

# A novel factor linking floral organ identity and growth revealed by characterization of the tomato mutant unfinished flower development (ufd)

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### *Conflict of interest statement*

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

### *Author contribution statement*

S.P-P. and R.L. were involved in experimental design and interpretation of data. S.P-P., M.Q. and A.O-A, performed experiments and analyzed data; S.P-P., M.Q., F.J.Y-L. and R.L. wrote, reviewed and edited the manuscript; J.C., T.A. and E.G. supervised experiments and provided critical review and editing of the manuscript. All authors contributed to editing and approving the final version of the manuscript.

### *Keywords*

flower development, organ growth, phytohormones, Transcriptome, *Solanum lycopersicum* L., UFD gene

### *Abstract*

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Floral organogenesis requires coordinated interactions between genes specifying floral organ identity and those regulating growth and size of developing floral organs. With the aim to isolate regulatory genes linking both developmental processes (i.e. floral organ identity and growth) in the tomato model species, a novel mutant altered in the formation of floral organs was isolated and characterized. Under normal growth conditions, floral organ primordia of mutant plants were correctly initiated, however, they were unable to complete their development impeding the formation of mature and fertile flowers. Thus, the growth of floral buds was blocked at an early stage of development; therefore, we named this mutant as unfinished flower development (ufd). Genetic analysis performed in a segregating population of 543 plants showed that the abnormal phenotype was controlled by a recessive mutation of a single nuclear gene. Global gene expression analysis confirmed that several MADS-box genes regulating floral identity as well as other genes participating in cell division and different hormonal pathways were affected in their expression patterns in ufd mutant plants. Moreover, ufd mutant inflorescences showed higher hormone contents, particularly ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and strigol compared to wild type. Such results indicate that UFD may have a key function as positive regulator of the development of floral primordia once they have been initiated in the four floral whorls. This function should be performed by affecting the expression of floral organ identity and growth genes, together with hormonal signalling pathways.

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### *Ethics statement*

(Authors are required to state the ethical considerations of their study in the manuscript including for cases where the study was exempt from ethical approval procedures.)

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## Abstract

Floral organogenesis requires coordinated interactions between genes specifying floral organ identity and those regulating growth and size of developing floral organs. With the aim to isolate regulatory genes linking both developmental processes (i.e. floral organ identity and growth) in the tomato model species, a novel mutant altered in the formation of floral organs was isolated and characterized. Under normal growth conditions, floral organ primordia of mutant plants were correctly initiated, however, they were unable to complete their development impeding the formation of mature and fertile flowers. Thus, the growth of floral buds was blocked at an early stage of development; therefore, we named this mutant as *unfinished flower development (ufd)*. Genetic analysis performed in a segregating population of 543 plants showed that the abnormal phenotype was controlled by a recessive mutation of a single nuclear gene. Global gene expression analysis confirmed that several MADS-box genes regulating floral identity as well as other genes participating in cell division and different hormonal pathways were affected in their expression patterns in *ufd* mutant plants. Moreover, *ufd* mutant inflorescences showed higher hormone contents, particularly ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and strigol compared to wild type. Such results indicate that *UFD* may have a key function as positive regulator of the development of floral primordia once they have been initiated in the four floral whorls. This function should be performed by affecting the expression of floral organ identity and growth genes, together with hormonal signalling pathways.

**Key words:** flower development, organ growth, phytohormones, transcriptome, *Solanum lycopersicum* L., *UFD* gene

## 1. Introduction

Besides its importance as a major vegetable species, tomato (*Solanum lycopersicum* L.) has been considered as a species of choice for studying the genetic and hormonal regulation of reproductive development. However, most studies on this topic have focused on the development and ripening of tomato fruit (Lozano et al., 2009; Pesaresi et al., 2014; Azzi et al., 2015) and less attention has been given to the study of floral organogenesis compared to that given to other model plant species such as Arabidopsis, Antirrhinum and rice. In Arabidopsis, once the floral transition takes place, the shoot apical meristem (SAM) acquires an inflorescence meristem (IM) fate allowing the formation of floral meristems (FM) on its flanks. Genetic control of FM initiation includes, as a main measure, the acquisition of the floral identity by the activation of the FM identity genes *LEAFY* (*LFY*) and *APETALA1* (reviewed in Siriwardana and Lamb, 2012; Winter et al., 2015). Tomato plants exhibit a sympodial growth pattern where the SAM converts into an IM, and vegetative development continues from a specialized axillary meristem called sympodial meristem, which arises from the axil of the last leaf. Meanwhile, the IM produces a new IM on its side before differentiating into a FM. Reiteration of IMs that are arranged perpendicular to one another, results in the production of an inflorescence organized in a zig-zag pattern (reviewed in Lozano et al., 2009; Thouet et al., 2012). *FALSIFLORA* has been characterized as the *LFY* ortholog in tomato where it plays similar functions as *LFY* as a regulator of FM identity (Molinero-Rosales et al., 1999; Thouet et al., 2012). However, *MACROCALYX* (*MC*), the tomato *API* homolog, most likely regulates IM instead of FM fate in tomato (Vrebalov et al., 2002; Yuste-Lisbona et al., 2016).

Once FM has been determined, floral organ identity is established from specific meristematic domains, which result in the initiation of sepal, petal, stamen and carpel primordia in the four whorls forming the flowers of most angiosperms. This process is regulated by several overlapping gene functions which act in a combinatorial fashion according to the ABC(DE) model (Coen and Meyerowitz, 1991; Causier et al., 2010). Most of the ABC identity functions are carried out by members of the homeotic MADS-box gene family. They encode transcriptional factors, which form part of multimeric protein complexes capable of binding to the target genes responsible for the morphological features of mature floral organs (Honma and Goto, 2001; Smaczniak et al., 2012). In tomato, phenotypic characterization and expression pattern analyses performed in floral homeotic mutants and transgenic plants with affected MADS-box gene expression seem to confirm the ABC(DE) model. Thus, the *MC* A-class gene is, amongst others, involved in sepal development (Vrebalov et al., 2002; Yuste-Lisbona et al., 2016). B-class gene function is carried out by two paralogous of *APETALA3*, i.e. *Tomato AP3* (*TAP3*; syn. *STAMENLESS* (*SL*)) and *Tomato MADS box 6* (*TM6*), and two *PISTILLATA* (*PI*) homologues, i.e. *Tomato PISTILLATA* (*TPI*; syn. *SIGLO2*) and *Solanum lycopersicum GLOBOSA* (*SIGLO*; syn. *SIGLO1*, *LePI*, *TPIB*) (Pnueli et al., 1991; Kramer et al., 1998; Busi et al., 2003; de Martino et al., 2006; Leseberg et al., 2008; Geuten and Irish, 2010; Quinet et al., 2014). The *TOMATO AGAMOUS 1* (*TAG1*)

gene specifies stamen and carpel identity (Pnueli et al., 1994a) and together with *TOMATO AGAMOUS LIKE-1* (*TAGL1*; syn. *ARLEQUIN* (*ALQ*) (Vrebalov et al., 2009; Gimenez et al., 2010), represent C-class gene function, while *TM5* (Pnueli et al., 1994b) and *TM29* (syn. *TAGL2*; Ampomah-Dwamena et al., 2002; Busi et al., 2003) have been proposed as E-class genes. The *TAGL1* gene represents D class gene function in tomato and is involved in flower and mainly in fruit development (Busi et al., 2003).

Floral organ development includes not only the meristematic signals of the ABC(DE) network which indicate the correct place where a tissue or organ will differentiate from the FM, but also changes in cell proliferation leading to the growth of floral organ primordia. This program initiates in response to meristematic signals and interacts with the organ identity genes (Reinhardt, 2005; Dornelas et al., 2011; Wellmer et al., 2014). As a result, each organ primordia grows initially largely through cell division, followed by a period of rapid directional cell expansion to sculpt its final size and shape (Anastasiou and Lenhard, 2007; Bögre et al., 2008). Members from the ovate family protein (OFP) have been characterized as regulators of cell proliferation. Thus, *OVATE*-like proteins act as transcriptional repressors in Arabidopsis, mainly affecting the expression of *GIBBERELIN 20 OXIDASE 1*, a key player in the gibberellin biosynthesis, causing a reduction in cell elongation (Hackbusch et al., 2005; Wang et al., 2007, 2011). In tomato, the *OVATE* gene acts as a negative regulator of growth, since the loss-of-function *ovate* mutant exhibits elongated carpels (Liu et al., 2002). Likewise, significant changes in cell division are due to a mutation in the tomato cell cycle-control gene *FRUIT WEIGHT 2.2* (*FW2.2*), which encodes a negative regulator of this process (Fray et al., 2000). On the contrary, *SUN* is a member of the IQD family of calmodulin-binding proteins and acts as a positive regulator of growth in tomato, since its high expression level is associated with increases in cell division (Xiao et al., 2008, 2009).

Phytohormones participate in the initiation and outgrowth of floral organ primordia such as auxins that play a key role in establishing the largely invariant arrangement of the different types of floral organs in the floral whorls (Wellmer et al., 2014). The unraveling of the genetic network and molecular mechanisms which control the dynamics of flower development, and particularly, the link between identity and growth of floral organs, is an important goal of plant biology currently (Jaeger et al., 2013) and some of the players involved in this process have begun to be identified in Arabidopsis (reviewed in Dornelas et al., 2011; Ó'Maoiléidigh et al., 2014; Wellmer et al., 2014). Extensive genetic and molecular analyses have shown that the homeotic MADS box proteins involved in the ABC model act as master regulators of flower development, and function not only in organ identity determination but also during organ morphogenesis (reviewed in Dornelas et al., 2011; Wellmer et al., 2014). Recently, genome-wide approaches have led to substantial progresses in discovering genes that act downstream of the signaling cascade initiated by MADS domain proteins (Wellmer et al., 2004, 2006; Kaufmann et al., 2009, 2010; Yant et al., 2010; Wuest et al., 2012; O'Maoileidigh et al., 2013; Pajoro et al., 2014). These experiments resulted in the identification of hundreds of genes whose expression depends on the activity of the floral organ identity factors. However, the

number of targets they indirectly control seems to be considerably larger than the number of direct targets, implying that floral organ formation must involve the activities of additional transcriptional regulators. Focusing on the identity of targeted genes showed that many of them code for transcription factors or are involved in hormone biosynthesis and signaling (Dornelas et al., 2011; Wellmer et al., 2014). Thus, the floral organ identity factors appear to mediate floral organogenesis by controlling the expression of other regulatory genes, whereas the formation of organ primordia and the differentiation of floral organs are thought to be partially mediated by different phytohormones. Most phytohormones were indeed shown to be involved in floral organ development in *Arabidopsis* (Chandler, 2011; Marsch-Martínez and de Folter, 2016; Yuan and Zhang, 2016). Petal development is mainly regulated by auxin and gibberellin homeostasis and by jasmonic acid via the transcription factor *BIGPETAL* (Chandler, 2011). During stamen development, jasmonic acid interacts with auxins and gibberellins to regulate anther development and pollen maturation (Plackett et al., 2011; Yuan and Zhang, 2016). Gibberellins also control filament elongation (Plackett et al., 2011). In addition to these major regulators of stamen development, ethylene has been shown to be involved in stamen initiation and cytokinins and brassinosteroids are required for proper pollen development (Chandler, 2011). Regarding gynoecium development, auxins promote carpel initiation, gynoecium growth and proper style and stigma formation while cytokinins participate in the development of the carpel margin meristem and derived tissues as well as in the valve margin formation (Marsch-Martínez and de Folter, 2016). Finally, after fertilization, synthesis and signaling of gibberellins are induced to trigger fruit growth (Gallego-Giraldo et al., 2014). However, although progress has been made in the recent years in the understanding of the hormonal control of floral organ development, we are not yet at the stage of having precise networks between hormone signalling pathways and floral organ identity and building gene interactions for the different floral organ developmental stages.

The genetic mechanisms underlying floral organ growth remain largely unknown, particularly in tomato, where genes specifically involved in this developmental process have not been discovered hitherto. The tomato *unfinished flower development* (*ufd*) mutant has been isolated from an EMS mutant collection of tomato (cv. MoneyMaker). The *ufd* flowers are unable to complete the normal growth of floral organ primordia, even though they were correctly initiated in their corresponding floral whorls. We recently characterized the genetic interactions of *ufd* with other tomato mutants affected in different phases of inflorescence and flower development, which showed that *UFD* could play a pivotal role between inflorescence architecture and flower initiation genes (Poyatos-Pertíñez et al. 2016). In this paper we further characterized *ufd* mutant to gain insight in the role of *UFD* during reproductive development of tomato. Transcriptome analysis indicated that *UFD* has a key role in the genetic control of floral organogenesis by affecting the expression of floral organ identity and cell cycle genes, and plant hormones. To provide additional insights into the regulatory interactions of *UFD* with floral organ identity and growth genes, expression analyses were carried out in *ufd* and wild type (WT) flowers during inflorescence development. Moreover, the complete

hormonal profile was investigated in *ufd* flowering shoot apices to highlight the involvement of *UFD* in hormonal metabolism and the role of hormones in tomato flower development. All together our results indicated that *UFD* has a key function as positive regulator of floral organ identity and growth, presumably by regulating transcriptional activity and hormonal signalling pathways.

## **2. Materials and methods**

### **2.1 Plant material and growth conditions**

The *ufd* mutant was isolated from an M2 population of tomato (*Solanum lycopersicum* L., cv. MoneyMaker), which was generated by mutagenesis with ethyl methanesulphonate (EMS). Genetic analysis of *ufd* mutation was initially performed on 20 plants of the original M2 family, and subsequently in a population composed of 543 M3 plants obtained by selfing a single heterozygous M2 plant (genotype *+/ufd*). Phenotype segregations were verified by a  $\chi^2$  test.

Seeds were germinated in plastic pots filled with sphagnum, peat/coco, peat/vermiculite substrate mixture (3:2:1). Plants were grown under standard greenhouse conditions, 25°C daytime maximum to 10°C night minimum temperature under approximately 14h natural light. All plants received regular watering and fertilizer treatments.

### **2.2 Microscope analysis**

Fresh plant tissues (inflorescences, flowers, floral buds and floral organs) were dissected and examined with a Nikon SMZ445 stereomicroscope to determine morphological and developmental characteristics. For histological studies, inflorescence samples were harvested and treated in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol) under partial vacuum for 2 h, then kept under room conditions for 20–24 h. Fixed samples were stored in 70% (v/v) ethanol at 4–7°C until further preparation. For light microscopy, tissue samples were embedded in paraffin (Paraplast plus, Sigma-Aldrich) and 8  $\mu$ m-thick sections were prepared on a Leica RM2145 microtome and counterstained using eosin. Later, paraffin was removed and tissue sections were dehydrated in an ethanol series, stained in a 1% Blue Toluidine solution and analysed and photographed with a Nikon Optiphot2 microscope. Scanning electron microscopy (SEM) analysis was carried out as follows: samples were fixed in FAEG (3.7% formaldehyde, 5% acetic acid, 50% ethanol and 0.5% glutaraldehyde) and kept in 70% ethanol. Subsequently, samples were dehydrated in an ethanol series, critical-point dried in liquid CO<sub>2</sub>, sputter-coated with gold palladium, analysed and photographed with a Hitachi S-3500N scanning electronic microscopy machine at an accelerating voltage of 10kv.

### **2.3 Transcriptome analysis by microarray hybridization**

In order to compare the gene expression profiles in wild type and *ufd* inflorescences, RNA samples were obtained from pooled flowering shoot apices harvested on 40-day-old plants of each wild type and *ufd* genotypes. Three independent biological replicates per genotype were performed, each one with at least 20 inflorescence apices (4-5 mm

long). The six RNA samples were reverse transcribed and labelled with Cy5. A common reference RNA sample was made by mixing aliquots of RNA from the six different samples labelled with Cy3. An aliquot of the common reference sample was mixed with each labelled test cDNA sample and the subsequent probes were then hybridized to TOM2 oligo-arrays as previously described (Lytovchenko et al., 2011). The use of the same reference sample for each slide hybridization allowed direct comparison of all hybridization experiments. To detect differentially expressed genes, a one-way ANOVA was carried out to compare the mean Lowess-normalized values for a gene between experimental groups (wild type and *ufd*). A *P* value cutoff of 0.05 was used to identify genes as differentially expressed between experimental groups. A hierarchical clustering was constructed using Pearson correlation coefficient as a similarity metric and the logarithm ratios of the fold change values for each gene in each experimental group as the input data.

## 2.4 Quantitative real-time PCR analysis

Gene expression analyses were performed by quantitative real-time PCR (qRT-PCR) experiments on wild type and mutant inflorescences at 3 stages of development: (i) flowering shoot apex containing the last initiated inflorescence, where the oldest flower buds was around stage 4 (according to Brukhin et al., 2003); (ii) type 1 inflorescence (IF1) corresponded to the penultimate initiated inflorescence, including flower buds from stages 6 to 12 in WT and up to stage 5 in *ufd*, since the growth of floral buds is arrested at this stage; and (iii) type 2 inflorescence (IF2) that is the third inflorescence from the top, which contained floral buds from stages 8 to 18 in WT while in the *ufd* IF2 only included flower buds up to stage 5. Total RNA was isolated using the Trizol Kit (Invitrogen) according to the manufacturer's recommendations. Contaminating DNA was removed using the DNA-free<sup>TM</sup> kit (Ambion) and then first strand cDNA was synthesized by means of a M-MLV Reverse transcriptase kit (Invitrogen). The reaction was performed according to the recommended protocol by using an oligo (dT) primer and 1 µg RNA. Selected primers specific to each examined gene were used for expression analysis (Supplementary Table 1). qRT-PCR reactions were performed with the SYBR Green PCR Master Mix kit (Applied Biosystems) according to the manufacturer's instructions in a 7300 Real-Time PCR System (Applied Biosystems; www.appliedbiosystems.com). qRT-PCRs were performed with three biological and two technical replicates for each cDNA sample. Data collection and analysis of expression levels were performed using the 7300 System Sequence Detection Software v1.2 (Applied Biosystem; www.appliedbiosystems.com). Results were processed using the  $\Delta\Delta C_t$  calculation method, expressed in arbitrary units and normalised by comparison to the housekeeping *UBIQUITINE3* (*UBI3*) gene and then to the value of the WT control. Significant differences between data sample means were determined by Student's t-test ( $P < 0.05$ ).

## 2.5 Hormonal analysis

Concentrations of the endogenous phytohormones including cytokinins, auxins, gibberellins, salicylic acid, jasmonic acid, brassinosteroids, abscisic acid, ethylene

precursor 1-aminocyclopropane-1-carboxylic acid (ACC), benzoic acid, and their metabolites were determined in developing inflorescences (pooled flowering shoot apices harvested on 40-day-old plants) of *ufd* and WT plants according to Quinet et al. (2014). Phytohormones were extracted using 50-100 mg lyophilised plant material from three biological replicates with methanol/ formic acid/water (15:1:4, by volume) and were subsequently purified by the dual-mode solid-phase method according to Dobrev and Kaminek (2002). The hormonal analysis and quantification were performed by HPLC (Ultimate 3000, Dionex, Sunnyvale, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP; Applied Biosystems, Foster City, CA, USA) using a multilevel calibration graph with 2H-labelled internal standards. Normality distributions and homoscedasticity were verified using Shapiro-Wilk and Levene's test respectively and data were transformed when required. ANOVA and Student's test were performed to evaluate the effects of the genotypes on the hormonal composition using SAS 9.2.

### 3. Results

#### 3.1 Phenotype and genetic characterization of *ufd* mutant

A screening was performed in an ethyl methanesulfonate (EMS)-induced tomato mutant collection with the aim to isolate new regulatory genes involved in flower development, particularly focused on those mutant phenotypes affected in floral organ identity and growth. As result, a mutant was identified and named *unfinished flower development* (*ufd*) as both their inflorescences and flowers showed a severe reduction in size and were unable to complete their normal development (Figure 1). As wild type tomato plants (cv. Moneymaker), the primary shoot of *ufd* mutant plants developed an initial vegetative segment composed by 6-7 leaves before the formation of the first inflorescence, and the subsequent plant growth continued through the development of an indeterminate number of sympodial segments, each composed of three leaves and a terminal inflorescence (Figure 1A-C, E). No differences between *ufd* and WT plants were observed in vegetative growth and flowering time (Figure 1 and Supplementary Table 2). After undergoing floral transition, *ufd* plants initiated the development of the inflorescence by the formation of the successive flowers in a zig-zag pattern, as normally occur in wild type tomato plants (Figure 1D, F). Nevertheless, once floral buds were initiated, they were unable to follow a normal growth thus impeding the formation of mature flowers. In addition, the number of flowers per inflorescence was smaller in *ufd* than in the WT plants ( $5.21 \pm 0.89$  and  $7.83 \pm 0.89$  flowers in *ufd* and WT, respectively,  $P < 0.05$ ).

A genetic analysis was performed in an M2 segregating population, where the *ufd* mutant phenotype was observed in one quarter of plants (15 WT: 5 *ufd*), suggesting a monogenic and recessive inheritance pattern. As the mutation resulted in sterile plants, WT individuals of the same M2 family were selfed, and an M3 segregating population was obtained from a single heterozygous M2 individual. Phenotypic characterization of 543 plants of this M3 progeny revealed a 3:1 ratio for the wild type : mutant phenotype (391

: 152,  $\chi^2 = 2.59$ ,  $P = 0.11$ ), which corroborated that *ufd* phenotype was caused by a monogenic and recessive mutation.

### 3.2 Floral ontogeny of *ufd* mutant

A detailed analysis of floral organogenesis was carried out in *ufd* mutant plants by scanning electron microscopy (SEM) of floral buds at several developmental stages (Figure 2), according to those described by Brukhin et al. (2003). At early stages (from stage 3 to stage 5), *ufd* floral buds were found to develop similarly to those of the wild type background (Figure 2A, B, F, G). As the development progressed, organ primordia were initiated at the expected positions in the corresponding floral whorls. Sepal primordia first appeared in a helical pattern followed by petal, while stamen and carpel primordia alternated with respect to the preceding whorl primordia as described previously (see review of Lozano et al., 2009). Slightly later, and coinciding with the end of stage 5 and stage 6 of tomato flower development (FIGURE 2C, H), the growth of floral buds is blocked in *ufd* plants (FIGURE 2I-J) while WT flowers continued to develop (Figure 2D-E), therefore this was considered the “most adult stage” of *ufd* flower development. At this stage, sepal primordia forming the first whorl ceased their development when they were still fused (Figure 2H, I), and trichomes appeared in a lower density on the sepal abaxial surface but restricted to a reduced area around the apical zone (Figure 2D, I). Petal primordia (second whorl) also stopped their development and remained as fused organs as expected for this developmental stage. They did not elongate enough to cover stamen primordia and trichomes were not observed on their abaxial surface (Figure 2H). The third floral whorl in adult *ufd* flowers consisted of dome-shaped stamen primordia, which reached a size comparatively higher than the petals, while carpel primordia led the formation of one or two faint cavities in the innermost whorl (Figure 2H). Moreover, no morphological differences were found in the epidermal cells of mutant floral organs or in their whorled disposition, indicating that *ufd* mutation does not affect morphology or phyllotaxis of floral organs (Figure 2D-E, I-J).

### 3.3 Transcriptome analysis of *ufd* mutant

To have a wider perspective of the transcriptional changes promoted by the *ufd* mutation during floral development, a global gene expression analysis was performed using the tomato - TOM2 microarray. With this purpose, three biological replicates of the wild type and *ufd* transcriptomes were compared against each other through the use of a common reference sample in each dual hybridization experiment. After filtering out redundant spots and selecting the ones which were significantly expressed between any of the two samples, a total of 325 transcripts were found to show differential expression in their mRNA levels in *ufd*, corresponding to 4.4 % of all spots (7380) which passed quality controls.

The differentially expressed genes (DEGs) identified from the *ufd* vs. WT comparison were assigned to different functional categories (Supplementary Figure 1) according to the Tomato Functional Genomics Database (TFGD, <http://ted.bti.cornell.edu/>). Cellular component categories such as membrane, ribosome and mitochondrion were more

represented in the up-regulated gene dataset while the thylakoid category was enriched in the down-regulated gene dataset (Supplementary Figure 1A). With regard to their molecular function, the percentage of transcripts classified under categories linked to structural molecule activity and RNA binding was greater in the up-regulated gene dataset (Supplementary Figure 1B). Categories such as protein binding, transcription factor activity, DNA binding, enzyme regulator activity and hydrolase activity were more richly represented in the down-regulated gene dataset (Supplementary Figure 1B).

In order to identify genes sharing a similar expression pattern, the whole set of 325 DEGs was clustered into eight clusters (numbered 1-8) according to their differential expression using the Pearson clustering algorithm (Figure 3 and Supplementary Table 3). The genes with altered expression levels in *ufd* samples consisted of two large clusters (clusters 1 and 2) and several smaller clusters. The largest cluster (cluster 1, 29%) included genes repressed both in wild type and *ufd* mutant samples when compared with the common reference, although at a lower level in the later one. Among others, this cluster included genes encoding transcription factors belonging to WRKY and bHLH families, and others involved in the ethylene pathway such as *ETHYLENE RESPONSE FACTOR 1* and *Lycopersicon esculentum Jasmonic Acid 2 (LEJA2)*. Cluster 2 (20%) and cluster 4 (6%) included genes showing minor down-regulation in the WT and slight up-regulation in the mutant, although the former showed either greater down-regulation in the WT while the latter showed greater up-regulation in the mutant. Cluster 2 included the elongation factor 1- $\alpha$  and some transcription factors, whereas cluster 4 included genes encoding transcription factors of AP2 and WRKY families, together with the *OVATE* gene involved in tomato fruit shape and several ethylene-related genes. Cluster 3 (10%) consisted of mostly constitutive genes showing a similar expression profile to that of cluster 2 and cluster 4. Some representatives of this cluster were genes encoding H2A and H2B histones and protein kinases. Genes included in cluster 7 (14%) showed major up-regulation in wild type inflorescences and minor up-regulation in the mutant ones. Some floral organogenesis-specific genes most of them belonging to the MADS-box family, such as *TDR4*, *TDR6*, *LeMADS1*, *TM29* and putative orthologs of *CRABS CLAW (CRC)* and *pMADS4* were found in this cluster, together with putative orthologs of *AUXIN RESPONSE FACTOR 8 (ARF8)*, *ARF6* and the *PHITOENE SYNTHASE* gene involved in the synthesis of carotenoids. Cluster 5 (5%) were also represented by genes up-regulated both in wild type and *ufd* inflorescences, although differences in the expression level favoured the *ufd* inflorescences in this cluster, which was mostly composed by constitutive genes encoding ribosomal proteins.

The loss-of-function phenotype of the *ufd* mutant, which agreed with its recessive inheritance, led us to focus our attention on two additional clusters (Figure 3 and Supplementary Table 3). Cluster 8 (11%) included genes significantly up-regulated in wild type inflorescences but strongly down-regulated in *ufd* mutant ones, among them those encoding MADS-box transcription factors such as *MC* and a *SIGLO1*, as well as other putative orthologs of flowering-related genes as *CYTOKININ OXIDASE 3 (CKX3)*, *ARGONAUTE*, *ARF1* and an ethylene-responsive transcription factor *RAP2*. Similarly,

cluster 6 (5%) contained genes, which were also up-regulated in WT, although at a lower level than in cluster 8, and greatly down-regulated in the *ufd* mutant. Some genes involved in abiotic stresses and in auxin response such as *ARF10* were included in this cluster.

Among the 325 DEGs, a total of 31 and 13 showed more than two-fold up- and two-fold down-regulation in the *ufd* mutant transcriptome, respectively (Supplementary Table 4 and 5). The retrieved set of 31 up-regulated genes appeared not to be significantly enriched in any particular biological or molecular functional category, neither that corresponding to transcriptional regulatory nor developmental processes (Supplementary Table 4). In this set four transcription factors were encountered, three of them belonging to the WRKY family together with the putative orthologue of the *BRASSINOSTEROID ENHANCED EXPRESSION 3* bHLH transcription factor. Genes also included in this set code for an elongation factor-1 alpha, a calcium-dependent protein kinase, a cytochrome P450, a 2-oxoglutarate-dependent dioxygenase involved in ethylene synthesis, and *LEJA2*. Most of the remaining up-regulated genes were involved in either biotic or abiotic stress responses. The set that was down-regulated in *ufd* inflorescences was particularly enriched in genes encoding for transcription factors of the MADS-box family (Supplementary Table 5), including *SIGLO1*, *TM29*, *MC*, *TDR6* and a putative ortholog of *pMADS4*. An additional transcription factor encountered was the putative orthologue of *Nicotiana tabacum* *CRC*. The rest of the down-regulated genes included *CKX3*, chymotrypsin inhibitor jasmonic-induced protein 21 (JIP1), flower-specific gamma-thionin-like protein/acidic protein precursor and wound-induced proteinase inhibitor 1.

All together, these results reveal that *UFD* may particularly regulate genes coding for transcription factors required for floral organ development and genes involved in hormone signalling (Figure 3). Some of these genes were investigated by qRT-PCR in order to confirm microarray expression data and to better characterize the *UFD* function.

### 3.4 Expression of genes involved in floral organ identity and growth

The expression of floral organ identity genes *MC* (A-class), *SL* (B-class), *TAG1* and *TAGL1* (C-class) and *TM5* and *TM29* (E-class) were analysed by qRT-PCR in *ufd* mutant inflorescences containing floral buds at increasing developmental stages (Figure 4). At early stages of flower development *MC* transcript abundance was slightly lower in the *ufd* mutant compared to WT (Figure 4A). Later, expression of *MC* increased significantly from flowering shoot apex stage in both WT and *ufd*, although it was down-regulated in late wild type inflorescences (IF2 stage) but continued to increase in *ufd* inflorescences (Figure 4A). Expression of the floral identity genes *SL*, *TAG1* and *TAGL1* increased as the inflorescence developed in wild type plants while the expression of these genes was strongly repressed in the *ufd* mutant independently of the inflorescence developmental stage (Figure 4B-D). A reduced expression of the B- and C-class genes in the *ufd* mutant compared to the WT confirmed the microarray observations. Similarly, *TM5* and *TM29*, whose transcript levels increased during the floral development, were significantly down-regulated in *ufd* inflorescences (Figure 4E, F).

Likewise, regulatory genes *FW2.2*, *OVATE*, and *SUN*, all of them involved in floral organ growth, were analysed by qRT-PCR (Figure 5). Non-significance differences between WT and *ufd* were found for these three genes at the apex and young inflorescence (IF1) stages; however, they were differentially expressed at IF2 stage ( $P < 0.05$ ). Thereby, the negative regulators of cell division *FW2.2* and *OVATE* were both overexpressed in *ufd* compared with wild type plants (Figure 5A, B), whereas the expression of *SUN*, a positive regulator of organ growth, was repressed in the *ufd* mutant plants (Figure 5C).

### 3.5 Endogenous hormone concentration in the *ufd* mutant

Microarray results indicated that *ufd* mutation affected transcriptional activity of genes involved in hormone signalling. In order to check if hormone content was also altered in *ufd* mutant plants, we investigated the hormonal profile in *ufd* flowering shoot apices. The *ufd* mutation did not affect the abscisic acid (Figure 6A), salicylic acid (Figure 6C) and the jasmonate (Figure 6D) concentrations of tomato apices. The strigol (Figure 6F) and ACC (Figure 6G) concentrations were higher in the apices of the *ufd* mutant compared to the wild type. Differences between genotypes also depended on the hormone forms. Regarding the auxins, no significant differences between genotypes were observed for the total auxin concentration (Figure 6B) but free indole-3-acetic acid (IAA,  $128 \pm 14$  vs.  $72 \pm 9$  pmol/g) and conjugated IAA ( $15 \pm 2$  vs.  $8 \pm 0.4$  pmol/g and  $2.8 \pm 0.2$  vs.  $1.1 \pm 0.1$  pmol/g for IAA-aspartate and IAA-glutamate, respectively) were detected at higher concentration in *ufd* than wild type flowering apices ( $P < 0.05$ ; Supplementary Table 6). On the same way, total gibberellin content did not differ between genotypes (Figure 6E) but the concentration of GA19 was higher in the *ufd* mutant ( $7.4 \pm 0.4$  vs.  $5.7 \pm 0.4$  pmol/g,  $P < 0.05$ ; Supplementary Table 6). At the cytokinins level, the main significant differences between genotypes were observed for the phosphate forms of trans-zeatin (trans-zeatin riboside monophosphate,  $P < 0.0001$ ) and cis-zeatin (cis-zeatin riboside monophosphate,  $P < 0.001$ ), which were respectively more than three ( $11.0 \pm 1.2$  vs.  $3.4 \pm 0.6$  pmol/g) and two times ( $2.7 \pm 0.2$  vs.  $1.2 \pm 0.3$  pmol/g) higher in the *ufd* mutant compared to WT (Supplementary Table 6).

## 4. Discussion

### 4.1 The *ufd* mutation affects floral organ growth

The tomato *unfinished flower development* (*ufd*) mutant has been isolated from an EMS mutant collection of tomato (cv. Moneymaker), and further characterized in this study. This mutant shows a singular phenotype that affects the growth of floral organs, in such way that they are unable to complete their development. Consequently, flower development is blocked at early stage (stage 5-6 according to Brukhin et al., 2003), when all floral organ primordia have been initiated in their appropriate whorls. The screening of a large segregating population proved that the *ufd* mutation is inherited as recessive and it does not affect other vegetative or reproductive traits. The *UFD* function is thus restricted to floral organogenesis, a developmental process where *UFD* is crucial for

making fully developed floral organs, although it is not required for the proper initiation of floral organ primordia.

Mutations in genes affecting floral organ identity and growth usually resulted in homeotic conversion of floral whorls or in abnormal development of the floral organs but did not stop their development at primordia stage (for a review, see Lozano et al., 2009; Causier et al., 2010; Mizzotti et al., 2014; Lombardo and Yoshida, 2015). Even a total loss of floral organ identity in *Arabidopsis* flowers resulted in the conversion of sepals, petals, stamens and carpels in leaf-like organs as observed in the *sep1-4* quadruple mutant or in mutants combining mutations in all floral homeotic genes (Bowman et al., 1991; Pelaz et al., 2000; Ditta et al., 2004). In tomato, floral organ identity was partially or completely lost when representative genes of MADS-box classes A (*MC*), B (*SL*, *TM6*, *TPI* and *TPIB*), C (*TAG1*) and E (*TM5* and *TM29*) were mutated or down-regulated (Lozano et al., 2009; Geuten and Irish, 2010; Quinet et al., 2014; Yuste-Lisbona et al., 2016), promoting homeotic changes in the floral organs where these gene functions are required. Additional floral whorls or floral organs were also observed in antisense *TM5* plants and ectopic shoots with partially developed leaves and secondary flowers emerged from the fruit in antisense *TM29* plants (Pnueli et al., 1994b; Ampomah-Dwamena et al., 2002). Furthermore, tomato mutations that altered regulatory genes involved in the activity and size of the FM as *INFLORESCENCE MERISTEM ACTIVITY* and the tomato homologous to *CLAVATA* genes have recently been isolated and further characterized (Sicard et al., 2008; Xu et al., 2015). Once again, the corresponding mutations affected inflorescence architecture and the number of floral organs, although alterations in floral organ identity and growth were not reported. To our knowledge, no other single mutants similar to *ufd* have been previously reported in tomato or in other model plant species such as *Arabidopsis*, *Antirrhinum* and rice. Only the flowers of the *Arabidopsis arf6 arf8* double mutant showed a similar phenotype to the *ufd* mutant. Loss of *ARF6* or *ARF8* in single mutants caused delayed stamen filament elongation and delayed anther dehiscence leading to decreased self-pollination, whereas floral organ growth was arrested in *arf6 arf8* double mutant resulting in the formation of infertile closed buds (Nagpal et al., 2005). However, *arf6 arf8* floral buds were blocked after carpel primordia were initiated, at a later developmental stage compared to *ufd* floral buds (Figure 2H). Therefore, it suggests that characterization of *ufd* mutation could reveal a novel regulatory gene specifically required for the normal growth of floral organs in tomato. Hopefully, the cloning of *UFD* gene, which is currently underway, will shed light on its function during flower development.

#### **4.2 UFD plays an essential role in flower development by affecting the expression of floral organ identity and growth genes**

It is generally accepted that once the FM has been specified, FM identity genes activate floral organ identity genes, which in turn specify the four different types of floral organs (reviewed in O'Maoiléidigh et al., 2014; Wellmer et al., 2014). However, after floral organ primordia are initiated, identity and growth of differentiated cells must be specifically coordinated to promote a full development of floral organs. Expression levels

of ABC genes are slightly lower in *ufd* mutant plants at the onset of flower development, but later during flower development B- and C-class genes were significantly repressed. Previous reports have proved that the ABC genes play pivotal roles not only determining floral organ identity at early stages of flower development but they are expressed during late stages of floral organogenesis indicating that their functions are needed throughout floral development. Moreover, several pieces of evidence have highlighted the close link between the identity and the final size of floral organs at the end stages of flower development (reviewed in Dornelas et al., 2011; Wellmer et al., 2014). However, genetic and molecular mechanisms coordinating differentiation and growth of specific cells that constitute floral organs still remain unknown (Dornelas et al., 2011). Results reported in the present study suggest that *UFD* gene may be involved in the genetic network linking both developmental processes in tomato. Transcriptomic and expression analyses performed in *ufd* mutant showed that the expression levels of ABC identity genes were smaller than those observed in WT plants during late stages of flower development, suggesting that floral organ primordia, which are unable to grow and complete their development, do not maintain their identity. Furthermore, the failure to grow floral organ primordia in *ufd* plants is supported by the up-regulation of genes involved in the repression of cell division, as *FW2.2* and *OVATE*. Accordingly, *SUN* is down-regulated in *ufd* inflorescences as expected from a positive regulator of floral organ growth. Overall, the results obtained suggested that identity and growth are parts of the same developmental program leading to floral organ formation, and that *UFD* may regulate both ABC and cell division genes. It is also reasonable to think that organ cells have lost their identity due to their incapacity to proliferate and grow, and therefore, *UFD* could be a target of ABC identity genes. Under this genetic scenario, *UFD* would participate in a positive feed-back regulatory pathway enabling the maintenance of the identity of a given floral organ and the differentiation and growth of specific cells composing this organ. Such a hypothesis would be in accordance with the autoregulatory feedback mechanism, which involves MADS-box and other interacting transcription factors (Dornelas et al., 2011). However, other hierarchical relationships between *UFD* and organ identity genes cannot be discarded until *UFD* is cloned and its functional role in establishing floral organ identity is further determined.

Expression results proved that *UFD* gene is required for the transcriptional activity of the B-, C- and E-class genes leading to further development of the floral organ primordia mainly in the three inner whorls. Interestingly, expression of *MC* in *ufd* floral buds did not differ significantly from that of WT up until the late stages of inflorescence development, when *MC* was up-regulated. In addition, sepals of *ufd* floral buds displayed a normal development and appeared to be more elongated than the remaining floral organs when they were initiated, however their growth was also arrested at the beginning of floral organogenesis. Sepals were suggested as the default state of floral organs and it was proposed that sepal identity might be a consequence of the specification of floral meristems through the activities of the FM identity genes (Causier et al., 2010; Wellmer et al., 2014). It is true that the first whorl of tomato flowers seems to have a special status since mutations in *MC* only change sepal identity (Vrebalov et al., 2002; Yuste-Lisbona

et al., 2016) while mutations of B- and C-class genes affect floral organs of two consecutive floral whorls and loss-of-function of E-class genes alter the three inner whorls (Pnueli et al., 1994b; Ampomah-Dwamena et al., 2002). In this context, *UFD* expression should be differently regulated in sepals and in the three innermost floral whorls. Our qRT-PCR results confirmed that *MC* expression was observed in *ufd* mutant while B-, C- and E-class genes remained down-regulated during inflorescence and flower development. Moreover, *MC* expression continued to increase during inflorescence development in the *ufd* mutant while it decreased in the WT. That *UFD* represses the *MC* expression during floral organ development and activates the B- and C-class genes for proper development of petals, stamens and carpels should not be excluded.

#### **4.3 *UFD* participates in the hormone signalling pathways involved in floral development**

The formation of organ primordia and the differentiation of floral organs are thought to be mediated by the activities of different phytohormones (Chandler, 2011; Wellmer et al., 2014). In accordance, transcriptomic analyses in *Arabidopsis* revealed that genes involved in FM and floral organ identity as well as in cell division regulate the expression of many genes involved in hormone responses and metabolism (Kaufmann et al., 2009, 2010; Yant et al., 2010; Wuest et al., 2012; O'Maoileidigh et al., 2013; Winter et al., 2015). In the same way, our results on transcriptome profile showed that genes related to jasmonic acid, cytokinin and auxin signalling were down-regulated in *ufd* mutant inflorescences while genes involved in ethylene response were up-regulated suggesting that hormone response could be mediated by *UFD* transcriptional activity. In tomato plants, insensitivity to hormones often resulted in floral organ defects. Jasmonic acid insensitivity leads to a female sterile phenotype and defects in stamen development in the *jai1-1* mutant (Li et al., 2004). This phenotype is partly due to a premature expression of ethylene signalling regulators showing that jasmonic acid and ethylene could have an antagonist effect during flower development (Dobritsch et al., 2015). Down-regulation of *ARF6* and *ARF8* resulted in shortened petals, stamens and styles and female sterility (Liu et al., 2014), some of these phenotypes being also attributed to reduced jasmonate production or signalling (Nagpal et al., 2005). Nevertheless, the way in which floral transcription factors and hormones interact to control floral organ growth remained largely unknown. Recently, it has been suggested that *SEPALLATA 3* (*SEP3*) could regulate floral organ development by modulating auxin response in *Arabidopsis* (Kaufmann et al., 2009). *SEP3* is a member of a SEP subfamily of MADS-box genes needed to specify the identity of FM and floral organs through the formation of multimeric complexes with ABC proteins (Immink et al., 2009). Therefore, *SEP3* could be a linking factor between identity and hormonal signals required for floral organ growth. The fact that expression of *TM29*, a tomato member of *SEP* family, is severely repressed in *UFD* would support this hypothesis.

Moreover, our results showed that the *ufd* mutation not only affects the expression of genes involved in hormone response but also affects the inflorescence hormonal profile. The production of ACC, strigol and some forms of auxins, gibberellins and cytokinins

were increased in *ufd* compared to WT. Modification of the hormonal profile was previously reported in B-class tomato mutants *sl* and *sl-2* (Sawhney, 1974; Singh et al., 1992; Singh and Sawhney, 1998, Quinet et al., 2014). These observations might indicate that hormones play a role in proper development of floral organs. In Arabidopsis, the initiation and outgrowth of floral primordia and floral organ initiation depend on the activity of several phytohormones, mainly auxins (Chandler, 2011; Wellmer et al., 2014). Cytokinins and gibberellins also interact with auxins at this level, e.g. in the determination of boundaries between floral organ primordia (Ding et al., 2015). Involvement of these hormones at the early stage of flower development could explain the higher concentration of IAA and some cytokinin forms in the *ufd* mutant flowering apices. Strigolactones were recently shown to play a hormonal role in plant development (de Saint-Germain et al., 2013) and the high strigol concentration in the *ufd* mutant flowering shoot apex suggests a potential role in flower development in tomato. Further investigations will be required to unravel the hormonal crosstalk underlying floral organ growth in tomato and how it interacts with *UFD*.

Summarizing, *UFD* is a novel regulator of the tomato floral organogenesis that ensures the proper development of floral organ primordia by affecting the expression of ABC(DE) transcription factors and genes involved in cell division and hormone pathways.

#### **Conflict of interest statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

#### **Author contributions**

S.P-P. and R.L. were involved in experimental design and interpretation of data. S.P-P., M.Q. and A.O-A, performed experiments and analyzed data; S.P-P., M.Q., F.J.Y-L. and R.L. wrote, reviewed and edited the manuscript; J.C., T.A. and E.G. supervised experiments and provided critical review and editing of the manuscript. All authors contributed to editing and approving the final version of the manuscript.

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## References

- Ampomah-Dwamena, C., Morris, B.A., Sutherland, P., Veit, B., and Yao, J.L. (2002) Down-regulation of *TM29*, a tomato *SEPALLATA* homolog, causes parthenocarpic fruit development and floral reversion. *Plant Physiol.* 130, 605-617. doi: 10.1104/pp.005223
- Anastasiou, E., and Lenhard, M. (2007) Growing up to one's standard. *Curr. Opin. Plant Biol.* 10, 63-69. doi: 10.1016/j.pbi.2006.11.002
- Azzi, L., Deluche, C., Gévaudant, F., Frangne, N., Delmas, F., Hernould, M., et al. (2015) Fruit growth-related genes in tomato. *J. Exp. Bot.* 66, 1075-1086. doi: 10.1093/jxb/eru527
- Bögre, L., Magyar, Z., and López-Juez, E. (2008) New clues to organ size control in plants. *Genome Biol.* 9, 226. doi: 10.1186/gb-2008-9-7-226
- Bowman, J.L., Drews, G.N., and Meyerowitz, E.M. (1991) Expression of the Arabidopsis floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. *Plant Cell* 3, 749-758. doi: 10.1105/tpc.3.8.749
- Brukhin, V., Hernould, M., Gonzalez, N., Chevalier, C., and Mouras, A. (2003) Flower development schedule in tomato *Lycopersicon esculentum* cv. sweet cherry. *Sex. Plant Reprod.* 15, 311-320. doi:10.1007/s00497-003-0167-7
- Busi, M., Bustamante, C., D'Angelo, C., Hidalgo-Cuevas, M., Boggio, S.B., Valle, E.M., et al. (2003) MADS-box genes expressed during tomato seed and fruit development. *Plant Mol. Biol.* 52, 801-815. doi:10.1023/A:1025001402838
- Causier, B., Schwarz-Sommer, Z., and Davies, B. (2010) Floral organ identity: 20 years of ABCs. *Semin. Cell Dev. Biol.* 21, 73-79. doi: 10.1016/j.semcdb.2009.10.005
- Chandler, J.W. (2011) The hormonal regulation of flower development. *J. Plant Growth Regul.* 30, 242-254. doi: 10.1007/s00344-010-9180-x
- Coen, E.S., and Meyerowitz, E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature* 353, 31-37. doi: 10.1038/353031a0
- de Martino, G., Pan, I., Emmanuel, E., Levy, A., and Irish, V.F. (2006) Functional analyses of two tomato *APETALA3* genes demonstrate diversification in their roles in regulating floral development. *Plant Cell* 18, 1833-1845. doi: 10.1105/tpc.106.042978
- de Saint Germain, A., Bonhomme, S., Boyer, F.D., and Rameau, C. (2013) Novel insights into strigolactone distribution and signalling. *Curr. Opin. Plant Biol.* 16, 583-589. doi: 10.1016/j.pbi.2013.06.007
- Ding, L., Yan, S., Jiang, L., Zhao, W., Ning, K., Zhao, J., et al. (2015) *HANABA TARANU* (*HAN*) bridges meristem and organ primordia boundaries through *PINHEAD*, *JAGGED*, *BLADE-ON-PETIOLE2* and *CYTOKININ OXIDASE 3* during flower development in *Arabidopsis*. *PLoS Genet.* 11, e1005479. doi: 10.1371/journal.pgen.1005479
- Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., and Yanofsky, M.F. (2004) The *SEP4* gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. *Curr. Biol.* 14, 1935-1940. doi: 10.1016/j.cub.2004.10.028

- Dobrev, P.I., and Kamínek, M. (2002) Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid phase extraction. *J. Chromatogr. A* 950, 21-29. doi: 10.1016/S0021-9673(02)00024-9
- Dobritsch, S., Weyhe, M., Schubert, R., Dindas, J., Hause, G., Kopka, J., et al. (2015) Dissection of jasmonate functions in tomato stamen development by transcriptome and metabolome analyses. *BMC Biol.* 13, 28. doi: 10.1186/s12915-015-0135-3
- Dornelas, M.C., Patreze, C.M., Angenent, G.C., and Immink, R.G.M. (2011) MADS: the missing link between identity and growth? *Trends Plant Sci.* 16, 89-97. doi: 10.1016/j.tplants.2010.11.003
- Frery, A., Nesbitt, T.C., Frery, A., Grandillo, S., van der Knaap, E., Cong, B., et al. (2000) fw2.2: a quantitative trait locus key to the evolution of tomato fruit size. *Science* 289, 85-88. doi: 10.1126/science.289.5476.85
- Gallego-Giraldo, C., Hu, J., Urbez, C., Gomez, M.D., Sun, T.P., and Perez-Amador, M.A. (2014) Role of the gibberellin receptors *GID1* during fruit-set in *Arabidopsis*. *Plant J.* 79, 1020-1032. doi: 10.1111/tpj.12603
- Geuten, K., and Irish, V. (2010) Hidden variability of floral homeotic B genes in Solanaceae provides a molecular basis for the evolution of novel functions. *Plant Cell* 22, 2562-2578. doi: 10.1105/tpc.110.076026
- Gimenez, E., Pineda, B., Capel, J., Antón, M.T., Atarés, A., Pérez-Martín, F., et al. (2010) Functional analysis of the *Arlequin* mutant corroborates the essential role of the *ARLEQUIN/TAGL1* gene during reproductive development of tomato. *PLoS ONE* 5, e14427. doi: 10.1371/journal.pone.0014427
- Hackbusch, J., Richter, K., Müller, J., Salamini, F., and Uhrig, J.F. (2005) A central role of *Arabidopsis thaliana* ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. *Proc. Natl. Acad. Sci. USA* 102, 4908-4912. doi: 10.1073/pnas.0501181102
- Honma, T., and Goto, K. (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* 409, 525-529. doi: 10.1038/35054083
- Immink, R.G., Tonaco, I.A., de Folter, S., Shchennikova, A., van Dijk, A.D., Busscher-Lange, J., et al. (2009) SEPALLATA3: the 'glue' for MADS box transcription factor complex formation. *Genome Biol.* 10, R24. doi: 10.1186/gb-2009-10-2-r24
- Jaeger, K.E., Pullen, N., Lamzin, S., Morris, R.J., and Wigge, P.A. (2013) Interlocking feedback loops govern the dynamic behavior of the floral transition in *Arabidopsis*. *Plant Cell* 25, 820-833. doi: 10.1105/tpc.113.109355
- Kaufmann, K., Muino, J.M., Jauregui, R., Airoidi, C.A., Smaczniak, C., Krajewski, P., et al. (2009) Target genes of the MADS transcription factor SEPALLATA3: integration of developmental and hormonal pathways in the *Arabidopsis* flower. *PLoS Biol.* 7, e1000090. doi: 10.1371/journal.pbio.1000090
- Kaufmann, K., Wellmer, F., Muiño, J.M., Ferrier, T., Wuest, S.E., Kumar, V., et al. (2010) Orchestration of floral initiation by APETALA1. *Science* 328, 85-89. doi: 10.1126/science.1185244

749 Kramer, E.M., Dorit, R.L., and Irish, V.F. (1998) Molecular evolution of genes  
750 controlling petal and stamen development: duplication and divergence within the  
751 *APETALA3* and *PISTILLATA* MADS-box gene lineages. *Genetics* 149, 765-83.

752 Leseberg, C.H., Eissler, C.L., Wang, X., Johns, M.A., Duvall, M.R., and Mao, L. (2008)  
753 Interaction study of MADS-domain proteins in tomato. *J. Exp. Bot.* 59, 2253-  
754 2265. doi: 10.1093/jxb/ern094

755 Li, L., McCaig, B., Wingerd, B., Wang, J., Whaton, M., Pichersky, E., et al. (2004) The  
756 tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal  
757 control of seed maturation, jasmonate-signaled defense responses, and glandular  
758 trichome development. *Plant Cell* 16, 126-143. doi: 10.1105/tpc.017954

759 Liu, J., Van Eck, J., Cong, B., and Tanksley, S.D. (2002) A new class of regulatory genes  
760 underlying the cause of pear-shaped tomato fruit. *Proc. Natl. Acad. Sci. USA* 99,  
761 13302-13306. doi: 10.1073/pnas.162485999

762 Liu, N., Wu, S., Van Houten, J., Wang, Y., Ding, B., Fei, Z., et al. (2014) Down-  
763 regulation of *AUXIN RESPONSE FACTORS 6* and *8* by microRNA 167 leads to  
764 floral development defects and female sterility in tomato. *J. Exp. Bot.* 65, 2507-  
765 2520. doi: 10.1093/jxb/eru141

766 Lombardo, F., and Yoshida, H. (2015) Interpreting lemma and palea homologies: a point  
767 of view from rice floral mutants. *Front. Plant Sci.* 6, 61. doi:  
768 10.3389/fpls.2015.00061

769 Lozano, R., Gimenez, E., Cara, B., Capel, J., and Angosto, T. (2009) Genetic analysis of  
770 reproductive development in tomato. *Int. J. Dev. Biol.* 53, 1635-48. doi:  
771 10.1387/ijdb.072440rl

772 Lytovchenko, A., Eickmeier, I., Pons, C., Osorio, S., Szecowka, M., Lehmberg, K., et al.  
773 (2011) Tomato fruit photosynthesis is seemingly unimportant in primary  
774 metabolism and ripening but plays a considerable role in seed development. *Plant*  
775 *Physiol.* 157, 1650-1663. doi: 10.1104/pp.111.186874

776 Marsch-Martínez, N., and de Folter, S. (2016) Hormonal control of the development of  
777 the gynoecium. *Curr. Opin. Plant Biol.* 29, 104-114. doi:  
778 10.1016/j.pbi.2015.12.006

779 Mizzotti, C., Gallianina, B.M., and Masieroa, S. (2014) The backstage of the ABC model:  
780 the *Antirrhinum majus* contribution. *Plant Biosyst.* 148, 176-186. doi:  
781 10.1080/11263504.2013.877531

782 Molinero-Rosales, N., Jamilena, M., Zurita, S., Gomez, P., Capel, J., and Lozano, R.  
783 (1999) *FALSIFLORA*, the tomato orthologue of *FLORICAULA* and *LEAFY*,  
784 controls flowering time and floral meristem identity. *Plant J.* 20, 685-693. doi:  
785 10.1046/j.1365-313X.1999.00641.x

786 Nagpal, P., Ellis, C.M., Weber, H., Ploense, S.E., Barkawi, L.S., Guilfoyle, T.J., et al.  
787 (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid  
788 production and flower maturation. *Development* 132, 4107-4118. doi:  
789 10.1242/dev.01955

790 Ó'Maoiléidigh, D.S., Graciet, E., and Wellmer, F. (2014) Gene networks controlling  
791 *Arabidopsis thaliana* flower development. *New Phytol.* 201, 16-30. doi:  
792 10.1111/nph.12444

- Ó'Maoiléidigh, D.S., Wuest, S.E., Rae, L., Raganelli, A., Ryan, P.T., Kwasniewska, K., et al. (2013) Control of reproductive floral organ identity specification in *Arabidopsis* by the C function regulator AGAMOUS. *Plant Cell* 25, 2482-2503. doi: 10.1105/tpc.113.113209
- Pajoro, A., Madrigal, P., Muiño, J.M., Matus, J.T., Jin, J., Mecchia, M.A., et al. (2014) Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development. *Genome Biol.* 15, R41. doi: 10.1186/gb-2014-15-3-r41
- Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E., and Yanofsky, M.F. (2000) B and C floral organ identity functions require *SEPALLATA* MADS-box genes. *Nature* 405, 200-203. doi: 10.1038/35012103
- Pesaresi, P., Mizzotti, C., Colombo, M., and Masiero, S. (2014) Genetic regulation and structural changes during tomato fruit development and ripening. *Front. Plant Sci.* 5, 124. doi: 10.3389/fpls.2014.00124
- Plackett, A.R., Thomas, S.G., Wilson, Z.A., and Hedden, P. (2011) Gibberellin control of stamen development: a fertile field. *Trends Plant Sci.* 16, 568-578. doi: 10.1016/j.tplants.2011.06.007
- Pnueli, L., Abu-Abeid, M., Zamir, D., Nacken, W., Schwarz-Sommer, Z., and Lifschitz, E. (1991) The MADS box gene family in tomato: temporal expression during floral development, conserved secondary structures and homology with homeotic genes from *Antirrhinum* and *Arabidopsis*. *Plant J.* 1, 255-66. doi: 10.1111/j.1365-313X.1991.00255.x
- Pnueli, L., Hareven, D., Broday, L., Hurwitz, C., and Lifschitz, E. (1994b) The *TM5* MADS box gene mediates organ differentiation in the three inner whorls of tomato flowers. *Plant Cell* 6, 175-186. doi: 10.1105/tpc.6.2.175
- Pnueli, L., Hareven, D., Rounsley, S., Yanofsky, M., and Lifschitz, E. (1994a) Isolation of the tomato *AGAMOUS* gene *TAG1* and analysis of its homeotic role in transgenic plants. *Plant Cell* 6, 163-173. doi: 10.1105/tpc.6.2.163
- Poyatos-Pertíñez, S., Quinet, M., Ortíz-Atienza, A., Bretones, S., Yuste-Lisbona, F.J., and Lozano, R. (2016) Genetic interactions of the *unfinished flower development* (*ufd*) mutant support a significant role of the tomato *UFD* gene in regulating floral organogenesis. *Plant Reprod.* doi: 10.1007/s00497-016-0286-6
- Quinet, M., Bataille, G., Dobrev, P.I., Capel, C., Gómez, P., Capel, J., et al. (2014) Transcriptional and hormonal regulation of petal and stamen development by *STAMENLESS*, the tomato (*Solanum lycopersicum* L.) orthologue to the B-class *APETALA3* gene. *J. Exp. Bot.* 65, 2243-2256. doi: 10.1093/jxb/eru089
- Rheinhardt, D. (2005) Regulation of phyllotaxis. *Int. J. Dev. Biol.* 49, 539-546. doi: 10.1387/ijdb.041922dr
- Sawhney, V.K. (1974) Morphogenesis of the *stamenless-2* mutant in tomato. III. Relative levels of gibberellins in the normal and mutant plants. *J. Exp. Bot.* 25, 1004-1009. doi: 10.1093/jxb/25.6.1004
- Sicard, A., Petit, J., Mouras, A., Chevalier, C., and Hernould, M. (2008) Meristem activity during flower and ovule development is controlled by the mini zinc finger gene

836 *INHIBITOR OF MERISTEM ACTIVITY* in tomato. *Plant J.* 55, 415-427. doi:  
837 10.1111/j.1365-313X.2008.03520.x

838  
839 Singh, S., and Sawhney, V.K. (1998) Absciscic acid in a male sterile tomato mutant and  
840 its regulation by low temperature. *J. Exp. Bot.* 49, 199-203. doi:  
841 10.1093/jexbot/49.319.199

842 Singh, S., Sawhney, V.K., and Pearce, D.W. (1992) Temperature effects on endogenous  
843 indole-3-acetic acid levels in leaves and stamens of the normal and male sterile  
844 'stamenless-2' mutant of tomato (*Lycopersicon esculentum* Mill.). *Plant Cell*  
845 *Environ.* 15, 373-377. doi: 10.1111/j.1365-3040.1992.tb00986.x

846 Siriwardana, N.S., and Lamb, R.S. (2012) The poetry of reproduction: the role of *LEAFY*  
847 in *Arabidopsis thaliana* flower formation. *Int. J. Dev. Biol.* 56, 207-221. doi:  
848 10.1387/ijdb.113450ns

849 Smaczniak, C., Immink, R.G., Muino, J.M., Blanvillain, R., Busscher, M., Busscher-  
850 Lange, J., et al. (2012) Characterization of MADS-domain transcription factor  
851 complexes in *Arabidopsis* flower development. *Proc. Natl. Acad. Sci. USA* 109,  
852 1560-1565. doi: 10.1073/pnas.1112871109

853 Sugawara, S., Mashiguchi, K., Tanaka, K., Hishiyama, S., Sakai, T., Hanada, K., et al.  
854 (2015) Distinct characteristics of indole-3-acetic acid and phenylacetic acid, two  
855 common auxins in plants. *Plant Cell Physiol.* 56, 1641-1654. doi:  
856 10.1093/pcp/pcv088

857 Thouet, J., Quinet, M., Lutts, S., Kinet, J.M., and Périlleux, C. (2012) Repression of floral  
858 meristem fate is crucial in shaping tomato inflorescence. *PLoS ONE* 7, e31096.  
859 doi: 10.1371/journal.pone.0031096

860 Vrebalov, J., Pan, I.L., Arroyo, A.J.M., McQuinn, R., Chung, M., Poole, M., et al. (2009)  
861 Fleshy fruit expansion and ripening are regulated by the tomato *SHATTERPROOF*  
862 gene *TAGL1*. *Plant Cell* 21, 3041-3062. doi: 10.1105/tpc.109.066936

863 Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., et al.  
864 (2002) A MADS-box gene necessary for fruit ripening at the tomato *Ripening-*  
865 *Inhibitor (Rin)* locus. *Science* 296, 343-346. doi: 10.1126/science.1068181

866 Wang, S., Chang, Y., Guo, J., and Chen, J.G. (2007) Arabidopsis Ovate Family Protein 1  
867 is a transcriptional repressor that suppresses cell elongation. *Plant J.* 50, 858-872.  
868 doi: 10.1111/j.1365-313X.2007.03096.x

869 Wang, S., Chang, Y., Guo, J., Zeng, Q., Ellis, B.E., and Chen, J.G. (2011) Arabidopsis  
870 Ovate family proteins, a novel transcriptional repressor family, control multiple  
871 aspects of plant growth and development. *PLoS ONE* 6, e23896. doi:  
872 10.1371/journal.pone.0023896

873 Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J.L., and Meyerowitz, E.M.  
874 (2006) Genome-wide analysis of gene expression during early *Arabidopsis* flower  
875 development. *PLoS Genet.* 2, e117. doi: 10.1371/journal.pgen.0020117.eor

876 Wellmer, F., Graciet, E., and Riechmann, J.L. (2014) Specification of floral organs in  
877 *Arabidopsis*. *J. Exp. Bot.* 65, 1-9. doi: 10.1093/jxb/ert385

- Wellmer, F., Riechmann, J.L., Alves-Ferreira, M., and Meyerowitz, E.M. (2004) Genome-wide analysis of spatial gene expression in Arabidopsis flowers. *Plant Cell* 16, 1314-1326. doi: 10.1105/tpc.021741
- Winter, C.M., Yamaguchi, N., Wub, M.F., and Wagner, D. (2015) Transcriptional programs regulated by both LEAFY and APETALA1 at the time of flower formation. *Physiol. Plant.* 155, 55-73. doi: 10.1111/ppl.12357
- Wuest, S.E., Ó'Maoiléidigh, D.S., Rae, L., Kwasniewska, K., Raganelli, A., Hanczaryk, K., et al. (2012) Molecular basis for the specification of floral organs by APETALA3 and PISTILLATA. *Proc. Natl. Acad. Sci. USA* 109, 13452-13457. doi: 10.1073/pnas.1207075109
- Xiao, H., Jiang, N., Schaffner, E.K., Stockinger, E.J., and van der Knaap, E. (2008) A retrotransposon-mediated gene duplication underlies morphological variation of tomato fruit. *Science* 319, 1527-1530. doi: 10.1126/science.1153040
- Xiao, H., Radovich, C., Welty, N., Hsu, J., Li, D., Meulia, T., et al. (2009) Integration of tomato reproductive developmental landmarks and expression profiles, and the effect of *SUN* on fruit shape. *BMC Plant Biol.* 9, 49. doi: 10.1186/1471-2229-9-49
- Xu, C., Liberatore, K.L., MacAlister, C.A., Huang, Z., Chu, Y.H., Jiang, K., et al. (2015) A cascade of arabinosyltransferases controls shoot meristem size in tomato. *Nat. Genet.* 47, 784-792. doi: 10.1038/ng.3309
- Yant, L., Mathieu, J., Dinh, T.T., Ott, F., Lanz, C., Wollmann, H., et al. (2010) Orchestration of the floral transition and floral development in *Arabidopsis* by the bifunctional transcription factor APETALA2. *Plant Cell* 22, 2156-2170. doi: 10.1105/tpc.110.075606
- Yuan, Z., and Zhang, D. (2015) Roles of jasmonate signalling in plant inflorescence and flower development. *Curr. Opin. Plant Biol.* 27, 44-51. doi: 10.1016/j.pbi.2015.05.024
- Yuste-Lisbona, F.J., Quinet, M., Fernández-Lozano, A., Pineda, B., Moreno, V., Angosto, T., et al. (2016) Characterization of vegetative inflorescence (*mc-vin*) mutant provides new insight into the role of *MACROCALYX* in regulating inflorescence development of tomato. *Sci. Rep.* 6, 18796. doi: 10.1038/srep18796

## Figure Legends

**Figure 1** Structure of wild type and *unfinished flower development* (*ufd*) plants grown under standard conditions. **(A, B)** Diagrammatic representation of wild type (WT) and mutant tomato plant growth. Green bars represent initial vegetative segment. Sympodial segments are in black. Yellow arrows represent sympodial meristems. Circles symbolize flowers, lines are leaves and black arrows represent developed axillary shoots. **(C, D)** Morphology of a wild type **(C)** and an *ufd* mutant plant **(E)**. White arrows indicate inflorescence position. The insets show a closer view of the inflorescences. **(D, F)** Adult inflorescences of the wild type **(D)** and the *ufd* mutant **(F)**. White bars show inflorescence size.

**Figure 2** Scanning electron microscopy (SEM) analysis and histological microscopy of floral development from wild type and *unfinished flower development* (*ufd*) mutant plants. **(A-E)** Wild type floral buds. **(F-J)** Mutant flowers. Wild type **(A)** and *ufd* **(F)** floral buds at stage 3, corresponding to stamen primordia initiation (most sepals were removed). Wild type **(B)** and *ufd* **(G)** floral buds at stage 5, when carpel primordia emerge and the distinct ovary cavities are visible (sepals removed). **(C)** Wild type flower at stage 6, when petals bend down to cover reproductive organs and the carpels grow up but are still unfused (sepals removed). **(H)** floral bud at the most adult stage observed in *ufd* mutant plants (sepals removed). **(D)** Wild type inflorescence with floral buds at stage 6 (f1) and 5 (f2). **i** *ufd* inflorescence showing the most developed floral bud at stage 5 (f1) and others at earlier stages (f2-f4). **(E)** Wild type inflorescence with flowers at different development stages (1, 2, 3 and 4). **(J)** *ufd* inflorescence with all flowers showing the same development stage (floral organs stop their development at primordia stage). All developmental stages are classified according to Brukhin et al. (2003). s, sepal; p, petal; st, stamen; c, carpel. Scale bars are 200  $\mu$ m **(A-C, F-H)**, 500  $\mu$ m **(D-E, I-J)**.

**Figure 3** Expression distribution of genes differentially expressed in *unfinished flower development* (*ufd*) and wild type (WT) tomato flowering shoot apices. **(A)** Hierarchical clustering of all genes differentially expressed in the *ufd* mutant compared to WT (see also Supplementary Table 3). **(B, C)** Heat-map of MADS genes and genes involved in hormone signaling, respectively. Color scale indicates logarithm ratio of the fold change in mutant compared with WT. Red color represents genes showing higher expression level and green color indicates reduced expression.

**Figure 4** Quantitative real-time PCR determination of expression of **(A)** *MACROCALYX* (*MC*), **(B)** *STAMENLESS* (*SL*), **(C)** *TOMATO AGAMOUS 1* (*TAG1*), **(D)** *TOMATO AGAMOUS-LIKE 1* (*TAGL1*), **(E)** *TOMATO MADS BOX 5* (*TM5*) and **(F)** *TOMATO MADS BOX 29* (*TM29*) in the wild type (WT) and the *unfinished flower development* (*ufd*) mutant flowering inflorescences along their development. Inflorescence developmental stages (i.e. Apex, IF1, and IF2) are described in Material and Methods section. Error bars show the standard deviation of three independent biological replicates; \* significant differences at  $P < 0.05$ .

**Figure 5** Quantitative real-time PCR determination of expression of (A) *FRUIT WEIGHT 2.2 (FW2.2)*, (B) *OVATE* (C) and *SUN* in the wild type (WT) and the *unfinished flower development (ufd)* mutant flowering inflorescences along their development. Inflorescence developmental stages (i.e. Apex, IF1, and IF2) are described in Material and Methods section. Error bars show the standard deviation of three independent biological replicates; ns, no statistically significant differences, \* significant differences at  $P < 0.05$ .

**Figure 6** Impact of the *unfinished flower development (ufd)* mutation on the endogenous hormone content of flowering shoot apices. (A) Total abscisic acid (ABA). (B) Total auxins. (C) Salicylic acid. (D) Total jasmonates. (E) Total gibberellins. (F) Strigol. (G) Ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). (H) Total cytokinins. Error bars show the standard deviation of three independent biological replicates; ns, no statistically significant differences, \* significant differences at  $P < 0.05$ , \* \* significant differences at  $P < 0.01$ . Details of the different forms inside phytohormone groups are presented in Supplementary Table 6.

Figure 1.TIF

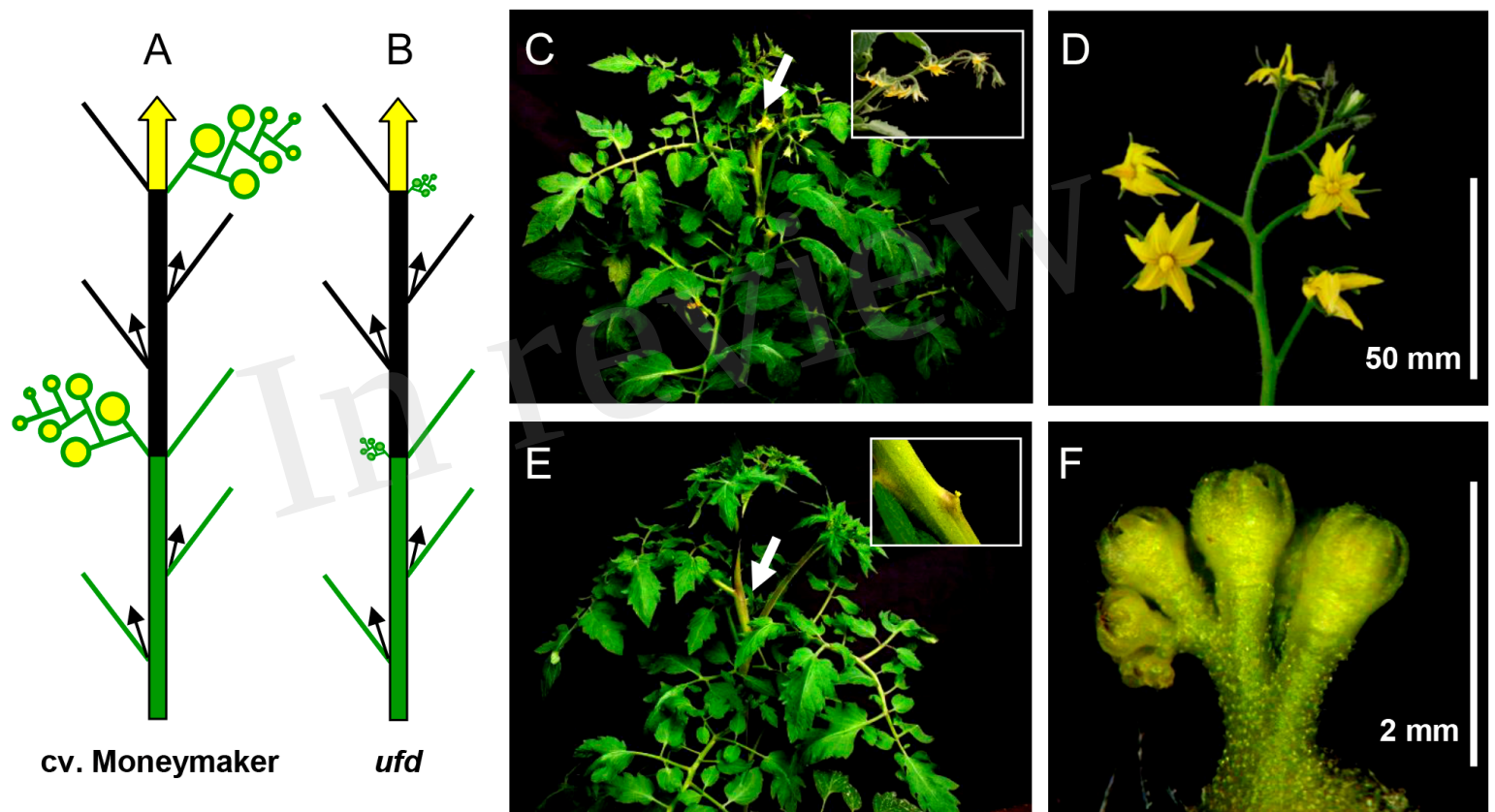


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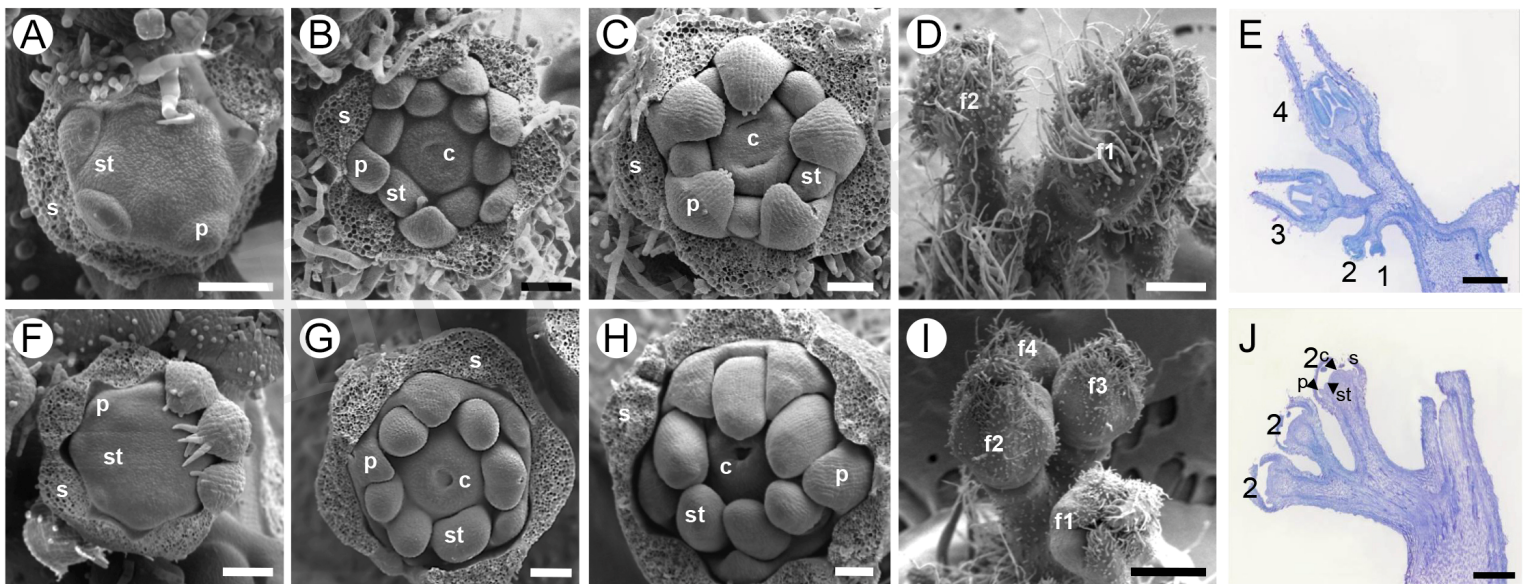


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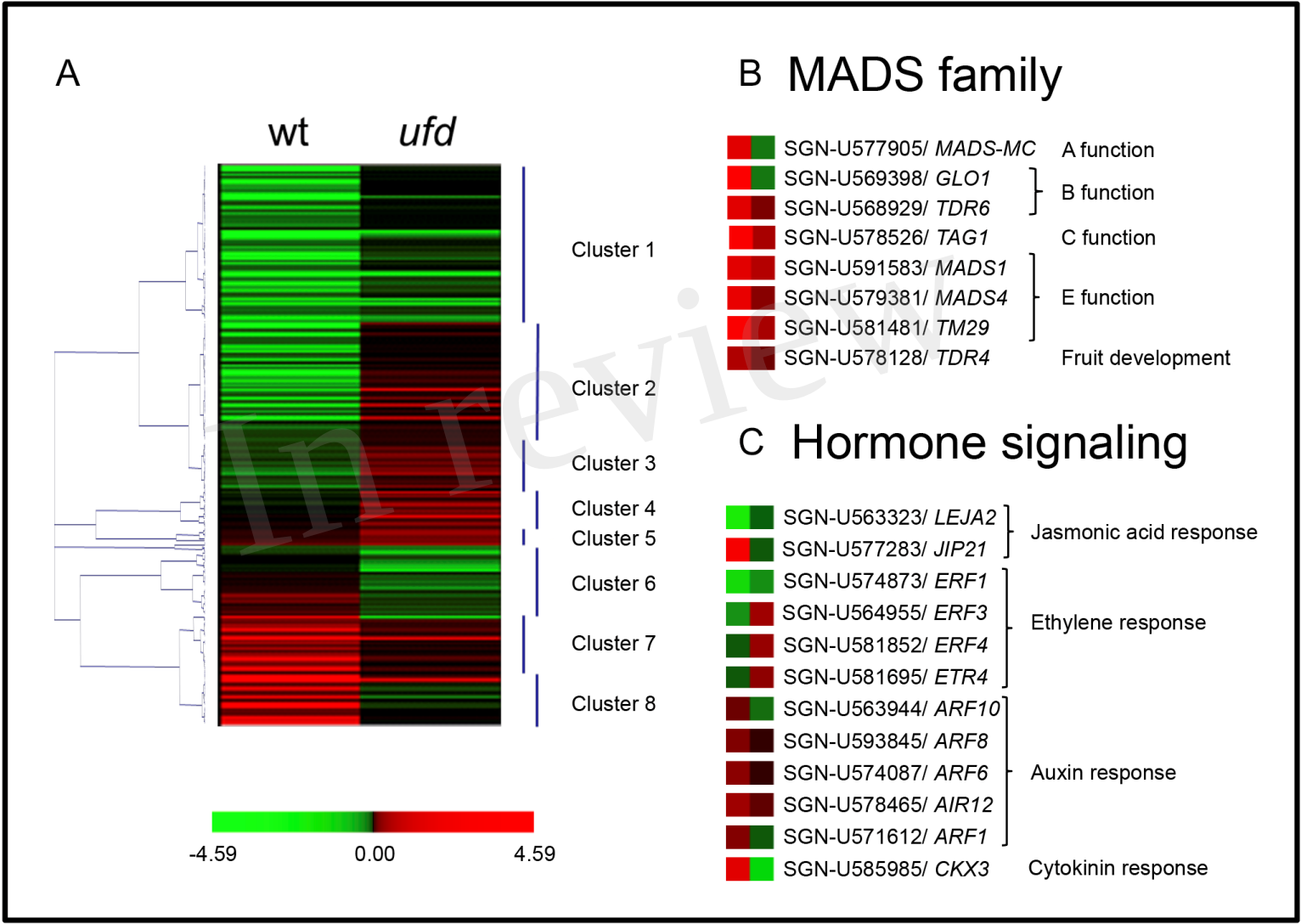


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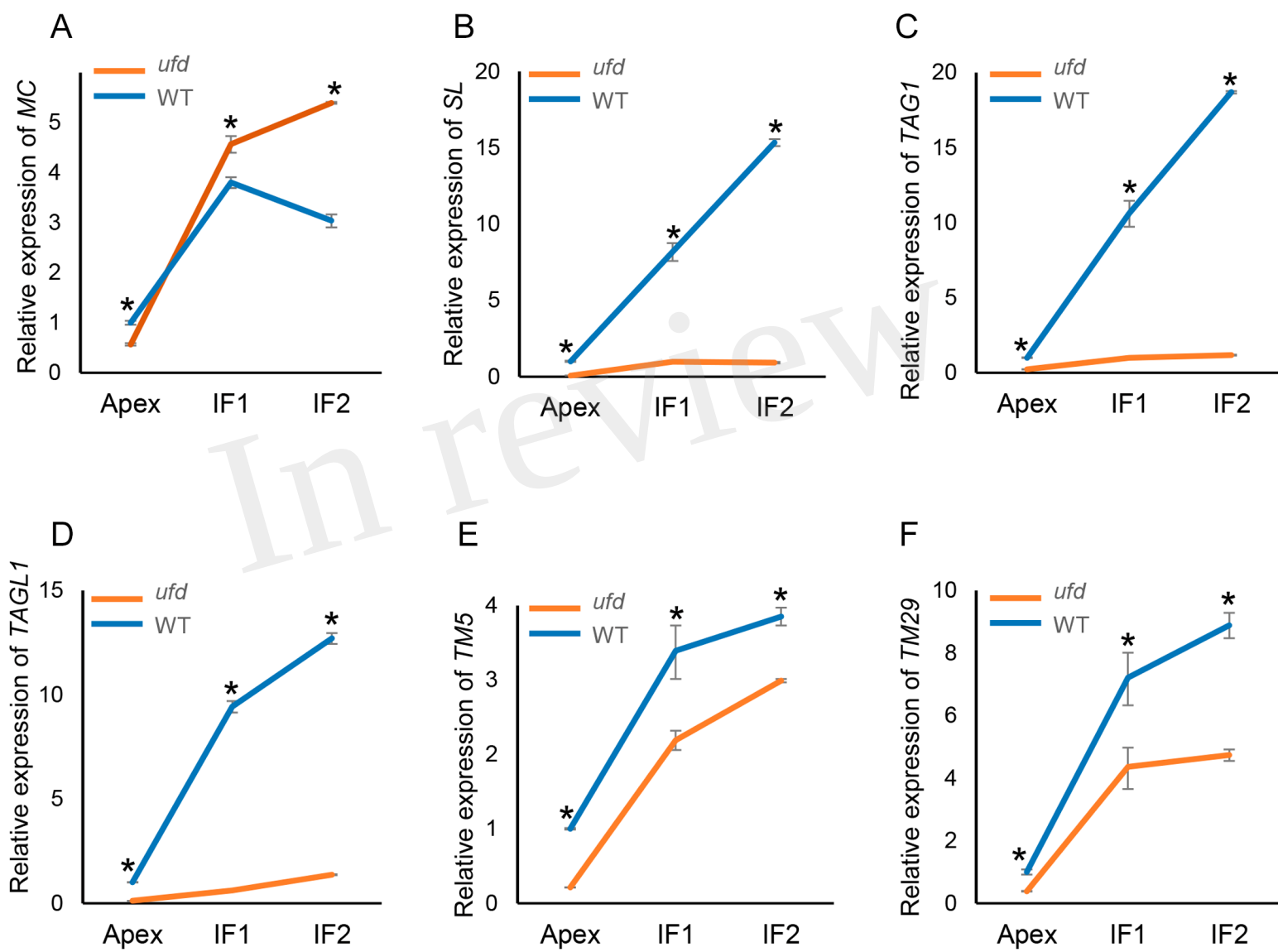


Figure 5.TIF

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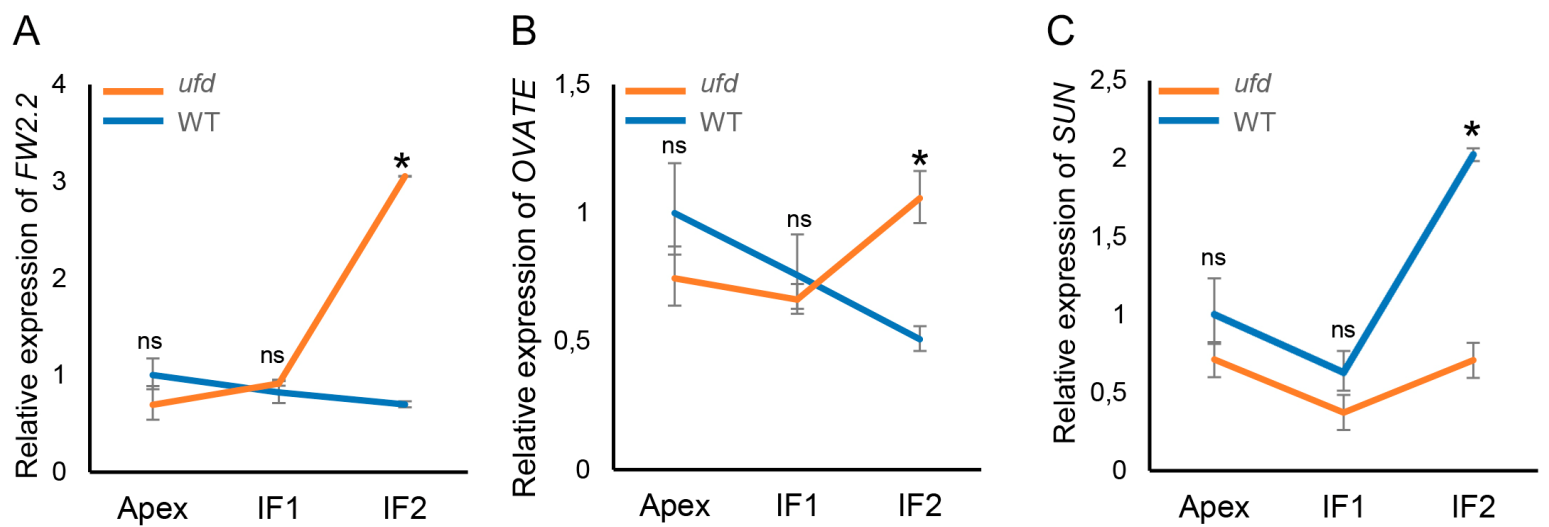


Figure 6.TIF

