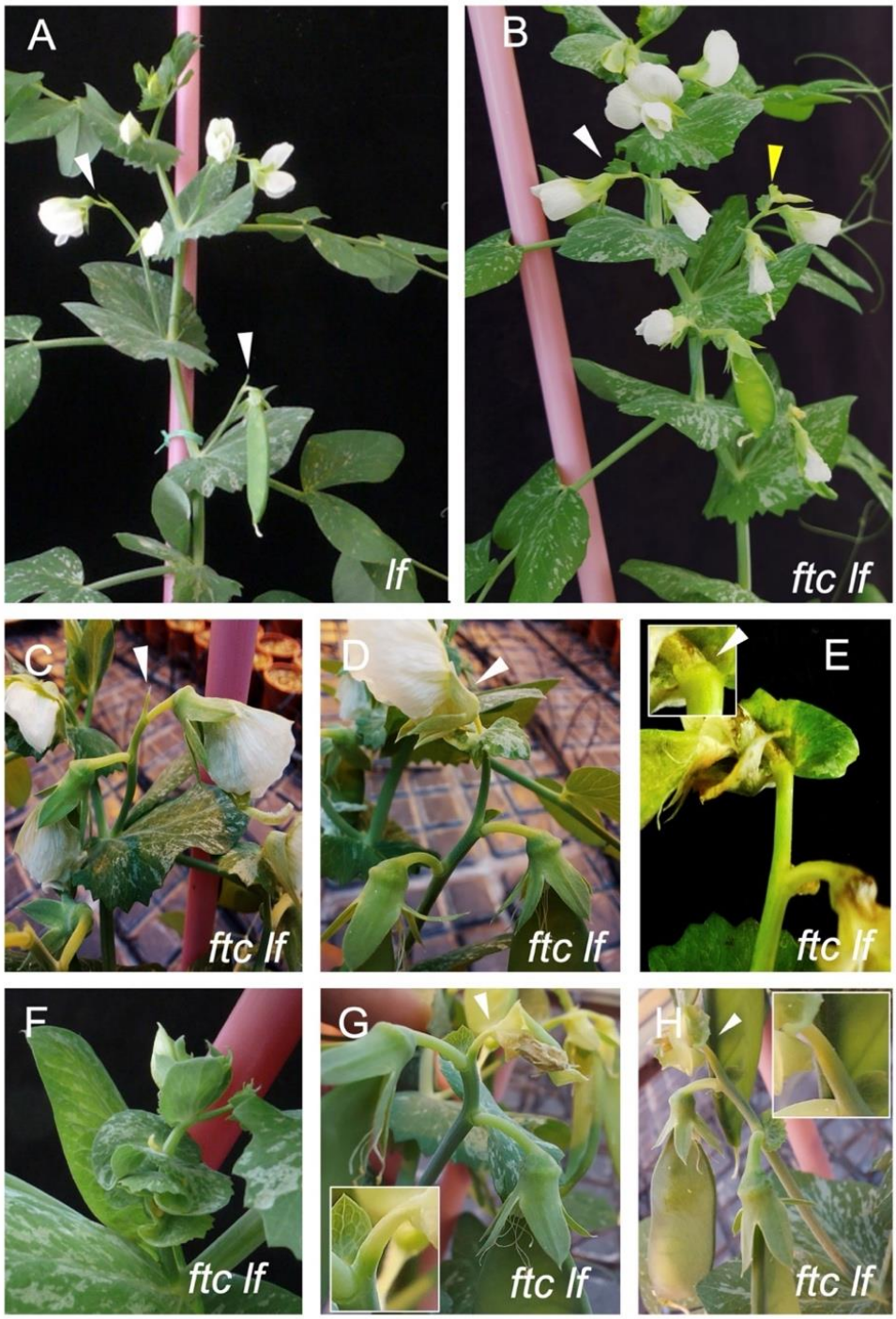

Figure 3. Phenotype of pea *ftc-2 lf-22* double mutant. (A) Inflorescence of *lf-22* mutant showing wild-type I2s with 1-2 flowers and ending in a normal stub (arrowheads). **(B**) Inflorescence of *ftc-2 lf-22* double mutant showing a stipule-like structure at the base of a flower (white arrowhead) and I2 ending in a flower (stub-flower; yellow arrowhead). **(C)** Detail of I2 from *ftc-2 lf-22* double mutant with a normal stub (arrowhead). **(D)** I2 of a *ftc-2 lf-22* double mutant plant with three normal flowers (three-flower-I2). Arrowhead points the third flower that has a stipule-like structure at its base. **(E)** Dissected three-flower-I2 where partial removal of the stipule-like structure allows to observe the rudimentary stub, a ring of tissue at the base of the floral pedicel (arrowhead), showed with more detail in the close up in the inset. **(F)** Branch-I2 structure in a *ftc-2 lf-22* double mutant plant. **(G-H)** Stub-flower (arrowheads) structures in *ftc-2 lf-22* double mutant. The inset show close-up views of the flowers replacing the stubs.

Table 1. Frequency of abnormal I2 structures in mutant combinations with *ftc*.

Genotype	NFI[a]	RNN[b]	WT-I2[c]		Stb-Fw[d]		Ax-Fw[e]		3FwI2[f]		Bch-I2[g]	
			I2 %	PI %	I2 %	PI %	I2 %	PI %	I2 %	PI %	I2 %	PI %
WT	16.6 ± 0.8	4.0 ± 1.2	100	100	0	0	0	0	0	0	0	0
ftc	18.9 ± 0.8	2.0 ± 0.5	100	100	0	0	0	0	0	0	0	0
lf	6.3 ± 0.5	10.1 ± 1.3	100	100	0	0	0	0	0	0	0	0
det	17.0 ± 0.0	1.1 ± 0.4	95	87	5	13	0	0	0	0	0	0
det lf	6.3 ± 0.5	3.4 ± 0.5	76	12,5	24	62,5	12	37,5	0	0	0	0
ftc lf	10.4 ± 1.5	6.1 ± 1.1	77	30	16	70	0	0	4,9	30	5,5	28,6
ftc det	18.5 ± 0.5	4.2 ± 0.4	7	0	29	90	64	100	0	0	0	0
ftc det lf	11.2 ± 1.1	5.5 ± 0.9	64	0	18	100	18	100	0	0	12	38

[a]**NFI:** Node of floral initiation (mean ± standard deviation); [b]**RNN**: Reproductive node number (mean ± standard deviation); [c]**WT-I2**: Wild-type-like I2; [d]**Stb-Fw**: I2 with stub-flowers; [e]**Ax-Fw**: Axilar/terminal flower; [f]**3FwI2**: Three-flower I2; [g]**Bch-I2**: Branch-I2. [h]**I2 %** refers to total number of I2s; [i]**PI %** refers to number of plants presenting at least one of these structures.10 plants were analysed per genotype, except for *lf-22*, *det-3* and *det-3 lf-22*, where only 8 plants were characterized. Branch-I2 data were recorded in an independent experiment (10 plants per genotype) that showed comparable results for the other I2 structures to the experiment presented in this table.

Characterization of double *ftc det* mutant

Both *DET (PsTFL1a)* and *LF (PsTFL1c)* are functional homologues of *TFL1* in *Pisum sativum*. Contrary to *LF*, mutations in *DET*/*PsTFL1a* do not affect flowering time, but they affect inflorescence architecture. As it has been described in previous studies, in *det* mutants, after the floral transition, the shoot apical meristem fails to maintain primary inflorescence (I1) identity, and the I1 is replaced by a I2 inflorescence (Foucher et al, 2003; Singer et al, 1990; Figure 4A). This ectopic I2 converts the indeterminate inflorescence of pea wild-type plants into a determinate inflorescence (Figure 4A).

Because of the appearance of defects in the I2 structures of the *ftc-2 lf-22* double mutant, a new mutant combination, *ftc-2 det-3*, was generated and characterized. The *ftc-2 det-3* plants displayed severe defects in the reproductive structures. Although this mutant combination was able to produce a certain number of normal I2s (around 7%; Table 1; Figure 4C), most of the I2s developed as stub-flowers (29%; Table 1) or axillar/terminal flowers, arising directly from the axillary meristem and thus not borne at the I2 (64%; Table 1; Figure 4 B, E, F, G). As *ftc-2 lf-22*, the *ftc-2 det-3* plants often developed stipule-like organs at the base of the flower, resembling bracts (Figure 3B, D, E; Figure 4D-G), indicating that *FTc* might be also repressing bract initiation, in combination with *LF* or *DET*. The stub-flowers developed more frequently in the first reproductive nodes, at a slightly higher frequency than in *ftc-2 lf-22*, while axillary flowers were mainly found in higher nodes (Figure 4B, F, G).

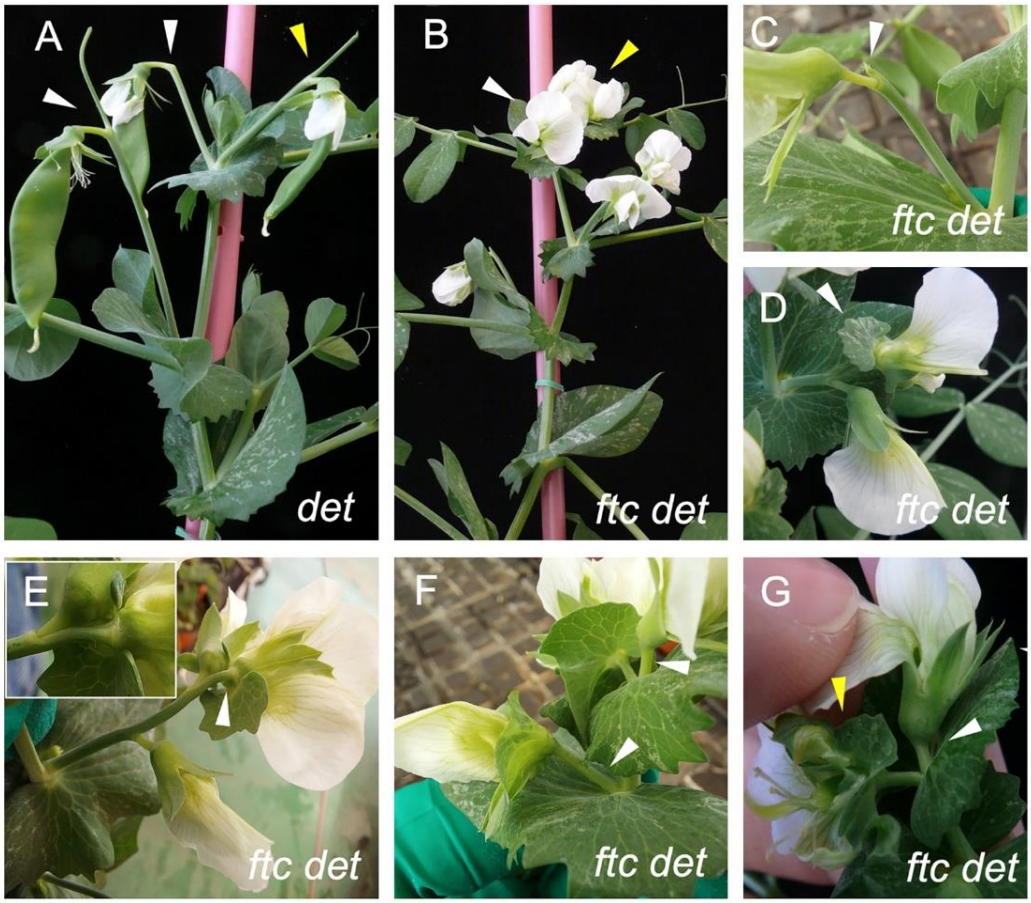

Figure 4. Phenotype of pea *ftc-2 det-3* double mutant. (A) Inflorescence of single *det-3* mutant plant, showing lateral (white arrowheads) and terminal I2s (yellow arrowhead). **(B)** Inflorescence of *ftc-2 det-3* double mutant, with axillary (white arrowhead) and terminal (yellow arrowhead) flowers. **(C)** Detail of a normal I2, with a stub (arrowhead), in a *ftc-2 det-3* double mutant plant. **(D)** Normal I2 s in a *ftc-2 det-3* double mutant with stipule-like organs (arrowhead). **(E)** Stub-flower (arrowhead), subtended by stipule-like structure, in a *ftc-2 det-3* double mutant I2. The inset shows a close-up view of the stub-flower, showing the absence of the I2 stub. **(F-G)** Axillary (white arrowheads) and terminal (yellow arrowhead) flower structures, some of them subtended by stipule-like organs, in *ftc-2 det-3* double mutant.

Moreover, in all *ftc-2 det-3* plants the I1 differentiated eventually into a terminal flower instead of developing in a terminal I2, as found in *det* single mutants (Table 1), and strongly resembling the *veg1 det* double mutant phenotype (Singer, 1999; Berbel et al., 2012). Interestingly, the branch-I2 structures that developed in the *ftc-2 lf-22* double mutant were not observed in this *ftc-2 det-3* background, suggesting that this trait could be specific of the *LF/FTc* interaction.

The novel and synergistic phenotypes observed in *ftc-2 det-3* mutants indicate that *FTc* and *DET* likely participate in common pathways directing inflorescence development in pea. Moreover, the lack of terminal secondary inflorescences (I2) in *ftc-2 det-3* that are consistently replaced with terminal flower structures as in *det veg1* mutants, also suggests

that *FTc* might be a possible regulator of *VEG1* in the development of the secondary inflorescences.

Characterization of triple *ftc det lf* mutant

To better understand the genetic interactions of *DET*, *LF* and *FTc* in the regulation of inflorescence development, we generated and characterized the double mutant *det-3 lf-22* and the triple mutant *ftc-2 det-3 lf-22*.

Double *det-2 lf-22* mutant showed the expected additive phenotype of early flowering and determinate growth (Figure 5A; Table A; Foucher et al, 2003). In addition, in *det-2 lf-22* plants most of the secondary inflorescences developed normally, as occurs in *lf-22* and *det-3* single mutants. However, 24% of the flowering nodes that developed in the double mutant carried stub-flowers (Figure 5A, C; Table 1), and in a lower proportion (12%; Table 1A), these plants also showed axillary/terminal flowers (Figure 5A, B; Table 1), indicating that this trait is not exclusively associated with *ftc* combinations, especially in the case of the stub-flower structures, which appeared in a slightly higher proportion than in other combinations. However, the axillary flower frequency was significantly lower than in the *ftc-2 det-2* combination (Table 1).

Triple *ftc-2 det-3 lf-22* had an intermediate phenotype between *ftc-2 lf-22* and *ftc-2 det-3*, except for these plants showing the most extreme phenotype regarding the formation of ectopic stipule-like bracts, that were observed in around 90% of the plants (Figure 5E, F, H). Triple *ftc-2 det-3 lf-22* produced most of the structures observed at *ftc-2 det-3* and *ftc-2 lf-22* double mutant combinations: stub-flowers, axillar/terminal flowers, branch-I2 and stipule-like bracts. The percentage of stub-flowers among total I2s was 18%, intermediate between *ftc-2 lf-22* and *ftc-2 det-3* (Figure 5F; Table 1; frequency of stub-flowers formation per node: *ftc-2 det-3* > *ftc-2 lf-22 det-3* > *ftc-2 lf-22*). Similarly, axillary flower formation in *ftc-2 det-3 lf-22* plants was occasionally observed, but at a much lesser proportion than in the double *ftc-2 det-3* mutants, while still being slightly higher than in *det-3 lf-22* (frequency of axillary flowers formation per node: *ftc-2 det-3* > *ftc-2 lf-22 det-3* > *ftc-2 lf-22*).

The intermediate phenotype of *ftc-2 lf-22 det-3* suggests a stronger association of axillary flowers (and to a lesser extent, of stub-flowers) with the *ftc-2 det-3* combination, while branch-I2 structures being closely associated with *ftc-2 lf-22*. This could indicate that *DET* and *LF*, although both being homologues of the Arabidopsis *TFL1* gene, could be playing antagonistic roles to some extent in pea plants.

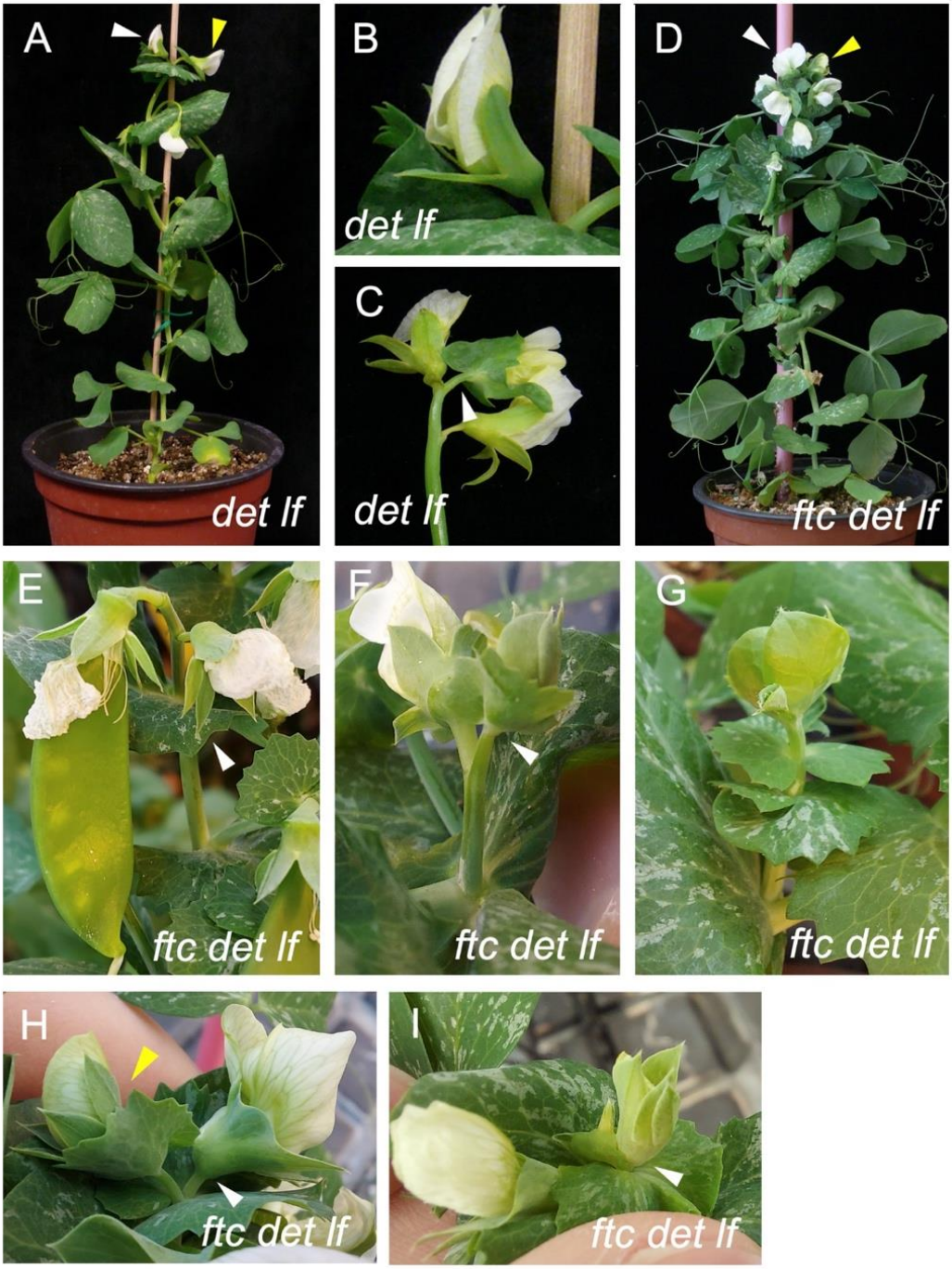

Figure 5. Phenotype of pea *ftc-2 det-3 lf-22* triple mutant. (A) *det-3 lf-22* double mutant plant with axillary (white arrowhead) and terminal (yellow arrowhead) flower structures. **(B)** Detail of an axillary flower in a double *det-3 lf-22* mutant. **(C)** Detail of stub-flower (arrowhead) in a double *det lf* mutant plant**. (D)** *ftc-2 det-3 lf-22* triple mutant plant with axillary (white arrowhead) and terminal (yellow arrowhead) flower structures. **(E)** Stipule-like structure in a I2 of a *ftc-2 det-3 lf-22* triple mutant. **(F)** Stub-flower (arrowhead), subtended by a stipule like-structure, in a *ftc-2 det-3 lf-22* triple mutant I2. **(G)** branch-I2 structure in a *ftc-2 det-3 lf-22* triple mutant. **(H-I)** Axillary (white arrowhead) and terminal (yellow arrowhead) flower structures *in ftc-2 det-3 lf-22* triple mutant. Terminal flower in H is subtended by a stipule-like organ.

DISCUSSION

Our work seeks to investigate the role of *FTc* in pathways that regulate flowering and the development of the compound inflorescence of legumes. We have isolated and characterized *FTc* mutant alleles. In addition, we have characterized *FTc* expression in the inflorescence and its genetic interaction with *LF* and *DET* genes, key regulators of flowering and inflorescence architecture. We have observed a clear role of *FTc* in promoting flowering (Figure 1B), like other members of the *FT* family. However, additional analyses of genetic interactions have revealed that *FT* also has a role in controlling the development of secondary inflorescence meristems (I2).

PsFTc is a gene belonging to the *FTc* subclade of the FT/PEBP family in pea. In Arabidopsis, the role of *FT* genes (*FT-TSF*) as floral inductors in photoinductive conditions is well known (Abe et al. 2005; Yamaguchi et al., 2005; Jaeger and Wigge, 2007; Jang et al., 2009). FT is part of a florigen mobile signal that is initially expressed in leaves. FT protein accumulates in the leaf in photoperiod inductive conditions and moves to the SAM where it activates floral genes like *AP1* and *FUL* (Schmid et al., 2003; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005). In pea, the functions of *FT* homologues have not been fully clarified, partly due to the higher complexity deriving from an extended number of *FT* genes in this species (from two to six). Although some pea *FT* genes behave as *AtFT*, as it is the case of *FTb2*, which is expressed specifically at leaves under photoinductive conditions (similar to *AtFT*), some appear to have gained new roles or have been sub-functionalized with novel modified expression domains and possibly novel functions in pea (Hecht et al., 2011; Weller and Ortega, 2015). *FTa1*, which is expressed at both leaves and apex, and specially *FTc*, expressed specifically at apex, are examples of this.

As pointed out before, the expression of *FTc*, specifically in the I2 meristem after the floral transition, has not been previously reported for a *FT* gene (Wigge et al., 2005; Corbesier et al, 2007) and raises some questions about its function, suggesting a possible role in the regulation of pea inflorescence architecture. ISH experiments showed that *FTc* expression overlaps with that of *VEG1*, gene responsible of I2 meristem specification, essential for development of I2 and the compound inflorescence. Moreover, mutations in *FTc* occasionally produced abnormal I2 structures. *VEG1/PsFULc* is a *FUL-like* gene with no functionally related homologues outside legumes (Berbel et al., 2012; Ping et al, 2014; Cheng et al, 2018). Arabidopsis *FT* promotes *FUL* expression (Teper-Bamnolker and Samach, 2005), therefore it sounds plausible that *FTc* also induces *VEG1/PsFULc* in a more complex genetic pathway, as it is the case of pea.

To investigate further the genetic pathways in which *FTc* could be involved, we analysed the genetic interaction of *FTc* with *LF/TFL1c* and *DET/TFL1a*, which also encode PEBPs, like *FTc*, but these proteins have a repressor role in floral induction and inflorescence determination, respectively (Singer, 1999; Foucher et al., 2003; Berbel et al., 2012). The frequency of abnormal I2 structures in *ftc-2* mutants increased when combined with the *lf-22* mutation (Table1). However, the highest frequency of abnormal structures was observed in *ftc-2 det-3* double mutants, which displayed several determined-like structures: 64% of the I2s are replaced by axillary or terminal flowers and 29% of the I2s differentiated into stub-flowers, that somehow could be considered equivalent to terminal flower structures. Together with the fact that *det-3* mutant essentially does not show abnormal I2 structures (only 5% of I2 transformed into stub-flowers; Table 1), this strongly suggests that the floral meristem identity gene *PIM* is directly activated at the I2 meristem of *ftc-2 det-3*, similarly to what has been reported for *det veg1* double mutants, and which is caused by the loss of function of *DET* and *VEG1*, repressors of *PIM* (Berbel et al., 2012). Moreover, the presence of stub-flowers suggests that in *ftc-2 det-3* double mutant *PIM* eventually becomes activated at the I2, as the stub (usually a residual structure of the indeterminate I2) is converted into a flower. This could indicate that in the *ftc-2 det-3* double mutant the expression level of *VEG1* is reduced due to the lack of *FTc*, indicating that *FTc* probably activates *VEG1* expression in the I2.

ftc-2 lf-22 also formed stub-flowers (Figure 3G, H) although those appeared less frequently than in *ftc-2 det-3* (16%). In addition, *ftc-2 lf-22* showed indetermined structures, non-present at *ftc-2 det-3*: three-flower-I2 (5%), and branch-I2. With our current knowledge, it is difficult to explain the formation of these indetermined structures, however it might indicate a role of *LF* in the repression of *VEG1* and possibly of *PIM*.

ftc-2 det-3 lf-22 combination showed fewer determinate structures than the double mutant *det-3 lf-22* that again could indicate that the loss of *LF* function causes an increase in *VEG1* expression. On the other hand, it could also be possible that *PIM* is more highly expressed because of the *lf-22* mutation, which would counteract for the absence of *DET* and the decreased *VEG1* expression due to the absence of *FTc*. In addition, *det-3 lf-22* displayed a similar phenotype to *ftc-2 det-3 lf-22* regarding the frequency of stub-flowers and axillary/terminal flower structures (Table 1), which suggest that a similar mechanism is operating in this background. On the other hand, no abnormal I2s were observed in the single mutants, suggesting robustness in the pea inflorescence development network.

In summary our data suggest a model in which *FTc* contributes to control pea inflorescence architecture, upon floral transition, by inducing expression of *VEG1*, which in turns specify

the formation of I2 meristem. We propose the possibility of *LF* acting as repressor of *VEG1* and perhaps *PIM*, in an opposite way to that of *FTc* (Figure 6).

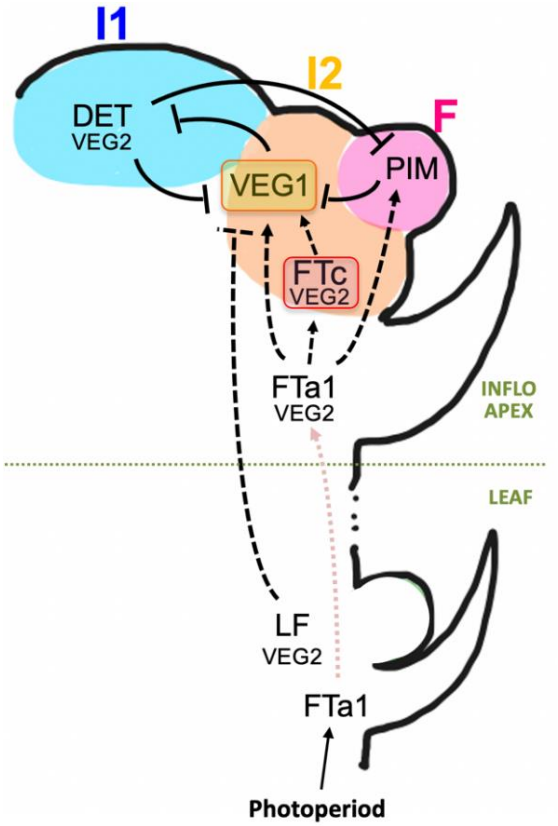

Figure 6. Genetic model for the role of *FTc* in inflorescence development in pea. Dashed lines represent likely genetic interactions that require further experimental support. Arrows represent induction and blocked arrows represent repression. Red dashed line from FTa1 represents its movement from the leaf to the meristem.

Though further studies will be necessary to confirm this genetic model, our results strongly indicate a regulatory relationship between *FTc* and the meristem identity genes that control the development of the legume compound inflorescence. Moreover, *FTc* is specifically expressed at the I2 meristems, which makes it a good candidate to mediate between pathways that control the onset of flowering and the activation of *VEG1*. Finally, our results also suggest that the increased number of *FT* genes in legumes has allowed subfunctionalization of *FTc* to contribute to the development of the more complex inflorescence in legumes. Thus, *FTc*, rather than being expressed in leaves and acting as a mobile signal that upregulates floral genes during floral induction (Wigge et al., 2005; Abe et al., 2005; Corbesier et al, 2007; Hecht et al., 2011), is expressed in the I2 meristem where it would has adopted the role of upregulating *VEG1*, which specifies I2 meristem identity; therefore, FTc is essential for the formation of the compound inflorescence in legumes.

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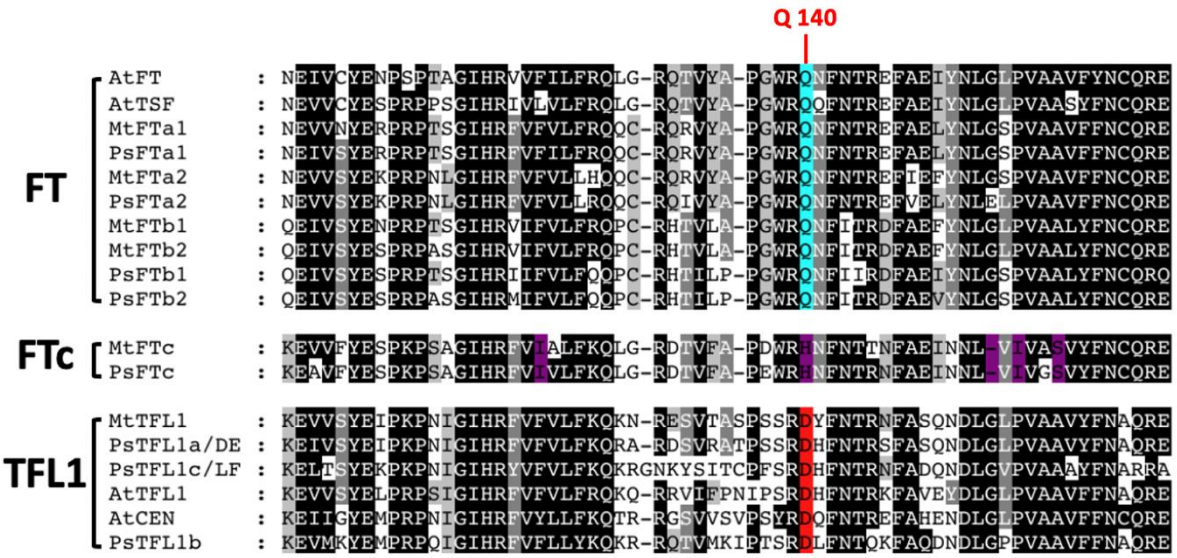
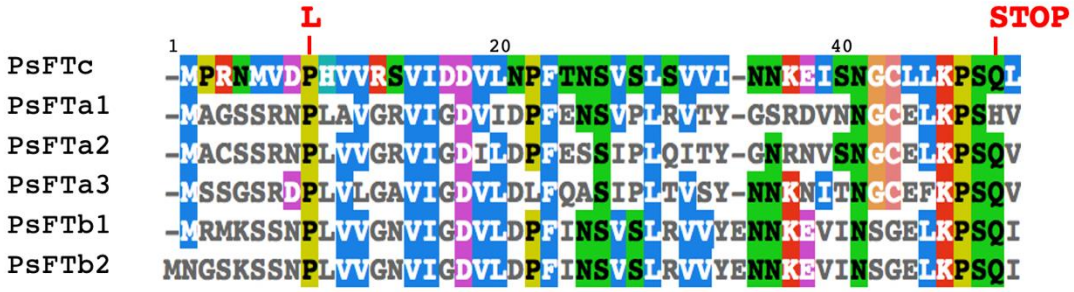
SUPPLEMENTARY FIGURE 1

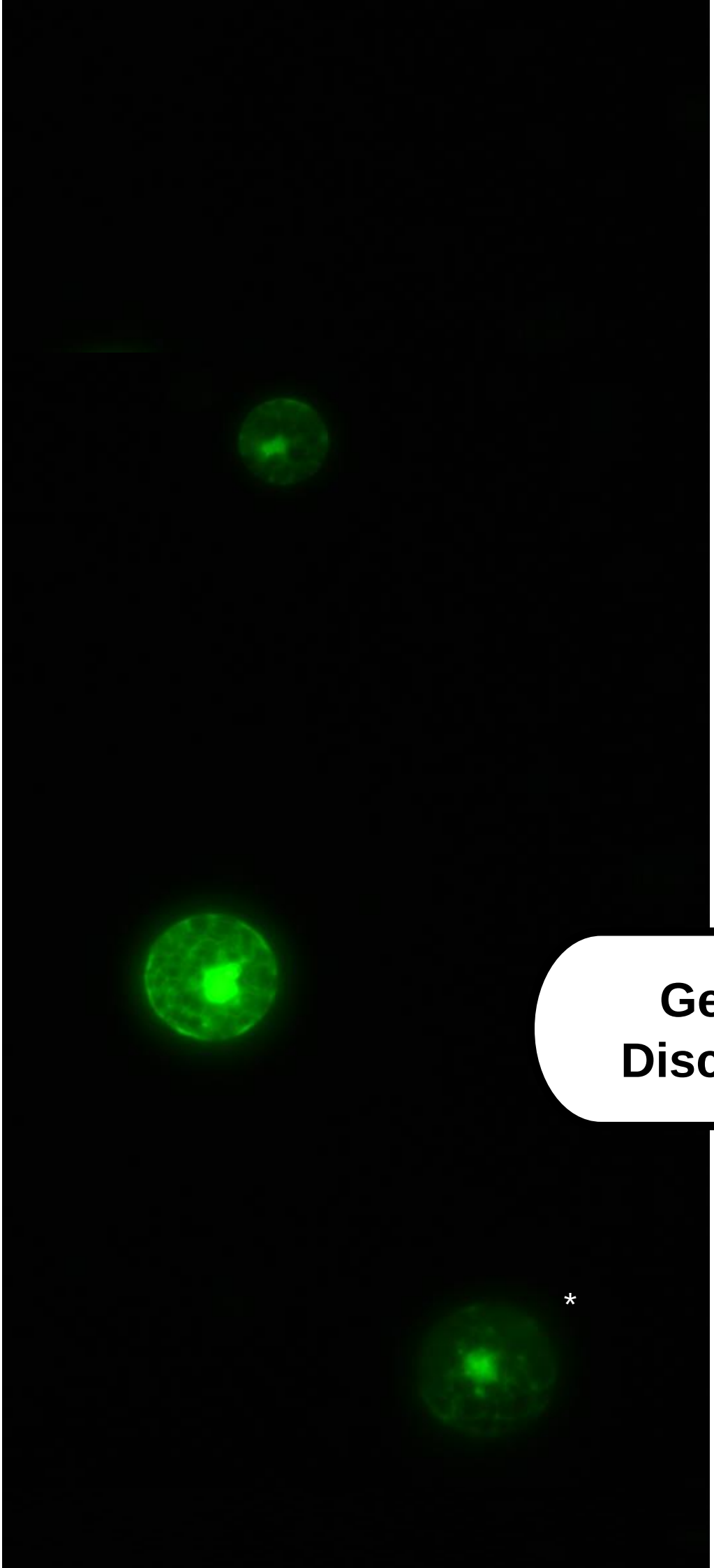


Supplementary Figure 1. Sequence aligment of a fragment (exon four) of the PsFTc protein with FT and TFL1 proteins from diferent plant species. At: *Arabidopsis thaliana*; Mt: *Medicago truncatula*; Ps: *Pisum sativum*. Gln140, highly conserved residue among FT proteins is highlighted.

SUPPLEMENTARY FIGURE 2



Supplementary Figure 2. Sequence aligment of the first fifty amino acid residues of the pea FT proteins. The Pro8 residue is conserved in all the pea FT proteins as well as in moss FTs. Pro8 is changed to Leu in the *ft-1* mutant allele and a stop codon replaces the Gln47 residue in the *ft-2* mutant allele.



General Discussion

* This image corresponds to pea transfected protoplasts with 35S::GFP and 35S::VEG1-VP16-GR constructs

This work is mainly focused on the development of the legume secondary inflorescences (I2) and on *VEG1*, which is a key controller of that process. The I2 meristem is probably an evolutionary novelty in legumes since these structures are not present in simple inflorescences, where flowers develop directly from the main primary inflorescence (I1; Benlloch et al., 2015). The I2 meristem can be considered as an intermediate meristem placed between the I1 meristem, and the floral meristem. Elucidating the control of I2 development is essential to understand the formation of the compound inflorescence and, consequently, the architecture complexity of legumes. Control of I2 development is also a relevant subject because inflorescence architecture determines the production of flowers and fruits and, therefore, strongly influences yield and yield stability of crops (Guo et al., 2020; Basu et al., 2021; Wang et al., 2021).

Initially, it was shown that in pea *VEG1* / *PsFULc* specifies the identity of the I2 meristem (Berbel et al., 2012), but control of I2 meristem identity specification has been shown later to be conserved also in other legume species such as Medicago and soybean (Ping et al., 2014; Cheng et al., 2018). *VEG1* encodes a protein from the MADS transcription factor family and belongs to the AGL79 subclade of the AP1 / SQUA / FUL group (Berbel et al., 2012). Therefore, *VEG1* is not a completely novel gene, but it is related to other meristem genes such as *AP1*, C*AL* and *FUL* (Litt and Irish, 2003; Shan et al., 2007). Nevertheless, its Arabidopsis homologue, *AtAGL79*, does not seem to carry out a function related to the control of inflorescence architecture (Gao et al., 2018), suggesting that the function of *VEG1* might have arisen as consequence of sub-functionalization or neo-functionalization in legumes (Berbel et al., 2012), which again provides evidence for the novelty and relevance of *VEG1* for the development of the I2.

To address the study of the genetic pathways that control I2 meristem formation, we have followed two different strategies: first, going downstream, analysing the genes that are expressed in the I2, as possible targets of VEG1; and second, going upstream, focusing in this case on the genes that regulate the formation of the I2, which probably act as regulators of *VEG1* as well. Our study of this developmental question has been carried out entirely in pea (*Pisum sativum*), the species in which the first *veg1* mutant was identified (Gottschalk, 1979; Reid and Murfet, 1984). Moreover, the genetic network that controls the specification and spatial distribution of *VEG1* is well defined in pea, and a large number of genes that regulate flowering have been identified (Weller and Ortega, 2015).

The process underlying the development of I2 once it has been specified, including how *VEG1* regulates its development, is largely unknown (Benlloch et al., 2015). We designed a

strategy to identify genes that are expressed in the I2 meristem, likely transcriptional targets of *VEG1*, based on the comparison of transcriptomes from different mutants with defects in I2 development, that seems to have worked. As a result, we have identified a number of candidate genes that are expressed in the I2, including *PsHUB54*, which apparently contributes to the control of I2 meristem activity. Therefore, our approach could possibly be used with other combinations of mutants affected in meristem identity in order to identify genes that control the functioning of these meristems. On the other hand, due to the large number of putative targets that this work has identified, we have only been able to analyse in depth a small number of those candidate genes. Although the already selected genes are promising candidates, if we consider the criteria and the information on which we have relied for the selection (information either already available or from our experiments), it is likely that there are still quite many genes in our lists, with functions related to I2 development, that remain to be explored. In addition, it would be necessary to investigate further into the function of some of these genes already analysed in order to unravel with more accuracy their possible function in inflorescence development.

To this end, by expanding and combining this strategy with other complementary approaches in the future, we could improve the efficiency in the detection and selection of potential *VEG1* targets. One of these possible complementary strategies in which we are currently working is the TARGET approach (Transient Assay Reporting Genome-wide Effects of Transcription factors), a recently developed method that allows the identification of genes that respond to the activation of transcription factors in non-transformable species (Bargmann et al., 2013; Para et al., 2014). This technique consists of transfecting protoplasts with a plasmid that contains two expression cassettes: one with the transcription factor (TF) of interest fused to the glucocorticoid receptor (GR) domain, and the other one with a fluorescent marker (green fluorescent protein -GFP- in our case) that allows enrichment of fluorescent/transfected protoplast by cell-sorting (FACS; Bargmann et al., 2013). After that, treatment with dexamethasone (DEX) inductor and cycloheximide (CHX) inhibitor enable distinction of direct and indirect targets of the TF under study (Bargmann et al., 2013). We are currently working on this method, and we already have made progress on it by optimizing pea protoplast isolation and assaying protoplast transfection with a 35S::VEG1-VP16-GR fusion construct, where we have reached an efficiency slightly higher than 40%. Alternatively, we started trying DAP-seq, a recently published method that combines affinity purification of genomic DNA-protein complexes in vitro, and high-throughput sequencing of the eluted DNA fragments (Bartlett et al., 2017; Franco-Zorrilla and Prat, 2021). For that, we have generated an MBP-VEG1 fusion protein (fusion of VEG1 with the Maltose Binding Protein; Riggs, 2000), and we are currently optimizing its binding efficiency to DNA to reduce

background noise and increase the reliability of genomic analysis in order to identify VEG1 potential targets. Both methods are state-of-the-art and have solid scientific evidence that supports their effectiveness (Bargmann et al., 2013; Para et al., 2014; Bartlett et al., 2017; Franco-Zorilla and Prat, 2021), so we hope that they will work in the near future.

To search for upstream regulators of the genetic network that controls inflorescence meristem identity, we have used an approach based on candidate genes selected on the basis of results from previous works in pea and on putative functional conservation suggested by studies in other species. The spatial expression of meristem identity genes in the pea inflorescence apex is well known, with defined and mostly complementary domains for each of those genes (*DET, VEG1, PIM*), which are maintained by negative regulatory interactions among them (Taylor et al, 2002; Foucher et al.; 2003; Berbel et al., 2012; Benlloch et al., 2015). However, little is known about how these expression patterns are initially established. Therefore, identifying new elements that induce the expression of meristem identity genes is another unsolved question in the control of legume inflorescence development. In our work, we have chosen to focus on the *FTc* gene mainly for two reasons: first, because FTs are factors that activate the floral transition in many species, promoting the expression of meristem identity genes such as *AP1*, *CAL*, *FUL* or *LFY* (Wigge et al., 2005; Jang et al., 2009) and second because *FTc* has a peculiar pattern of expression, associated to the onset of flowering and its expression is specifically located at the inflorescence apex (Hecht et al., 2011), which makes it a good candidate as a possible inducer of meristem identity gene expression.

In this thesis we have confirmed *FTc* as a likely positive regulator of *VEG1*. *FTc* has a very interesting expression pattern, as it is detected specifically in the I2 meristem, partly overlapping with *VEG1* but located more at inner layers of the I2 meristem, which is slightly reminiscent of the expression of *DET* in pea I1 meristems and of *TFL1* in the Arabidopsis inflorescence meristem (Bradley et al., 1997; Berbel et al., 2012). Moreover, in addition to the well-established long-range transport of the FT protein (the florigen) from the leaf to the inflorescence apex in Arabidopsis and in other species, the TFL1 protein has been also shown to move from the center to the periphery of the inflorescence meristem in Arabidopsis (Conti and Bradley, 2007; Goretti et al., 2020). Thus, it is possible that FTc is also a mobile factor within the I2 meristem, where it could activate *VEG1* expression. The positive regulatory interaction of *FTc* and *VEG1* somehow parallels other FT described roles, like AtFT directly regulating *AtFUL*, a gene closely related to the *VEG1* homolog *AtAGL79* (Hecht et al., 2011; Berbel et al., 2012), which suggest a possible conservation of the FT-MADS pathway.

An expansion in the number of *FT* genes has occurred in legumes (from two in Arabidopsis to six *FT* genes in pea) conforming three subclades: *FTa*, *FTb* and *FTc* (Hecht et al., 2011); and it is possible that this has fostered some degree of sub-functionalization and/or neo-

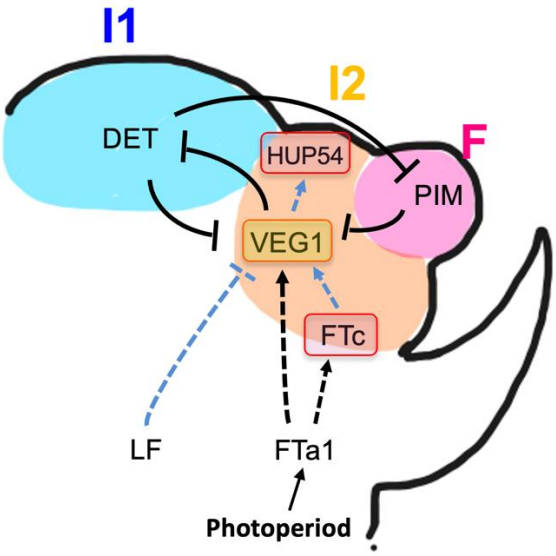


Figure 1. Model of the genetic network controlling inflorescence meristem specification in in the compound inflorescence of pea as suggested by this work. I1: primary inflorescence meristem; I2: secondary inflorescence meristem; F: floral meristem. Arrows represent induction and blocked arrows represent repression. Blue arrows indicate new interactions suggested by this work. Genes which have been subject of this work are highlighted in light red.

functionalization. *FTc* could have specialized as a specific regulator of *VEG1* and maybe of other aspects of I2 meristem development. The simple *ftc* mutant, although it has slight defects at secondary inflorescences, does not show a similar phenotype to *veg1* (Berbel et al., 2012), which suggests that it would not act as a major activator of *VEG1*. In contrast, the *gigas* mutant or the *vegetative2 (veg2)* mutant, which correspond to the loss-of-function of the *FTa1* and the homolog of *FD* genes, respectively, have phenotypes very similar to *veg1*. This supports a major role of GI/FT1a and VEG2 in the activation of *VEG1* expression (Hecht et al., 2011; Sussmilch et al., 2015). However, both *gi* and *veg2* mutants eventually flower in some conditions (Hecht et al., 2011; Sussmilch et al., 2015), indicating that they are not strictly required for activation of inflorescence meristem genes, and suggesting that other factors also contribute to these roles, possibly FTc, which in addition to contributing to activate *VEG1* expression, might also work by restricting the expression domain of *VEG1* to the I2 meristem.

The study of *FTc* has allowed us to identify it as a regulator of I2 development but it has also revealed several questions that we will address in the future and that today remain unanswered. We need to test our hypothesis of *VEG1* being regulated by FTc and in this

sense, one of the remaining tasks is to check the expression of *VEG1* in the *ftc* mutant, in order to elucidate whether *VEG1* spatial expression pattern or its level are affected. In addition, it would be important to determine if *FTc* expression is altered in the *veg1* mutant to explore possible additional regulatory interactions, as well as the expression of *VEG1*, *DET* and *PIM* in the mutant combinations generated in this work, to test the expanded genetic model for pea inflorescence development that we are proposing.

As a whole, the work of this thesis shows that the combination of classical genetic analysis together with transcriptomic-based system biology approaches, facilitated by the increasing availability of legume genome sequences, opens new ways to understand how the development of legume architecture is regulated.

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CONCLUSIONS

CONCLUSIONS

Our approach has allowed to us to deepen into the **study of the network that control the development of the pea secondary inflorescence** and to identify several candidates to be regulated by VEG1 and to be useful for legume breeding. Moreover, our results suggest that **an increased number of *FT* genes in legumes has allowed sub-functionalization of *FTc* to be involved in the control of I2 meristem identity**, and therefore, in the control of the development of the legume compound inflorescence. The specific conclusions extracted from this thesis can be summarised in the following statements:

1. We have generated a successful strategy to identify genes expressed in the I2 meristem of the pea inflorescence.

2. We have isolated several putative targets of VEG1. Among them *PsHUP54,* which is expressed in the I2 meristem and seems to control its activity. Silencing *PsHUP54* leads to plants that produce larger pods with almost double of seeds than the control, which places *PsHUP54* as a promising tool to improve yield in legumes.

3. *PsFTc* is specifically expressed in the pea I2 meristem. *PsFTc* contributes to the regulation of flowering time and is involved in the control of inflorescence meristem identity, in a process possibly mediated by *VEG1*.


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