Characterization of constricted fruit (ctf) Mutant Uncovers a Role for AtMYB117/LOF1 in Ovule and Fruit Development in Arabidopsis thaliana

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Abstract

Pistil and fruit morphogenesis is the result of a complex gene network that is not yet fully understood. A search for novel genes is needed to make a more comprehensive model of pistil and fruit development. Screening for mutants with alterations in fruit morphology generated by an activation tagging strategy resulted in the isolation of the ctf (constricted fruit) mutant. It is characterized by a) small and wrinkled fruits, with an enlarged replum, an amorphous structure of the septum and an irregular distribution of ovules and seeds; b) ectopic carpeloid structures in sepal bearing ovule-like structures and c) dwarf plants with curled rosette leaves. The overexpressed gene in ctf was AtMYB117, also named LOF1 (LATERAL ORGAN FUSION1). AtMYB117/LOF1 transcripts were localized in boundary regions of the vegetative shoot apical meristem and leaf primordia and in a group of cells in the axradial base of petioles and bracts. Transcripts were also detected in the boundaries between each of the four floral whorls and during pistil development in the inner of the medial ridges, the placenta, the base of the ovule primordia, the epidermis of the developing septum and the outer cell layers of the ovule funiculi. Analysis of changes of expression of pistil-related genes in the ctf mutant showed an enhancement of SHATTERPROOF1 (SHP1) and SHP2 expression. All these results suggest that AtMYB117/LOF1 is recruited by a variety of developmental programs for the establishment of boundary regions, including the development of floral organs and the initiation of ovule outgrowth.

Introduction

Meristem activity determines plant development. All above-ground organs, vegetative and reproductive, originate from the shoot apical meristem (SAM). When plants initiate flowering, the vegetative SAM is transformed into an inflorescence meristem (IM). The IM, in turn, generates a collection of undifferentiated cells called floral meristems that give rise to flowers. The flower contains reproductive structures, such as stamens and pistils that enclose the ovules which develop into seeds upon fertilization. Therefore, the fruit with mature seeds is the final product of a developmental process that gives rise to flowers. The ability to generate differentiated tissues indicates that the early embryo is meristematic and ovule primordia. The transition of meristem into meristem or differentiated tissue involves the formation of boundary layers of cells, also present between adjacent organs, with characteristics of both meristem and fully differentiated cells, such as reduced growth activity. These boundaries are considered reference points for the generation of new meristems and lateral organ primordia.

Arabidopsis fruit are siliques that originate from pistils constituted of two fused carpels, which are essentially modified leaves. Pistils are composed of the ovary (with ovules and multiple tissues: replum, septum, valves and valve margin), the style and the stigma at the top. Twenty developmental stages have been proposed between the formation of the flower buttress and silique dehiscence, providing common landmarks to describe developmental events. Flower primordia form at stage 2 while the onset of pistil development occurs at stage 6. At stage 7 the pistil grows as a hollow tube. At stage 8, early repla give rise to two medial ridges flanked on both sides by placental tissues. At stage 9, the septum forms when the medial ridges fuse and the placenta produces ovule primordia. The ability to generate differentiated tissues indicates that the early embryo is meristematic and ovule primordia. The transition of meristem into meristem or differentiated tissue involves the formation of boundary layers of cells, also present between adjacent organs, with characteristics of both meristem and fully differentiated cells, such as reduced growth activity. These boundaries are considered reference points for the generation of new meristems and lateral organ primordia.

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important components of flower development, a search for novel
genes is needed to form a more comprehensive model of pistil and
fruit development.

Identifying novel genes involved developmental processes often
requires bypassing the functional redundancy of genes constituting
a family. In this regard, an activation tagging strategy that consists
of the insertion of the Cauliflower Mosaic Virus (CaMV) 35S
enhancers, that act differently than the complete CaMV 35S
promoter, has been selected [6]. The activation tagging strategy
has been successfully used [7] as mutant phenotypes are caused by
overexpression rather than knock-out of the targeted gene.
Therefore, we used this strategy to search for genes involved in
pistil and fruit development in Arabidopsis. We selected a dominant
mutant showing altered fruit morphology that we named ctf for
constricted fruit. The overexpressed gene in ctf was AtMYB117, a
member of the MYB family of transcription factors [8,9].

AtMYB117 was recently described as LOF1 (LATERAL ORGAN
FUSION1), functioning in organ boundary specification, meristem
initiation and organ patterning [10]. GUS activity in an enhancer-
trap line suggests that AtMYB117/LOF1 expression is localized to
organ boundaries [10]. Additionally, in lof1-1 lines, a T-DNA
mutant with a partial phenotype, AtMYB117/LOF1 expression was
undetectable in both pedicel nodes and paracline junctions, in
conjunction with visible defects, but was only slightly reduced in
inflorescence apex where no phenotypic defects were observed.
These results [10], and the alterations in the morphology of the
fruit in ctf prompted us to examine in more detail the expression of
AtMYB117/LOF1 using in situ hybridization. In addition, we
generated an artificial microRNA (amiRNA) [11] to silence
AtMYB117/LOF1 and AtMYB105/LOF2 (closely related to
AtMYB117/LOF1) expression to study a possible role for AtMYB117/
LOF1 in the development of reproductive organs.

Results

Isolation and morphological characterization of ctf
mutants

To isolate Arabidopsis mutants with alterations in pistil and fruit
development we screened approximately 5000 activation-tagged
primary lines and identified a mutant which was named ctf,
according to its morphological characteristics, that is, a small and
wrinkled fruit (Figure 1A). T2 plants segregated for the constricted
fruit characteristic in a 1:2:1 ratio, suggesting a single insertion
locus. The fruit phenotype initially observed in heterozygous
plants became more severe in homozygous individuals, indicating
that the phenotype is determined by a semidominant allele
(Figure 1A), which is consistent with the overexpression of a gene
caused by CaMV 35S enhancers. Other remarkable characteris-
tics of homozygous ctf fruits were that the replum was wider and
the stigmatic papillae were more elongated (Figure 1B). The cells
of ctf style did not have the wax crenulations present in the wild
type style cells, and ctf style was composed of small and smooth
cells that did not have visible stomata (Figure 1B). ctf plants also
showed alterations in leaf, inflorescence and flower development.
Flowers had an altered morphology with abnormal separated
organs and upward curled leaves in the rosette (Figures 1C and D).
In addition, ctf plants were smaller than wild type with short
internodes and bushy appearance due to the development of
multiple stems and loss of apical dominance (Figure 1E).

Figure 1. Phenotype of the ctf mutant. (A) Fruits from wild type Ler and heterozygous (ht) or homozygous (hm) ctf mutants. (B) Scanning
electron micrograph of Ler and ctf mutant pistil at floral stage 12. (C) Flower from Ler and ctf mutant. (D) ctf plants showing upward curled rosette
leaves. (E) Wild type (right) and ctf mutant (left) plants. ctf and Ler plants were 8 weeks old. r, replum; s, stoma. Scale bars are 100 μm.
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Transverse sections of homozygous ctf fruit revealed an irregular distribution of seeds, an enlarged replum and an amorphous structure of the septum (Figures 2A–D). Frequently, the irregular septum filled almost the whole cavity of the fruit (Figure 2B). Longitudinal sections of Landsberg erecta (Ler) pistils showed the septum in the centre and a row of ovules in each locule (Figures 3A and C). In contrast, the ovules in ctf pistils were not organized in rows (Figures 3B, D and E), but were piled up on each other, which caused a distortion in the septum linearity. Whole-mount cleared ovaries were examined to compare the morphology of Ler and ctf ovules. Compared to wild type, the ovules in ctf ovaries developed closer to each other. The distance between ctf funiculi was smaller than between Ler funiculi (Figures 3F and I), and the ovules were forced to be distributed at various levels, as they did not fit in the same plane, possibly explaining why a row of ovules per carpel was not observed in ctf sections. In addition, ovules and seeds in ctf mutant were slightly larger than those in Ler plants (Figures 3F–I).

To thoroughly study the ctf flower morphology, ctf inflorescences were observed by cryo-scanning electron microscopy (cryo-SEM). ctf flowers in anthesis from 6-week old plants generated ectopic carpelloid structures (Figures 4A, C, E, G) that were not visible in flowers of Ler and younger ctf plants (Figure 4B). Stigmatic papillae (Figures 4D, F, H) and style-like regions (Figure 4F) developed on the edges of the sepals. The cells in these regions showed typical wax crenulations of style cells. The carpelloid sepals often appeared folded and, on the inner side, ovule-like structures developed (Figures 4D and H). Seventy-three percent (11/15) of analyzed ctf plants exhibited flowers with carpelloid sepals, while wild type Ler plants did not show this phenotype (data not shown).

To better characterize the carpelloid structures observed in sepals of ctf flowers, we searched for the presence of SPATULA (SPT) transcripts. SPT is a transcription factor that promotes growth of tissues arising from the carpel margins, including ovules, septum and transmitting tract, as well as style and stigma [12]. ctf plants were crossed with Ler plants carrying pSPT-6253:GUS, the SPT promoter region fused to a GUS reporter gene [13]. In ctf sepals, GUS expression was recorded in stigmatic papillae, ovules and in the edges of the sepals where the ovules arise (Figures S1A and B). These blue edges consisted of typical cells of transmitting tissue (Figure S1C). These cells could not be identified in the cryo-SEM images.

**Molecular characterization of ctf mutants**

Plasmid rescue revealed that the T-DNA containing the CaMV 35S enhancer in ctf plants was inserted into chromosome 1 between At1g26770 and At1g26780, the two immediately adjacent genes that are transcribed in the same orientation (Figure S2). The expression of At1g26770 and At1g26780 and three other proximal genes, At1g26760, At1g26790 and At1g26795, was analyzed by qRT-PCR. Of the five genes, only the expression of At1g26780 was highly increased in ctf mutants relative to wild type plants. At1g26780 encodes the transcriptional factor AtMYB117, a member of the R2R3 MYB gene family (subgroup 21) from Arabidopsis [8,9].

To confirm that the ctf phenotype was conferred by the activated expression of AtMYB117/LOF1, the AtMYB117/LOF1

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**Figure 2. Histological analysis of fruits in ctf.** Paraffin cross sections of Ler (A) and ctf (B) fruits stained with Alcian blue and Safranin O at late stage 17. Cryo-scanning electron micrographs of transverse sections of Ler (C) and ctf (D) fruits at early stage 17. f, funiculus; r, replum; sp, septum. Scale bars are 200 μm in A and B, and 300 μm in C and D.

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cDNA was introduced into Ler wild type plants under the control of the CaMV 35S promoter. Homozygous T2 lines of transgenic plants resembled ctf mutants with mild to severe phenotypes. Intensity of phenotype was correlated with the level of expression of the transgene, where plants with severe ctf phenotype had the highest level of AtMYB117/LOF1 transcript (data not shown).

Localization of the expression of AtMYB117/LOF1 gene in Ler plants

Lee et al. [10] analyzed the AtMYB117/LOF1 spatial expression using the enhancer-trap line ET4016. GUS activity was restricted to adaxial boundary regions and the base of floral organs. To confirm whether the ctf phenotype was caused by overexpression of AtMYB117/LOF1, we examined the expression of AtMYB117/LOF1 by RNA in situ hybridization in vegetative and reproductive tissues of Ler and ctf plants.

AtMYB117/LOF1 transcripts were detected throughout all stages of development in Ler plants. In young seedlings AtMYB117/LOF1 transcripts were observed at the boundaries between the vegetative SAM and leaf primordia (Figure 5A). On longitudinal sections through the inflorescence stem, AtMYB117/LOF1 transcripts were detected in a group of cells in the adaxial base of petioles and bracts (Figure 5B). Figure 5C shows floral meristems at stages 1–3 [4] arising on the flank of the IM. AtMYB117/LOF1 was expressed at the boundaries between the IM and floral meristems. This signal was maintained and later observed in the adaxial-basal side of petals (Figures 5B and C). Floral organ primordia are formed on the floral meristem where AtMYB117/LOF1 transcripts were observed at the boundary between sepal primordia and the floral meristem at stage 3 (Figure 5C). AtMYB117/LOF1 transcripts were detected at the boundaries between each of the four whorls (sepals, petals, stamens and pistil) beginning at floral stages 6 and 7 and continuing until stage 10 (Figure 5D). At stage 7, AtMYB117/LOF1 transcripts were also detected at the inner of the medial ridges in the pistil (Figure 5D). At stage 9, expression was observed in the placenta (Figure 5E) and in the base of the ovule primordia (Figure 5F). Later, signal became restricted to the epidermis of the developing septum that is derived from the medial ridge and to the outer cell layers of the ovule funiculi (Figures 5G–I). AtMYB117/LOF1 transcripts were not detected beyond stage 11.

Localization of the expression of AtMYB117/LOF1 gene in ctf plants

In ctf mutants, the expression of AtMYB117/LOF1 was expanded spatially and temporally relative to wild type Ler plants (Figure 6). The most remarkable differences were: 1) in seedlings, expression was observed in the adaxial side of hyponomous young

Figure 3. Morphological characteristics of ovules and seeds in ctf. Resin longitudinal sections of a Ler (A) and ctf (B) in anthesis. The transmitting tissue was stained with Alcian blue. Close-up view of Ler (C) and ctf (E) ovules, septum and transmitting tissue. Paraffin longitudinal section of ctf pistil in anthesis stained with Toluidine blue (D). Whole-mount cleared ovules and funiculi of a Ler (F) and ctf (I) pistil in anthesis. Note the distance between funiculi (stars indicate close funiculi and dotted lines mark the distance between separated funiculi). Ler (G) and ctf (H) seeds. f, funiculus; ov, ovule; sp, septum; tt, transmitting tissue; v, valve. Scale bars are 200 µm in A, B, F and I; 100 µm in C and E; and 500 µm in G and H.
leaves (Figure 6A); 2) in longitudinal sections of inflorescences, the signal filled the whole inflorescence meristem and stem (Figure 6B); 3) during flower development, expression was observed in the adaxial side of sepals and in cells around the stomium in stamens (Figure 6C); 4) in pistils, expression was localized to maturing ovules, the septum area close to the replum, the adaxial side (inner layers) of the valves (Figure 6D) and the style cells (Figures 6E, F). The signal was observed at least up to stage 13 (data not shown).

Analysis of expression of pistil development genes in ctf

The morphological characteristics of ctf fruit suggest that some of the genes that are known to control fruit development could be up or down-regulated in ctf mutants. To test this hypothesis, we analyzed the expression in inflorescences of SHATTERPROOF1 (SHP1) and SHP2, that specify valve margin identity and promote ovule, stigma, style and medial tissue development [14,15]; REPLUMLESS (RPL), that represses valve and valve margin development [16]; FRUITFULL (FUL), that is involved in valve cell development [17]; BREVIPEDICELLUS (BP), that was implicated in replum and valve margin development and ASYMMETRIC LEAVES1 (AS1) and AS2, that regulates mediolateral patterning of the fruit [18]. qRT-PCR analysis showed that only SHP1 and SHP2 transcript levels were remarkably increased in ctf (Figure 7A). SHP2-directed GUS expression, localized to valve margin in wild type Ler plants (Figure 7B), was extended to the whole pistil and also to sepals in ctf fruit (Figure 7C). The increased
expression in sepals correlated with the presence of carpelloid structures bearing ovules.

**AtMYB117/LOF1 silencing by amiRNA**

*lof1-1*, a T-DNA insertion mutant of *AtMYB117/LOF1*, had only a slight reduction of *AtMYB117/LOF1* transcript levels in inflorescence apex [10]. Therefore, as an alternative approach, we tried silencing *AtMYB117/LOF1* expression using an amiRNA strategy in Ler and Col-0 ecotypes. *AtMYB117/LOF1* is closely related to *AtMYB105/LOF2*, and both function redundantly in boundary formation [10]. The possibility that *AtMYB117/LOF1* and *AtMYB105/LOF2* could also have redundant function in reproductive tissues and organs, prompted us to generate an amiRNA against both genes according to the described criteria [11]. amiRNA117/105 plants were tested for expression levels of both target genes in inflorescences by qRT-PCR analysis. Several transgenic lines were selected according to *AtMYB117/LOF1*-amiRNA117/105 expression levels and were brought to the homozygous state. Expression levels of these lines are shown in Figure S3. The amiRNA silenced both endogenous *AtMYB117/LOF1* and *AtMYB105/LOF2* expression with stronger effects observed in Ler than in Col-0 ecotypes. However, none of the lines exhibited defects in inflorescence structure, and the observed phenotypes in the paracleade junctions in amiRNA117/105 plants were very similar to those described for *lof1-1* plants [10] (Figure S4). Funiculi with alterations in cellular morphology were also observed, but only for a few amiRNA117/105 plants (Figure S5).

**Discussion**

We have isolated a gain-of-function mutant, named *ctf*, characterized by alterations in fruit morphology. It showed wrinkled valves, stigma with more prominent papillae, atypical style cells, wider replum, septum with an irregular structure and stacked seeds. The overexpressed gene was identified as *AtMYB117*, and overexpression of *AtMYB117* cDNA in *Arabidopsis* Ler plants recapitulated the *ctf* phenotype. This gene, also named *LOF1*, was found to have a role in lateral organ separation and axillary meristem formation [10]. In *ctf* pistil and fruit our results demonstrate that *AtMYB117/LOF1* is expressed in the same tissues as in wild type fruit, but with higher intensity, and also in other tissues such as the style, inner layers of valves and mature ovules. Therefore, the alterations in fruit tissues might be explained by changes in the intensity of expression of *AtMYB117/LOF1*. CaMV 35S enhancers lead primarily to an enhancement of endogenous expression patterns, the resulting phenotype being a consequence of such an enhancement, as opposed to ectopic overexpression, reflecting the normal role of the activated gene [6].

A remarkable characteristic of *ctf* was the presence in sepals of ectopic carpelloid structures, including stigma, style, transmitting tissue and ovules. The identity of the ectopic tissues in *ctf* was confirmed by GUS activity directed by the *SPT* promoter, revealing the presence of transmitting tissue, not visible by cryo-SEM. Previous studies had shown that ectopic expression of *SHP1* and *SHP2*, involved in ovule, stigma, style and medial tissue development [15], is sufficient to induce the transformation of sepals into carpelloid organs bearing ovules [19,20]. The formation of ectopic carpelloid structures in *ctf* sepals could be explained by the effect of the enhancement of *AtMYB117/LOF1* expression on the regulation of *SHP1* and *SHP2*. Increased expression of *SHP1* and *SHP2* and an extended localization of *SHP2*:GUS activity to the whole pistil and sepals was observed in *ctf* plants, while it is normally localized to the valve margins in wild type Ler plants.

The *AtMYB117/LOF1* expression pattern obtained by RNA in situ hybridization extends the results obtained by Lee et al. [10],
using an enhancer-trap line, in regard to the role of AtMYB117/LOF1 in the establishment of organ boundaries. These authors detected AtMYB117/LOF1 expression in the adaxial boundary regions between the SAM and lateral organs during vegetative development and between the inflorescence meristem and flower primordia. We also observed AtMYB117/LOF1 transcripts in the adaxial boundary regions between each of the floral organs, in the boundaries of ovule primordia where they derive from the placenta, in the sepal and in the region of ovule primordia that will give rise to the funiculus. To examine a possible role of AtMYB117/LOF1 in the development of ovules, we used amiRNA to obtain a knock-down mutant. Total silencing of AtMYB117/LOF1 and AtMYB105/LOF2 genes in the inflorescence was not achieved in any of the amiRNA117/105 lines. In spite of the low levels of AtMYB117/LOF1 and AtMYB105/LOF2 transcripts, the flowers and fruits did not present clear morphological changes. However, we did observe alterations in cellular morphology in the funiculus of some plants. This result, together with the observations mentioned above on AtMYB117/LOF1 expression and the putative genetic relationship with SHP1 and 2, might suggest a functional role for AtMYB117/LOF1 in funiculus/ovule development. Further studies will be needed to understand if the AtMYB117/LOF1 gene is essential in ovule initiation and development and if SHP1 and SHP2 can be directly or indirectly regulated by the AtMYB117/LOF1.

AtMYB117/LOF1 has a similar expression pattern to that of lateral organ boundary genes like CUC2, a member of the NAC family of transcription factors [21], and JLO/LBD30, a member of the LBD gene family [22]. Like AtMYB117/LOF1, these boundary genes are also expressed in the base of ovule primordia. NAC, LBD, and GRAS gene families have been identified as regulators involved in the definition of the boundaries of lateral organ regions [23]. Some LBD proteins can interact with other partners bearing MYB domains, such as AtAS2/LBD6 and AtASL4/AtLOB, which interact with AtAS1/AtMYB91. Therefore, AtMYB117/LOF1 might be a partner in a multiprotein complex, with a role in different steps and processes of plant development involving lateral organ formation. Fine-scale regulation of developmental processes could be modulated by differential regulation of one or more components, allowing for a great degree of plasticity.

Concluding remarks

The characterization of the ctf mutant, the phenotype of amiRNA117/105 plants and the localization of AtMYB117/LOF1 transcripts in boundary regions throughout different stages of development in Arabidopsis, including the initiation of ovule...
outgrowth, suggest that this gene is recruited in a variety of vegetative and reproductive developmental programs for the establishment of boundary regions and for funiculus/ovule development.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana Landsberg erecta (Ler) and Columbia-0 (Col-0) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC, www.biosci.ohio-state.edu). T-DNA insertion line, lof-1 (N525235) in the Col-0 ecotype, was obtained from NASC, the European Arabidopsis Stock Center (http://arabidopsis.info/), and homozygous mutants were selected by PCR-based genotyping. Sequences of genotyping primers used are available upon request. The SHP2::GUS reporter line [24] was kindly provided by Cristina Ferrándiz (IBMCP, Spain) and the pSPT-6253::GUS reporter line [13] by David Smyth (Monash University, Australia). Arabidopsis seeds were surface-sterilized, sowed in MS [25] plates and stratified at 4°C for 3 days in darkness. Seed were germinated by incubation in growth chambers at 22°C under a 16-h light/0-h dark photoperiod for one week, and seedlings were then transferred to soil and grown to maturity in the same growth conditions.

Generation of activation-tagged transgenic plants

Arabidopsis transformation was performed as previously described using the activation-tagging binary vector plasmid pSIKO15 [6]. This plasmid was introduced into GV3101 Agrobacterium tumefaciens cells and plants were transformed via the floral dip method [26]. Transformed seedlings were selected in plates of MS supplemented with 330 μM ammonium glufoximate (HuKa). The screening was done by morphological observations throughout the development of the transformed plants for phenotypes such as fruit length and shape. Homozygous and heterozygous plants were identified by genotype analysis by PCR (Table S1).

Identification of the localization of the T-DNA insertions

The T-DNA insertion point in the ctf mutant was located by the plasmid rescue method [6]. Approximately, 1 μg of total genomic DNA was digested overnight with EcoRI. The digested DNA was ethanol precipitated, ligated overnight with T4 DNA ligase (New England Biolabs), and transformed into E. coli DH5α competent cells by electroporation. Ampicillin-resistant colonies were selected, and the rescued plasmids were purified, sequenced, and compared with the published Arabidopsis genome to determine T-DNA/genome junctions. qRT-PCR was carried out to test the expression level of genes around the predicted insertion point.

Recapitulation analysis

The full-length AtMYB117/LOF1 cDNA was isolated by PCR using gene-specific primers [11]. The corresponding artificial microRNA (amiRNA) approach

To knock down both AtMYB117/LOF1 and AtMYB105/LOF2, an amiRNA was generated as described in [11]. The corresponding amiRNA was PCR amplified (Table S1) according to the protocol at Web MicroRNA Designer2 (WMD2; http://wmd2.weigelworld.org) and cloned into the pENTR Directional TOPO vector (Invitrogen). The resulting clones were confirmed by restriction analysis and sequencing. cDNA was transferred to the destination vector pK2GW7 under the control of the CaMV 35S promoter (Plant Systems Biology, VIB-Ghent University, Belgium) for over expression in plants [27]. Destination plasmid was transformed to Agrobacterium tumefaciens GV3301 strain to transform Ler plants via the floral dip method. Transformed plants were selected on MS plates with 50 μM kanamycin. AtMYB117/LOF1 expression level in transformed plants was measured by qRT-PCR with specific primers (see below).

Gene expression analysis by qRT-PCR

Plant tissues were harvested, frozen with liquid N2, and stored at −80°C. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Genomic DNA was eliminated with 50 units of DNeasy (Qiagen) for 15 min at room temperature. cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). qRT-PCR analysis was carried out using the SYBR® GREEN PCR Master Mix (Applied Biosystems) in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as described in [29]. Primer sequences are indicated in the Table S1. In a single experiment, each sample was assayed in triplicate and the experiment was repeated twice, with similar results. ACT8 (At1g9240) and PPA2 Ser/Thr protein phosphatase 2A (At1g13320) were used as reference genes for normalization [30].
In situ hybridization

Seedlings and inflorescences were embedded, sectioned and hybridised as described [31]. Two different templates for AtMYB117/LOF1 were generated, a fragment of 223 bp containing the 5’ region of cDNA and an 847 bp fragment corresponding to the whole coding region of the AtMYB117/LOF1 cDNA. The short probe was specific for the endogenous AtMYB117/LOF1 gene and did not show any sequence similarity with AtMYB105/LOF2. Both fragments were cloned into the pGem-Teasy vector (Promega), and sense and antisense probes were synthesized using the corresponding SP6 and T7 RNA polymerases. After verifying that the two antisense probes gave identical results, we used the longer probe because it generated a more intense signal. Control experiments were performed with sense probes of AtMYB117/LOF1 and no significant signal was detected (data not shown).

Histological procedures

Plant tissues were fixed overnight in 4% (w/v) p-formaldehyde in 0.1 M sodium phosphate pH 7.2 with 0.05% (v/v) of Tween 20 at 4°C, dehydrated, and embedded in paraffin wax (Paraplast Plus) or Technovit 7100 resin as described [32]. Samples embedded in paraffin were sectioned on a rotary microtome at 8 μm and stained with 1% Alcian blue 8GX and 1% Safranine O in 50% ethanol or 0.02% Toluidine blue. Tissues embedded in resin were sectioned in a Reichert Jung Ultracut E microtome at 3 μm and stained with 1% Alcian blue 8GX. Pistils from Ler and ctf plants were harvested at anthesis, fixed, dehydrated and cleared with chloral hydrate according to [33]. Images were captured using a microscope Eclipse E600 (Nikon) equipped with Nomarski interference optics.

GUS staining

To monitor SPT and SHP2 expression, the pSPT-6253:GUS and SHP2-GUS reporter lines were crossed to ctf mutant. T2 segregants were genotyped for ctf and homozygous plants were stained for GUS activity. Inflorescences were fixed 30 min in 90% acetone, washed in staining buffer (50 mM sodium phosphate pH 7.0, 10 mM potassium ferricyanide, 10 mM potassium hexacyanoferrate, and 0.2% Triton X-100), and incubated overnight at 37°C in staining buffer supplemented with 0.1 mM X-GlcA (5-bromo-4-chloro-3-indolyl-b-D-glucuronide cyclohexylammonium). Samples were dehydrated to 70% (v/v) ethanol and observed in a stereoscopic microscope (Leica MZ16) and an Eclipse 600 microscope (Nikon) equipped with Nomarski interference optics.

cryo-SEM

Samples were harvested, mounted on SEM stubs attached to the specimen holder of a CT-1000C cryo-transfer system (Oxford Instruments) and frozen in liquid N2. The frozen specimens were transferred to the cryo-stage of a JEOL JSM-5410 scanning electron microscope, sublimated by controlled heating at −85°C and sputter coated with a thin film of gold. Finally, samples were observed at incident electron energy of 10 keV.

Supporting Information

Figure S1 Localization of SPATULA gene expression in ctf flowers. GUS expression driven by the SPT promoter in Ler (A) and ctf (B) flowers. Enlargement of the boxed carpelloid structure observed by Nomarski technique (C), pi, pistil, ov, ovule; se, sepal; tt, transmitting tissue. Scale bars are 100 μm. (TIF)

Figure S2 Localization of the insertion point of the T-DNA in the ctf mutant. (A) Detail of the genome region, showing the genes up- and down-stream of the insertion; overexpressed gene (Atf26780 AtMYB117/LOF1) in the ctf mutant is encircled. (B) Scheme of the activation tagging T-DNA and position of the restriction sites used for plasmid rescue. (TIF)

Figure S3 AtMYB117/LOF1 and AtMYB105/LOF2 expression analysis by qRT-PCR. Relative gene expression in inflorescence of amiRNA117/105 lines in ecotypes Ler and Col-0. Each experiment was carried out with three technical replicates and was repeated twice with similar results. Data (expression normalized to ACT8 and relative to the expression of the inflorescence of Ler or Col-0 in each case) are mean ± SD of a single experiment. (TIF)

Figure S4 Phenotypes of lof1-1 and amiRNA117/105 plants. Col-0 and lof1-1 plants (A). Ler and amiRNA117/105 (line 12) plants (B). Note fused paraclade junctions in both mutant plants (red arrows). (TIF)

Figure S5 Morphological characteristics of funiculi in amiRNA117/105 fruits. Cryo-scanning electron micrographs of funiculi of Ler and amiRNA117/105 fruits (A-D) at late stage 17. Scale bar is 50 μm. (TIF)

Table S1 Primers used in this work. (DOC)

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Author Contributions

Conceived and designed the experiments: MDG CU MAPA JC. Performed the experiments: MDG CU MAPA. Analyzed the data: MDG CU MAPA. Contributed reagents/materials/analysis tools: MDG CU. Wrote the paper: MDG CU JC.

References

1. Girin T, Sorefan K, Ostergaard L (2009) Meristematic sculpting in fruit development. J Exp Bot 60: 1493–1502.
2. Kelley DR, Gasser GS (2009) Ovule development: genetic trends and evolutionary considerations. Sexual plant reproduction 22: 229-234.
3. Aida M, Tasaka M (2006) Morphogenesis and patterning at the organ evolutionary considerations. Sexual plant reproduction 22: 229–234.
4. Roeder A, Yanofsky M (2006) Fruit development in Arabidopsis. The Arabidopsis book. Rockville, MD: The American Society of Plant Biologists.
5. Balanza V, Navarrete M, Trigueros M, Ferrandiz C (2006) Patterning the female side of Arabidopsis: the importance of hormones. J Exp Bot 57: 3457–3469.
6. Weigel D, Ahn JH, Blázquez MA, Borevitz JO, Christensen SK, et al. (2000) Activation tagging in Arabidopsis. Plant Physiology 122: 1003–1013.
7. Kurokumi T, Takahashi S, Kondou Y, Shinozaki K, Matsui M (2009) Phenome analysis in plant species using loss-of-function and gain-of-function mutants. Plant Cell Physiol 50: 1215–1221.
8. Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in Arabidopsis thaliana. Curr Opin Plant Biol 4: 447–456.
9. Dubos C, Stracke R, Grosewold E, Weisshaar B, Martin C, et al. (2010) MYB transcription factors in Arabidopsis. Trends Plant Sci 15: 373–381.
10. Lee DK, Geiser M, Springer PS (2009) LATERAL ORGAN FUSION1 and LATERAL ORGAN FUSION2 function in lateral organ separation and axillary meristem formation in Arabidopsis. Development 136: 2425–2432.
11. Schwab R, Osowski S, Rieger M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell 18: 1121–1133.

12. Heisler MG, Atkinson A, Bylstra YH, Walsh R, Smyth DR (2003) SPATULA, a gene that controls development of carpel margin tissues in Arabidopsis, encodes a bHLH protein. Development 128: 1089–1098.

13. Groszmann M, Bylstra Y, Lampugnani ER, Smyth DR (2010) Regulation of tissue-specific expression of SPATULA, a bHLH gene involved in carpel development, seedling germination, and lateral organ growth in Arabidopsis. J Exp Bot 61: 1495–1508.

14. Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, et al. (2000) SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. Nature 404: 766–770.

15. Colombo M, Brambilla V, Marcheselli R, Caporali E, Kater MM, et al. (2010) A new role for the SHATTERPROOF genes during Arabidopsis gynoecium development. Dev Biol 337: 294–302.

16. Roeder AH, Ferrandiz C, Yanofsky MF (2003) The role of the REPLUMLESS homeodomain protein in patterning the Arabidopsis fruit. Curr Biol 13: 1630–1635.

17. Gu Q, Ferrandiz C, Yanofsky MF, Martienssen R (1998) The fruitfull mads-box gene mediates cell differentiation during arabidopsis fruit development. Development 125: 1509–1517.

18. Alonso-Blanco C, Ripoll J Jr, Ochando I, Vera A, Ferrandiz C, et al. (2007) Common regulatory networks in leaf and fruit patterning revealed by mutations in the Arabidopsis ASYMMETRIC LEAVES1 gene. Development 134: 2663–2671.

19. Favarov R, Pinyopich A, Battaglia R, Kosuker M, Borghi L, et al. (2003) MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell 15: 2603–2611.

20. Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, et al. (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424: 85–88.

21. Ishida T, Aida M, Takada S, Tatsaka M (2000) Involvement of CUP-SHAPED COTYLEDON genes in gynoecium and ovule development in Arabidopsis thaliana. Plant Cell Physiol 41: 60–67.

22. Boergli L, Bureau M, Simon R (2007) Arabidopsis JAGGED LATERAL ORGANS is expressed in boundaries and coordinates KNOX and PIN activity. Plant Cell 19: 1795–1808.

23. Majer C, Hochholdinger F (2011) Defining the boundaries: structure and function of LOB domain proteins. Trends Plant Sci 16: 47–52.

24. Savidge B, Rounsley SD, Yanofsky MF (1995) Temporal relationship between the transcription of two Arabidopsis MADS box genes and the floral organ identity genes. Plant Cell 7: 721–733.

25. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497.

26. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743.

27. Karimi M, Inze D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7: 193–195.

28. Bensmihen S, To A, Lambert G, Krej T, Giraudat J, et al. (2004) Analysis of an activated AB5 allele using a new selection method for transgenic Arabidopsis seeds. FEBS Lett 561: 127–131.

29. Dorcy E, Urbez C, Blazquez MA, Carbonell J, Perez-Amador MA (2009) Fertilization-dependent auxin response in ovules triggers fruit development through the modulation of gibberellin metabolism in Arabidopsis. Plant J 58: 318–332.

30. Czechowski T, Stitt M, Ahmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139: 5–17.

31. Ferrandiz C, Sessions A (2002) Nonradioactive in situ hybridization. In Arabidopsis: A laboratory Manual. Weigel D, Glazebrook J. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. pp 195–203.

32. Gomez MD, Beltran JP, Canas LA (2004) The pea END1 promoter drives author-specific gene expression in different plant species. Planta 219: 967–981.

33. Christiansen S (2002) Cleared tissue for observation of vascular strands. In Arabidopsis: A laboratory Manual. Weigel D, Glazebrook J. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. pp 194–195.