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Manuel Ángel Piñeiro and José Antonio Jarillo, researchers at the Centro de Biotecnología y Genómica de Plantas UPM-INIA declare that the work contained in the PhD thesis titled: "Insights into the regulation of reproductive development in *Arabidopsis thaliana* by the chromatin protein EBS" by Dorota Natalia Komar:

1) has been carried out under our supervision;
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3) can be presented and defended in order to obtain a PhD degree;
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Madrid, …… 2018

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Manuel Ángel Piñeiro Galvín José Antonio Jarillo Quiroga

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This book is not only a detailed report of my PhD work. Behind the charts and scientific phrases, a totally different story can be found. A story of laughs and cries, hopes and disappointment, successes and failures. A story of meeting wonderful people, and not so great ones, of gaining new friends and being departed from the old ones. Of thousands skype talks, and billions missed ones, of difficult decisions and obvious choices. A story of learning: new skills, your limits, emotions known only from literary works. I truly hope that all the people that accompanied me through this journey know their role, but since traditionally this is a space to awkwardly express your gratitude, I will do it in here as well. And I am sorry, it will be cheesy.

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ABSTRACT

Plant development progresses through distinct phases including embryonic, vegetative, and reproductive, eventually followed by seed set and senescence. These developmental stages are characterized by specific spatiotemporal patterns of gene expression so that selective activation or silencing of genes enables differentiation of characteristic cell types for each developmental stage. The developmental switch from vegetative to reproductive growth is one of the most dramatic changes in plant's life, as successful reproduction requires not only the formation of the flowers, but also the change of plant architecture traits like internode elongation and branching to ensure the balance between optimal number of flowers and production of resources through photosynthesis. For that reason, the different phases of plant reproductive development are tightly regulated.

Chromatin organization is a key element in the regulation of gene expression and is responsible for on/off transcriptional states of genes in eukaryotic organisms. The Arabidopsis locus *EARLY BOLTING IN SHORT DAYS* (*EBS*) encodes a transcriptional regulator that is required for repression of the floral integrator gene *FT*. In addition, EBS specifically recognizes H3K4me2/3 marks through the PHD domain, and mutations in the *EBS* gene cause an increase in the levels of histone H3 acetylation in the regulatory sequences of *FT*. Furthermore, EBS binds regulatory regions of this master gene of flowering and interacts with histone deacetylases such as HDA6. Altogether these observations indicate that EBS is crucial to maintain a transcriptionally inactive state in the chromatin of its target genes.

In this work we have explored the role of EBS in the control of additional aspects of reproductive development in Arabidopsis. Following a detailed phenotypic analysis of the *ebs* mutant, we have unveiled the involvement of EBS in plant architecture control after bolting, floral meristem maintenance and function, as well as flower development and floral organ patterning. We have also characterized the impact of environmental conditions (temperature, photoperiod) on the expressivity of these traits. In addition, we have determined changes in Arabidopsis transcriptome that occur in the absence of a functional EBS protein, and propose *FT*, *FLC*, *AGL24*, *LFY*, *TFL1* and *SEP3* as candidate genes to mediate the phenotypic alterations observed in *ebs*. We have also analyzed the impact of EBS on chromatin features of these loci and concluded that the mechanism of *FT* repression mediated by EBS could also operate at least in some of these genes. However, additional mechanisms mediated by EBS regulating chromatin compaction and accessibility may occur. Finally, we have carried out genetic analyses to understand possible interactions between those candidates and *EBS*, making possible to elaborate working hypotheses to explain how these genes could mediate the effect of EBS on the control of inflorescence and flower production in Arabidopsis.

Altogether, the data presented in this work reveal that EBS represents a central hub in the control of reproductive growth, orchestrating the expression of different genes that need to be coordinately expressed to enable successful completion of the Arabidopsis developmental plan and ensure adequate production of fruits and seeds to safeguard efficient transmission of genetic information to the following generation.

RESUMEN

El desarrollo vegetal atraviesa por diversas fases incluidas la embrionaria, la vegetativa y la reproductiva, seguidas de la maduración de las semillas y senescencia. Estos estadios de desarrollo presentan patrones espacio-temporales de expresión génica característicos, de manera que la activación o el silenciamiento selectivo de genes permite la diferenciación de tipos celulares específicos para cada fase. El cambio de crecimiento vegetativo a reproductivo es una de las transiciones más dramáticas que tienen lugar en el ciclo de vida de las plantas, ya que requiere no solo la formación de flores sino también de cambios en caracteres relacionados con la arquitectura de la planta tales como la elongación de entrenudos y el patrón de ramificación para asegurar un balance óptimo entre el número de flores y la producción de recursos a través de la fotosíntesis. Por ello, las diferentes fases del desarrollo reproductivo vegetal están finamente reguladas.

La organización de la cromatina es clave en la regulación de la expresión génica y es responsable de los estados transcripcionales activos o inactivos de los genes en organismos eucarióticos. El locus *EARLY BOLTING IN SHORT DAYS* (*EBS*) de Arabidopsis codifica un regulador transcripcional necesario para la represión del integrador floral *FT*. Además, EBS reconoce específicamente marcas histónicas H3K4me3 a través de un dominio PHD, y mutaciones en el gen *EBS* causan un aumento en los niveles de acetilación de la histona H3 en regiones reguladoras de *FT*. Asimismo, EBS se une a secuencias reguladoras de este gen maestro de la floración e interacciona con histonas deacetilasas como HDA6. Todas estas observaciones indican que EBS es crucial para mantener un estado transcripcionalmente inactivo en la cromatina de sus genes diana.

En este trabajo hemos investigado el papel de EBS en el control de aspectos adicionales del desarrollo reproductivo de Arabidopsis. A través de un análisis fenotípico detallado hemos desvelado la implicación de EBS en el control de la arquitectura de la inflorescencia, así como en el mantenimiento y función de meristemos florales y el desarrollo de flores y órganos florales. Hemos caracterizado también el impacto que tienen diferentes condiciones ambientales (de temperatura y fotoperiodo) en la expresividad de estos caracteres. Además, hemos analizado los cambios transcripcionales globales que ocurren en Arabidopsis en ausencia de una proteína EBS funcional, y proponemos a los genes *FT, FLC, AGL24, LFY, TFL1* y *SEP3* como candidatos a mediar las alteraciones fenotípicas observadas en el mutante *ebs*. Hemos estudiado también el impacto de EBS en las características de la cromatina en estos loci. Esto nos ha permitido concluir que el mecanismo de represión de *FT* mediado por EBS podría funcionar también al menos en algunos de estos genes. Sin embargo, podrían existir mecanismos adicionales por los que EBS también modula la compactación y la accesibilidad de la cromatina. Finalmente, hemos llevado a cabo análisis genéticos para comprender las posibles interacciones entre esos genes candidatos y EBS, posibilitando la generación de hipótesis de trabajo que nos permitan explicar cómo estos genes podrían mediar el efecto de EBS en el control del desarrollo de la inflorescencia y las flores en Arabidopsis.

Estos datos revelan que EBS representa un nodo central en el control del desarrollo reproductivo, orquestando la expresión coordinada de genes necesarios para completar el desarrollo en Arabidopsis con el fin de asegurar una producción adecuada de frutos y semillas que garantice la transmisión de la información genética entre generaciones.

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ABBREVIATIONS

ATP: adenosine triphosphate

c-myc: MYC family tag peptide

cDNA: complementary deoxyribonucleic acid

ChIP: chromatin immunoprecipitation

co-IP: co immunoprecipitation

d: day

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

EDTA: ethylenediaminetetraacetic acid

EGTA: egtazic acid

fpkm: fragments per kilobase of transcript per million mapped reads

g: relative centrifugal force

GEO: Gene Expression Omnibus

GFP: Green fluorescent protein

GM: germination medium

GUS: β-glucuronidase

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

KDa: Kilodalton

LD: long day

lncRNA: long non-coding RNA

MOPS: 3-(N-morpholino) propanesulfonic acid

mRNA: messenger ribonucleic acid

MS: Murashige and Skoog/ mass spectrometry

NASC: Nottingham Arabidopsis Stock Center

ncRNA: non-coding RNA

PAGE: Polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

qPCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

SAM: shoot apical meristem

SD: short day

SDS: sodium dodecyl sulfate

SEM: Scanning Electron Microscope

tDNA: transferred DNA

TE: transposon element

TF: transcription factor

Tris: Tris(hydroxymethyl)aminomethane

TSS: transcription start site

UTR: untranslated region

WT: wild type

INTRODUCTION

Plant development and architecture is driven by meristems that initiate lateral organs on their flanks. Plant meristems are formed by groups of undifferentiated cells that divide and subsequently acquire cell identity to generate organs. While the shoot apical meristem (SAM) gives rise to all aerial organs throughout the life cycle of the plant, the root apical meristem is responsible for the generation of the whole root system.

The lifespan of plants is divided into several growth phases including vegetative and reproductive stages. The development of floral organs is crucial for the reproductive success of plant species, as it results in seed production and a new generation of plant individuals. Annual plants progress through a period of vegetative growth at early developmental stages and then transit to the reproductive stage and senescence in the same year. In contrast, perennial plants bear shoot meristems that once have reached sexual maturity, begin repeated transitions between vegetative and reproductive growth through consecutive years. In any case, this transition from vegetative to reproductive growth is strictly controlled and responds to both endogenous and environmental stimuli, particularly those that depend on seasonal change. The tight regulation of flowering time ensures that reproductive organs are formed at the appropriate season and is essential for reproductive success.

The main environmental factors influencing flowering initiation are temperature and day length, also referred to as photoperiod. In a temperate climate zone, a range of flowering times is found, although the majority of species flower between spring and autumn seasons as warm temperature is usually a prerequisite for flowering to occur. Global warming contributes to increased ambient temperature and has severe consequences for flowering. Diverse data suggest that the increase in ambient temperature will advance flowering in late-flowering plants and delay flowering in early-flowering plants (Fitter et al., 1995). Regarding photoperiodic cues, plants can be divided into short day (SD) plants that flower in the autumn when day length is getting shorter; long day (LD) plants that flower in the spring when days are getting longer; and day neutral plants (DN) that are insensitive to day length. Flowering is also sensitive to stress conditions such as drought, heat, cold, nutrient deficiency, pathogen attack, and also cues perceived as changed light quality like shading or overcrowding. Endogenous signals such as plant age and hormones are also very important to ensure that plants flower at maturity, when sufficient resources are accumulated to sustain floral development and seed set (Levy and Dean, 1998; Cerdan and Chory, 2003).

1. Genetic control of reproductive development in Arabidopsis

The model species *Arabidopsis thaliana* has been used in many studies to characterize a number of plant developmental processes, including reproductive development. Recent studies have shed light in the regulation of these processes in other plant species, but since this work was done in Arabidopsis, in this introduction we will mainly focus on the gene regulatory networks that control the transition of a vegetative shoot apical meristem into an inflorescence meristem, together with those necessary to specify floral meristem identity in this model species.

After germination, the seedling goes through a juvenile vegetative phase, where it is not competent to flower. This is followed by the transition to the adult vegetative phase, responsive to floral inductive signals. With the transition to flowering, the plant enters the reproductive phase. During the vegetative phase, the aerial parts of Arabidopsis consist mostly of leaves produced on the flanks of the SAM. The generation of leaves at this stage takes place without internode elongation, giving rise to a rosette. Upon induction of flowering, the formation of reproductive organs and internode elongation (bolting) are triggered, giving rise to the formation of an inflorescence. The shape of an inflorescence varies strongly among different species and has strong implications in reproductive success as it has an effect on pollination, fruit set and seed dispersal (Wyatt, 1982). Arabidopsis inflorescence is an indeterminate (does not produce a terminal flower) raceme. The apical meristem is able to grow (at least theoretically) indefinitely, because the undifferentiated character is kept in the stem cells located in its center and floral meristems continuously develop on its flanks. Each floral meristem gives rise to a separate flower, and flowers undergo self-pollination to create seed-bearing siliques (fruits). The entire life cycle of Arabidopsis is completed within few weeks or months depending on the accession and growing conditions.

The reproductive phase change not only leads to flower production but usually involves multiple other morphogenetic changes, including modifications of leaf shape (heteroblasty) and of plant architecture (Bernier, 1988). The architecture of the inflorescence depends on its branching pattern and on the relative position where flowers are formed. In Arabidopsis the inflorescence architecture is determinated by apical dominance, by which the main apical bud partially inhibits growth of lateral buds. The main, central stem of the plant serves as a pivotal axis for the creation of lateral organs. In the basal part of the inflorescence, cauline leaves and axillary meristems are originated. The apical zone, or shoot apex, bears the potential for indefinite growth, and generates individual flowers. Floral primordia are initiated at the rate of one every time interval, called a plastochron, and at a certain spatial position with constant angle around the stem and relative to the previous primordium. This array provides a clearly established pattern, called phyllotaxy, that determines the arrangement of the lateral organs in the stem. Axillary meristems lead to the formation of secondary branches (lateral stems) while axillary meristems formed on secondary branches lead to the formation of higher order branches. The formation of tertiary branches is very rare in wild type (WT) Arabidopsis plants grown in standard conditions. Beside the main stem, extra rosette stems, also called rosette inflorescences or rosette branches, can emerge. Apical dominance impells hierarchical development: flowers on the main stem mature first, then the maturation of the flowers on secondary branches and rosette stems occur (Alvarez et al., 1992).

1.1 Regulation of flowering time

Different regulatory circuits allow Arabidopsis plants to finely tune the time of flowering in response to environmental signals and endogenous cues. The use of genetic strategies has identified numerous genes involved in these pathways that modulate flowering time. In turn, the activity of these pathways converges at the level of a few so called floral integrators that include *FLOWERING LOCUS T* (*FT)*, *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *AGAMOUS LIKE 24 (AGL24)*.

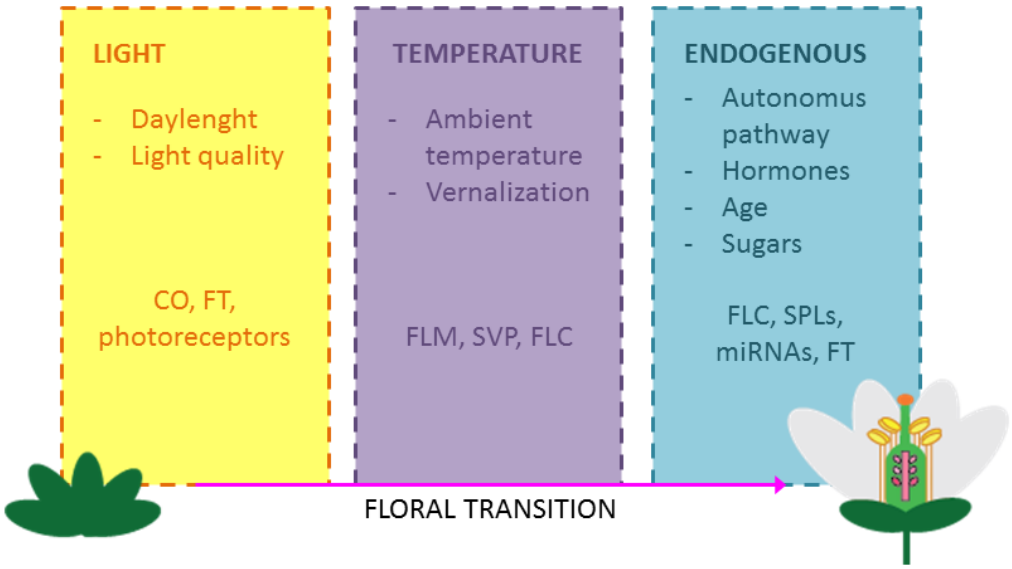

Fig.1. Environmental and endogenous factors controlling the phase transition from vegetative to reproductive state in the model species *Arabidopsis thaliana*. Some of the main players involved are indicated.

1.1.1 Environmental signals

1.1.1.1 Photoperiod

Plants coordinate flowering initiation with the most favorable time of the year by using a reliable indicator of seasonal change such as day length (Andres and Coupland, 2012). Although the flowering of *Arabidopsis* is stimulated by LD, this plant can also eventually flower in SD, making it a 'facultative LD plant'. For a proper photoperiodic flowering response, plant needs both a mechanism that allows sensing the light and an internal time-keeper that makes possible to measure the duration of the light period. The interaction of light sensed by different photoreceptors with the circadian clock, an internal pace-maker that generates an oscillation with a period of nearly 24 hours, allows plants to perceive day length. The gene *CONSTANS* (*CO*) plays a central role in photoperiod perception in Arabidopsis and *CO* expression is regulated by the circadian clock. As a result, *CO* mRNA cycles during the day and displays a different pattern under LD and SD. In inductive photoperiodic conditions, the peak of *CO* expression overlaps with the light period, whereas under non-inductive SD *CO* transcript is only present in the dark. Besides the circadian clock, additional factors participate in the transcriptional regulation of *CO*. In addition to this transcriptional regulation, CO protein stability is controlled by different factors that modulate its selective degradation. CO protein stability is highest in the late afternoon in LD and appears to be regulated by light signals perceived by phyA, phyB, cry1 and cry2 photoreceptors. To regulate CO protein stability, phyA and cry signaling pathways function antagonistically to phyB: phyA and cry signals protect CO protein from degradation whereas phyB signals promote degradation (Imaizumi and Kay, 2006; Shim and Imaizumi, 2015). CO degradation relies on the activity of the proteasome. The complex formed by the RING finger CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and SUPPRESSOR OF PHYTOCHROME A-105 (SPA) proteins degrades CO in the darkness, but this degradation is prevented in the evening by blue light (Jang et al., 2008). The E3

ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) is responsible for the morning degradation of CO (Lazaro et al., 2012).

CO is responsible for the regulation of the expression of the floral integrator genes *FT* and its homologue *TWIN SISTER OF FT* (*TSF*) in the vasculature of the leaves (Jarillo and Piñeiro, 2011). The tight transcriptional and posttranslational regulation causes that in winter, when days are short, the peak of *CO* mRNA occurs in the darkness when CO protein is being degraded. In this situation, there is no CO-dependent *FT* expression and flowering is delayed. In contrast, during spring day length increases sufficiently to make *CO* expression coincide with the light period, then active CO protein is produced, which induces *FT* gene expression and flowering initiation (Imaizumi and Kay, 2006).

1.1.1.2 Ambient temperature

Ambient growth temperatures also shape the timing of flowering. In general, most Arabidopsis accessions flower earlier when grown at higher temperatures, such as 23°C, than at lower temperatures, such as 16°C, while the flowering is further accelerated in temperatures around 27-28 °C (Balasubramanian et al., 2006). The MADS box transcription factor SHORT VEGETATIVE PHASE (SVP) appears to play a crucial role in this pathway because *svp* mutants are insensitive to changes in ambient temperature and flower early at both temperatures (16 °C and 23 °C) (Lee et al., 2007). Another MADS box protein, FLOWERING LOCUS M (FLM) is a key player in the flowering response to ambient temperature and functions as a partner of SVP. *FLM* is alternatively spliced in a temperature-dependent manner. While the FLM-ß variant is preferentially accumulated at low temperatures, the variant FLM-δ is expressed at high temperatures. The DNA-binding heterodimer of FLM-ß with SVP represses flowering by negatively regulating *FT* and *SOC1* expression. Conversely, FLM-δ is proposed to transfer SVP into an inactive complex and thereby indirectly promotes *FT* and *SOC1* expression and, consequently, flowering (Lutz et al., 2015).

Temperature-dependent interactions between nucleosomes and DNA have also been reported. It was shown that nucleosomes containing the histone variant H2A.Z are essential to perceive ambient temperature properly. Arabidopsis genotypes deficient in incorporating H2A.Z into nucleosomes phenocopy warm grown plants and show a constitutive warm temperature transcriptome (Kumar and Wigge, 2010). H2A.Z present in the *FT* promoter is also thermo-responsive. Eviction of H2A.Z-containing nucleosomes from *FT* chromatin at high temperatures promotes *FT* expression by increasing the accessibility of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) to the promoter region facilitating flowering induction (Kumar et al., 2012).

1.1.1.3 Vernalization

Vernalization represents the acquisition of the ability of a plant to flower by exposure to the prolonged winter cold. This reproductive strategy has been developed by many plant species to ensure vegetative growth over winter and reproductive development in favorable spring conditions (Whittaker and Dean, 2017; Hepworth et al., 2018). In Arabidopsis huge variation in response to vernalization exists throughout ecotypes, depending on their origin and the activity of dominant alleles of *FRIGIDA* (*FRI*) (Sanchez-Bermejo and Balasubramanian, 2016). FRI causes upregulation of the floral repressor gene *FLOWERING LOCUS C* (*FLC*) that

encodes another MADS box protein. Vernalization-responsive Arabidopsis accessions have increased *FLC* RNA and protein levels during initial stages of development (Michaels and Amasino, 1999). During winter (or vernalization) VERNALIZATION INSENSITIVE 3 (VIN3) and other PHD-containing proteins together with Polycomb Group (PcG) complexes repress *FLC* expression. This repressed state of *FLC* is maintained during warm periods by epigenetic silencing and is only reset in the following generation after meiosis (Crevillen and Dean, 2011).

In Arabidopsis, there are five close homologs of FLC named MADS AFFECTING FLOWERING (MAF) 1-5 (MAF1 corresponds to the abovementioned FLM). The genes encoding these transcription factors are arranged in tandem with *FLC* as a 22 kb cluster in chromosome 5, except for *FLM*, that is located in chromosome 1. FLC and its homologs seem to interact with each other forming nuclear complexes that act together in the inhibition of flowering (Gu et al. 2013). Interestingly, an FLC-independent vernalization response must exist since *flc* null mutants still have a vernalization-sensitive phenotype (Alexandre and Hennig, 2008). The MADS box transcription factor AGL19 is involved in that process (Schönrock et al 2006), although the downstream targets, except for *FT*, remain unidentified. Recent evidence shows that Arabidopsis histone deacetylase HDA9 regulates flowering time through direct repression of *AGL19* (Kim et al., 2013).

1.1.2 Plant endogenous signals

A number of endogenous pathways function independently of environmental signals to control flowering, and are related to the developmental state of the plant. These endogenous factors ensure that flowering does not occur prematurely, when plants are too young and not enough resources for flowering have been accumulated. These pathways are briefly discussed below.

1.1.2.1 The autonomous pathway

One of the best-known pathways of flowering regulation through endogenous signals is a pathway that reduces *FLC* mRNA in a vernalization-independent way. A number of representative mutants of this pathway such as *fca*, *fld*, *fpa*, *fve*, *fy*, *ld*, and *flk*, flower later than WT plants in all photoperiods due to increased levels of *FLC* expression. Rather than a hierarchical set of activities, the autonomous promotion pathway comprises genes with different biochemical functions that all share *FLC* as a target (Boss et al., 2004). Two sub-pathways have been studied in depth. One of them involves FCA, FPA, FLK and FY proteins, which interact to regulate the processing of *FLC* RNA. The second is responsible for chromatin-mediated regulation of *FLC* expression and involves the putative histone demethylase FLD, which interacts with the histone deacetylase-associated protein FVE and with LUMINIDEPENDS (LD), regulating *FLC* expression through H3 deacetylation and H3K4 demethylation processes (Cheng et al., 2017).

1.1.2.2 Plant hormones

Each of the currently known phytohormones (auxin, cytokinins, gibberellins (GAs), brassinosteroids, ethylene and abscisic acid) has been associated with flowering time control (Davies, 2010). However, a broader description is available only for some of them, like GAs. Flowering is promoted by the application of GA_3, and also in case of mutations causing

constitutive GA signaling (Jacobsen and Olszewski, 1993). This floral promotion is triggered by the activation of genes encoding the floral integrator genes *SOC1*, *FT* and *LEAFY* (*LFY*) (Mutasa-Göttgens and Hedden, 2009). On the other hand, mutations blocking GA signaling (*gai*) or GA biosynthesis (*ga1-3*) delay flowering, particularly under SD (Wilson et al., 1992), consistent with the central role ascribed to this plant hormone in flowering promotion under non-inductive photoperiods. Recent reports have also revealed additional roles for GAs in the activation of flowering in LDs. This hormone promotes the induction of *FT* and *TSF* in leaves and the expression of *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPLs)* genes both in leaves and in the SAM. Interestingly, even though gibberellins promote termination of vegetative growth and floral transition, they inhibit flower formation. GAs action has to be suppressed in later developmental stages through a negative feedback loop with *LFY* (Yamaguchi et al., 2014). The *LFY* gene is primarily activated by gibberellins, but later, the LFY protein activates a gibberellin catabolism gene what results in reduced GAs level. Then, gibberellin-sensitive DELLA proteins can be accumulated and together with SPLs promote the expression of the floral meristem identity gene *APETALA1* (*AP1*) (Yamaguchi et al., 2014).

Cytokinins also participate in the regulation of flowering. They overlap with GA in the induction of flowering, as exogenous application of cytokinins promotes the floral transition (Besnard-Wibaut, 1981). Moreover, they have been proposed to transmit root-to-shoot signals during the initiation of flowering, since application of cytokinins in the roots of plants growing in flowering delaying SD conditions strongly promotes the floral transition in the absence of any extra flowering stimulators, acting mainly at the level of *FD* and the *FT* paralogue *TSF* (D'Aloia et al., 2011).

1.1.2.3 Age pathway

Plants, similarly to animals, have a period of juvenility and the reproduction phase can only occur once the plant has reached an appropriate developmental stage. Two miRNAs: miR156 and miR172, as well as various transcription factors are involved in defining the timing of phase transitions in plants. *MiR156* is highly abundant at the juvenile stage, and its expression decreases progressively with age. This miRNA targets and cleaves the transcripts of particular *SPL* genes inhibiting their expression to prevent the juvenile-to-adult as well as the adult-to-reproductive transition. In contrast to *miRNA156*, *miRNA172* expression increases with age to promote phase transition and flowering initiation by repressing the expression of *AP2*-like genes that function to inhibit flowering initiation through *FT* repression. *SPLs* (at least *SPL9*) are involved in the activation of *miRNA172*. This opposite, but complementary function, suggests a strong regulatory relationship between both miRNAs (Wang, 2014).

1.1.2.4 Sugar signaling

Flowering and seed production are very energy-demanding processes. During recent years, carbon assimilation and sugar status in plants have emerged as very important regulatory elements during flowering (Moghaddam and Ende, 2013; Wingler, 2018). Arabidopsis plants deficient in the trehalose-6-phosphate-synthesizing enzyme TREHALOSE-6-PHOSPHATE SYNTHASE 1 (TPS1) flower extremely late. This enzyme is required for the expression of *FT* in leaves. Moreover, TPS1 reduces *miR156* expression, and consequently promotes the induction of flowering through SPL proteins (Wahl *et al.,* 2013). Additional studies are still

required to further clarify the role of sugar signaling in the regulation of reproductive development.

1.2 Floral integrators

The broad input information about internal factors and environmental cues that influence flowering time is integrated at the level of the expression of flowering master genes. Three of them especially worth mentioning in Arabidopsis are *FT* and *SOC1* that promote flowering, and *FLC* that inhibits the floral transition.

Based on a spatio-temporal working model, it has been proposed that flowering inducing signals mainly converge at the level of the expression of *FT* (Zeevaart, 2006). Interestingly *FT mRNA* is only expressed in leaves, but acts in the shoot apex. FT proteins seem to be part of the florigen, a mobile factor that responds to environmental stimuli perceived in the leaves and translate them into the flowering inducing information in the shoot apex. FT protein does not activate downstream genes in leaves, but is translocated to the SAM where it binds to the bZIP transcription factor FD (Abe et al., 2005). FD is present in the meristem even before *FT* expression, but is unable to trigger expression of flowering genes without FT. The FT-FD complex activates floral meristem identity genes at different levels: directly, by activation of *AP1* expression (Wigge et al., 2005), and indirectly, by the activation of *SOC1* expression. SOC1 integrates signals from the photoperiod, vernalization and autonomous floral induction pathways (Lee et al., 2000). It binds to the promoter of *AGL24*, activating its transcription. AGL24, in turn, promotes upregulation of *SOC1*. AGL24 and SOC1 proteins together activate the floral meristem identity gene *LFY* (Liu et al., 2008).

In contrast to the two floral integrators described above, FT and SOC1, which promote flowering, FLC is considered the main flowering repressor in Arabidopsis. As mentioned earlier, FLC ensures that flowering occurs in spring, after winter and when plants reach an appropriate developmental stage. FLC inhibits the initiation of flowering by binding to the genomic region of central flowering genes such as *FT*, *FD* and *SOC1*, and repressing their expression (Deng et al., 2011).

1.3 Arabidopsis inflorescence architecture

Most flowering plants produce multiple flowers in sequence from a reproductive shoot apex to form a flower spike (inforescence). As mentioned above, Arabidopsis develops an indeterminate, raceme type inflorescence (Prusinkiewicz et al. 2007), comprising individual lateral flowers arising immediately and sequentially from an apical inflorescence meristem. This indeterminate characteristic is gained through a complex network of positive and negative feedback loops (Toe et al., 2014). Flower meristem formation is initiated by increased expression of *AP1* and *LFY*. These genes, however, are not expressed equally throughout the SAM, but only at the flanks of the meristem. This strictly controlled expression pattern is regulated by the repressive action of TERMINAL FLOWER 1 (TFL1), expressed in the center of the inflorescence. TFL1 is a small protein homologous to phosphatidylethanolamine-binding proteins (PEBPs) belonging to the same protein family as FT. In fact, TFL1 and FT show a highly similar structure, and the change of only few amino acids nearby their binding pocket can make one of these proteins to acquire the function of the other (Hanzawa et al., 2005; Wang et al., 2017). This suggests that the mechanism of FT and TFL1 action is very similar and the

differences in their activity lay in their binding specificity. Due to TFL1 activity, *LFY* and *AP1* expression is repressed in the center of inflorescence apex, ensuring their presence on the outer zones and thus safeguarding the indeterminate identity of the core of the inflorescence (Fig 2). Consistent with this description, in *tfl1* mutants where the repressing role of TFL1 is absent, *LFY* and *AP1* expression occur in the central part of the inflorescence meristem, changing the identity of the apex into a terminal flower. In *35S::TFL1* plants reproductive development is impaired and an enlarged vegetative rosette with a long inflorescence stem and many lateral branches is formed (Liljegren et al., 1999; Goslin et al., 2017), further supporting an antagonistic function for TFL1, and LFY and AP1 proteins in the regulation of inflorescence architecture.

Floral meristem identity genes *LFY* and *AP1* promote establishment and maintenance of floral identity in newly formed floral primordia and without their activity, the floral primordia develop with inflorescence characteristics (Shannon and Meeks-Wagner, 1993). Both genes act to mutually activate each other´s expression, enhancing the maintenance of the floral meristem identity (Kaufmann et al., 2010; Benlloch et al., 2011), but they are also responsible for the repression of shoot meristem genes. In fact, repression of *AGL24* by LFY and AP1 is a crucial step in promoting flower development. Without such repression, continued *AGL24* expression in floral meristems is sufficient to cause floral reversion (return to previous developmental stages) regardless of the activation of floral organ identity genes (Yu et al., 2004). In addition, LFY and AP1 repress *TFL1* by direct binding to its promoter (Kaufmann et al., 2010; Winter et al., 2011), generating a negative regulatory feed-back loop that is essential for proper inflorescence development.

Fig.2. A working feedback loop model for inflorescence identity involving AP1, TFL1 and LFY. A) A schematic representation of *AP1*, *LFY* and *TFL1* expression domains in the SAM. B) Relationship between the proteins. TFL1 represses the expression of *AP1* and *LFY*, ensuring indeterminate growth of the inflorescence by preventing the conversion of the shoot apex into a floral meristem. *LFY* and *AP1* mutually induce their expression and repress TFL1 expression. *LFY* alone has an additional function promoting *TFL1* expression (Liljegren et al., 1999; Goslin et al., 2017).

Additional proteins have been implicated in floral meristem development. One example is XAANTAL2 (XAL2/AGL14), a factor necessary and sufficient to induce flowering. The regulation of this gene is important in floral meristem maintenance and determinacy (Perez -Ruiz et al., 2015). On this manner, *xal2* mutants are late flowering, particularly under SD conditions, while *XAL2* overexpressing plants display an early flowering phenotype, but their flowers have vegetative traits. Interestingly, inflorescences of *35S::XAL2* plants display higher expression levels of *LFY*, *AP1* and *TFL1* than WT plants (Perez -Ruiz et al., 2015). Other proteins involved in floral meristem development are PENNYWISE (PNY) and POUND-FOOLISH (PNF) (Smith et al., 2004). In *pny pnf* double mutants, meristem maintenance and flowering are impaired due to misregulation of lateral organ boundary genes and downregulation of floral meristem identity genes *LFY* and *AP1* (Smith et al., 2004; Khan et al.,

2015). Additionally, *pny* mutants show defects in internode elongation. As a consequence of this alteration, patterning of inflorescence and phyllotaxy are disturbed in these mutants (Smith and Hake, 2003). In Arabidopsis WT plants, flowers are produced at equal distances along the inflorescence stem forming a raceme with a 137.5° angle between flowers (Mirabet et al., 2012). One of the major signals associated with phyllotaxis is auxin. This plant hormone has been proposed as one of the main determinant of lateral organ initiation, with auxin response maxima correlating with incipient organ primordium initiation. In fact, *pin-formed 1* (*pin1*) mutants, which are unable to redistribute auxin polarly, have naked pin-like inflorescences devoid of any floral meristem (Gälweiler et al., 1998).

Branching pattern is another factor affecting inflorescence architecture. While flowers are generated in the apical part of Arabidopsis inflorescence, branches are formed in its basal part, and branch- and flower-producing regions are separated (Hempel and Feldman, 1994). Branches develop from axillary meristems localized in the axils (the junction between leaf and stem) of cauline leaves, or rosette leaves in case of rosette branches (Long and Barton, 1999). Branching patterns depend on a very important developmental decision: whether axillary buds grow out to give a branch or they remain small and dormant in the axils of the leaves. This decision relies on many environmental stimuli perceived in different regions of the plant and transduced into the axillary buds (Horvath et al., 2003). Key players regulating branching patterns are MORE AXILLARY GROWTH (MAX) genes, acting outside the axillary bud (Booker et al., 2005) and BRANCHED1 (BRC1), a key branching integrator gene, working inside the axillary bud (Aguilar-Martínez et al., 2007).

In sum, inflorescence architecture depends on the initiation of the bolting stem (bolting transition) and development of the lateral organs around its main axis: leaves, branches or flowers (flower transition). It is often considered that these two transitions are fully dependent on each other and occur in a successive manner (Suh et al., 2003, Teo et al., 2014). However, the phenotypes of different mutants like *pin1*, that bolts normally, but generate a naked, flower-lacking stem (Gälweiler et al., 1998), *lfy*, that produces numerous additional cauline leaves after bolting, and shows a delayed flower formation (Schultz and Haughn, 1991), and *acaulis* (stemless) mutants, suggest that in fact these bolting and flower transitions are partially separate processes. It seems that flowering and bolting transitions are indeed synchronized under flowering inducing conditions (warm, LD), but not in non-optimal conditions where flower and bolting nodes are temporally and spatially separated. This was depicted by applying photoperiodic perturbations to WT plants at different developmental stages. On this manner, it has been proposed that the bolting transition occurs after the determination of the floral nodes. Moreover, manipulation of the time when plants are exposed to photosynthetically active light also affected bolting and suggested that bolting time is not only determined by the initiation of the phase change, but also by other factors such as the production of sufficient biomass for optimal seed production (Pouteau and Albertini, 2009). From the conclusions drawn in this study a method to identify the optimal conditions for flowering has been proposed, which is when both transitions are maximally synchronized.

1.4 Emergence of the flowers. The ABC model of flower development and its modifications

Flowers are generated from determinate floral meristems that give rise to a fixed number of floral organs: sepals, petals, stamens and carpels. Arabidopsis flowers are composed of four sepals, four petals, six stamens and two fused carpels. These organs are arranged in concentric circles (whorls) from the outside to the center. Floral organs are determined by a group of regulatory genes, dubbed as floral organ identity genes. They were identified based on mutants showing homeotic transformations of the flower organs, that is a replacement of one floral organ by another (Bowman et al., 1989). The analysis of these homeotic mutants led to the conclusion that floral organ identity genes act in combinatorial manner (Smyth et al., 1990; Bowman et al., 1991; Bowman et al., 1993). Those studies gave birth to the best-known model in plant sciences, the ABC model of flower development. According to this model, the identity of different types of floral organs is provided by different sets of genes. Sepals are created by the action of the group A of genes –*AP1 AP2*; petals are created by the combined activity of A and B group genes – *AP3* and *PISTILLATA* (*PI*); stamens by the function of B group genes and the C group gene – *AGAMOUS* (*AG*); and carpels by the group C gene alone. Moreover, group A and group C genes act antagonistically, preventing each other's expression and restricting the expression domain of each class of genes. In the ideal group A mutants, sepals are replaced by carpels and petals are replaced by stamens. In the ideal group B mutants, sepals are created in the first two whorls and carpel in the two inner ones. In the group C mutants, stamens are replaced by petals and carpels are substituted by sepals (Smyth et al., 1990; Bowman et al., 1991; Bowman et al., 1993). Also, in C gene mutants the floral meristem becomes indeterminate giving rise to the phenotype of flower within a flower. In addition, the activity of a number of genes is required to set the boundaries between the domains of expression of the different organ identity genes. This class of floral development genes is usually referred to as cadastral genes.

The ABC model, despite being quite simple and attractive, was shown to present some shortcomings. All ABC genes, except for *AP2*, encode MIKC-type MADS-domain transcription factors (Irish, 2010). Soon, other members of same family of transcriptional regulators started to be analyzed based on their role in flower development. A group of genes closely related to *AG* has been proposed to contribute to function D. This group consists of *SEEDSTICK* (*STK*) and *SHATERPROOF* (*SHP*) *1* and *2* genes, and is involved in ovule formation (Pinyopich et al., 2003; Favaro et al., 2003). Another class of MIKC-type MADS-box protein comprise the *SEPALLATA* (*SEP*) *1-4* genes. *sep* single and double mutants exhibit no or only weak phenotypes. However, *sep1 sep2 sep3* triple mutant bears flowers in which all floral organs are substituted by sepals, and the flower itself becomes indeterminate. Quadruple mutant *sep1 sep2 sep3 sep4* displays even stronger phenotypic alterations, with all organs being replaced by leaves. The function of the *SEP* genes has been classified as a separate group of floral organ identity genes, the group E (Pelaz et al., 2000; Ditta et al., 2004, Zahn et al., 2005).

The ABCDE model harmonizes well genetic and molecular data. Nevertheless, the A function has often been thrown into question since the function for specifying perianth organ (sepals and petals) identity and antagonism of C function is difficult to separate from a more fundamental function performed by *AP1*, setting floral meristem identity. Even an early alternative to ABC model assumed the existence of A and B group complemented with a so-called 'floral ground', where AP1 would correspond to floral ground state and B and C group

genes would correspond to A and B of the alternative model. In this alternative model, the development of sepals, being so close to leaves, would represent a 'default state' of floral organs (Schwarz-Sommer et al., 1990). Another model (A)B(C) was suggested where (A) comprises the function of A and E class genes of ABCDE model. Having a closer look, A and E class genes are closely related in tested flowering plants, creating a phylogenetic superclade (Gramzow and Theissen, 2010). Also, some evidence provided by *AG*-like genes in extant gymnosperms and stem group seed plants (Gramzow et al., 2014) supports the combined function of C and D genes from the ABCDE model, giving a (C) class genes that specify reproductive organ identity and distinguishes reproductive (C) from non-reproductive organs (A)B.

The initial models for genetic control of flower development relied mostly on genetic analysis, but later studies started to reveal what is the exact molecular mechanism of action of the products of floral homeotic genes. First trace of evidence was provided by the study of Egea-Cortines et al., 1999, where they reported that orthologues of *AP3*, *PI* and *AP1* from *Antirrhinum majus* form ternary complexes via their C-termini. These multimeric complexes show increased DNA binding in gel-shift assays compared with individual hetero- or homodimers. Later on, class B proteins of Arabidopsis, PI and AP3, were reported to interact with AP1 or AG through SEP3 (Honma and Goto, 2001). The Floral Quartet Model states that there is at least one unique quaternary complex for the determination of the identity for each of the floral organs (Theissen and Saedler, 2001; Smaczniak et al., 2012) (Fig 3).

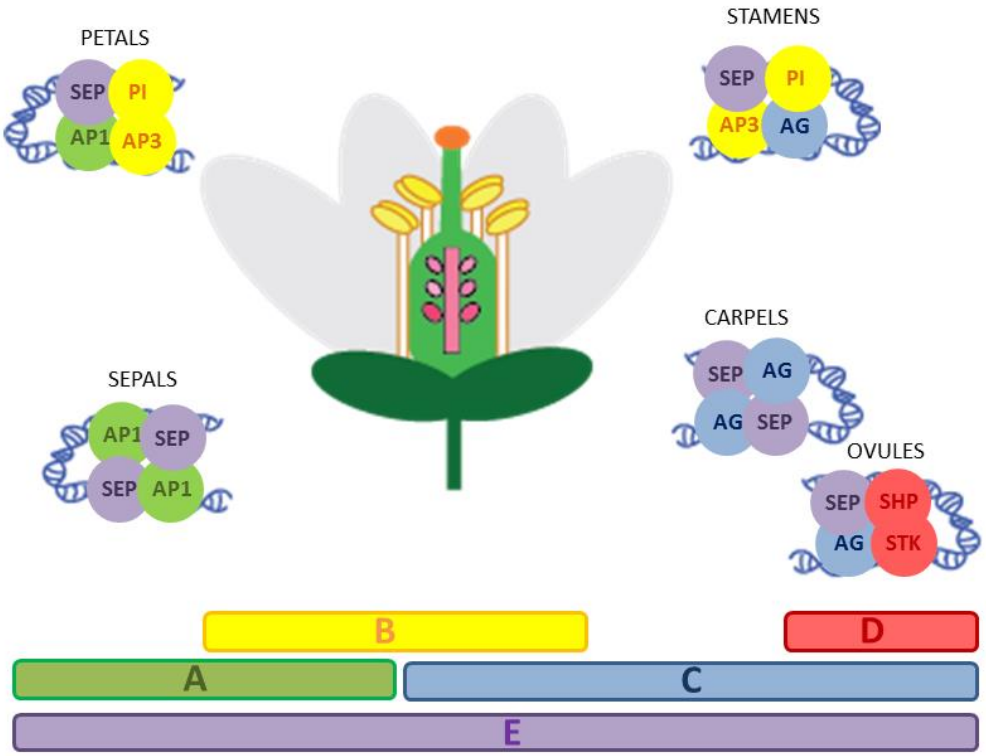

Fig.3. ABC model for the control of flower development. Quadruple complexes are needed for each organ to be created. A-class genes (*AP1* in Arabidopsis) are needed for sepal formation. B-class genes (*PI* and *AP3* in Arabidopsis) together with A-class genes are needed for the generation of petals and together with C-class genes are needed for stamens formation. C-class genes alone (*AG* in Arabidopsis) are needed for carpel identity. The ABC model has been expanded to E-class genes (*SEP1-4* in Arabidopsis) that are needed for the floral whorls definition and D-class genes (*STK* and *SHP* in Arabidopsis) that are needed for ovule formation (adapted from Theissen et al., 2016).

1.4.1 Reproductive reversion

In Arabidopsis, once triggered, the transition from vegetative to reproductive growth is irreversible. Plants flower even if inductive conditions no longer exist. This feature is called floral commitment and exists within many plant families (Tooke et al., 2005). Nevertheless, the return from floral to vegetative development has been observed in different plant species, as well as in some Arabidopsis mutants (Muller-Xing et al., 2014; McCullough et al., 2010; Melzer et al., 2008).

Reproductive reversion was classified as inflorescence and floral (Adrian et al., 2009). Inflorescence reversion occurs during or after inflorescence development and results in meristem reverting to vegetative growth. On the other hand, floral reversion happens later in development. As a result, incomplete flowers can be created, some floral organs can be substituted by leaves or, in more dramatic cases, floral meristem reverts to either inflorescence or vegetative meristem. Floral reversion was strongly connected with flowering in suboptimal conditions (as reviewed in Battey and Lyndon, 1990; Tooke at al., 2005), but the molecular mechanisms underlying this developmental abnormality remain unknown. For instance, *Arabidopsis suecica*, a close relative of *Arabidopsis thaliana*, displays photoperiod-dependent floral reversion in a subset of its flowers and occurs more frequently when flowering takes place under non-optimal conditions, suggesting environmental conditions as a cause of reversion (Asbe et al., 2015, McCullough et al., 2010). Also, some of the Arabidopsis mutants defective in homeotic genes, like *ag* or *lfy*, exhibit floral reversion (Mizukami and Ma, 1997; Okamuro et al., 1996). The computational modulation of data coming from *35S::XAL2* plants, together with other publicly available data suggested that meristems at various developmental stages represent different steady states (an unvarying condition of a process): the vegetative meristem represents one steady state, while inflorescence and floral meristem the others. With optimal expression of flowering genes, the transition flow vegetative meristem – inflorescence meristem – floral meristem is the most energetically-optimal scenario. If the balance in the expression of meristematic genes is disrupted by mutations or overexpression of some of the components, the flow can preferentially follow the direction from inflorescence meristem to the creation of inflorescence/floral meristem double identity cells (Pérez-Ruiz et al., 2015).

On the other hand, recent observations strongly suggest that the expression of *FT* in organs formed after the floral transition is needed to prevent reproductive reversion (Liu et al., 2014 and Satake et al., 2016). On this way, the *FT* defective mutant allele *ft-10* displayed a developmental reversion phenotype when grown under SD conditions. This reversion was observed in flowering shoots early after the formation of the first true flowers. The inflorescence meristem reverted to form vegetative side branches with rosette-like cauline leaves. All these phenotypic defects were suppressed by the introduction of a functional *FT* transgene in the *ft-10* mutant background (Liu et al., 2014). In addition, strong activation of *FLC* either in *A. thaliana* ecotype *Sy-0* or in PcG mutants also causes floral reversion by the repression of *FT* expression in the inflorescence (Müller-Xing et al., 2014). In addition, a computational model for the impact of FT (flowering signal) and sucrose (inflorescence signal) on floral reversion was established. The model predicted the diverse inflorescence structures, depending on the number of flowers and the value of the florigen threshold, and showed that: 1) the florigen threshold needs to be increased if the total floral stimuli are decreased by the knockout of genes involved in the determination of floral meristem identity; 2) the commitment

of the lateral organ to flower formation depends on the time when it was created; and 3) floral reversion enhances floral commitment of newly formed floral meristem, even if reverted meristems developing leaf-like organs do not produce the florigen (Satake et al., 2016).

Thus, floral reversion seems to be provoked by the imbalance of a finely tuned network of floral transition, floral meristem identity and floral organ identity genes, and can be produced either by non-inductive flowering conditions or gene misregulation caused by the mutation/overexpression of some important regulators of reproductive development.

2. Chromatin-mediated regulation of reproductive development in Arabidopsis

Cells of a multicellular organism are in general genetically identical, but structurally and functionally distinct owing to the differential expression of genes. Many of these differences in gene expression arise during development and cell proliferation (Holliday and Pugh, 1975; Jaenisch and Bird, 2003). In nuclei of all eukaryotic cells, DNA is highly folded and compacted by histone and non-histone proteins into a dynamic polymer called chromatin. Chromatin remodeling processes play a central role in the establishment of gene expression patterns that drive plant development (Jarillo et al., 2009). Additionally, chromatin structure provides a mechanism that ensures the stability of gene expression patterns throughout the mitotic cell divisions that take place in a specific cell line (Jaenisch and Bird, 2003). Distinct levels of chromatin organization are dependent on the structuring of nucleosomes, chromatin's basic subunits. Each nucleosome is formed by a central octamer of four core histone proteins: an H3-H4 tetramer and two H2A-H2B dimers, wrapped by almost two turns of superhelical DNA (Kornberg and Lorch, 1999). Nucleosomes are separated by the 'linker' histone H1. Histones are small proteins consisting of a globular domain and a more flexible and charged NH_2 terminus (so called 'histone tail'). Numerous studies have highlighted how chromatin dynamics is essential for the correct regulation of different components of the flowering pathways and the inflorescence and flower development processes (He, 2012; Wagner, 2017).

2.1 Chromatin-based mechanisms for the regulation of gene expression

Different mechanisms act on chromatin-based gene expression control, including chromatin conformation and nucleosome remodeling, subnuclear localization, chemical modification of histones, replacement of histones by variants and DNA methylation.

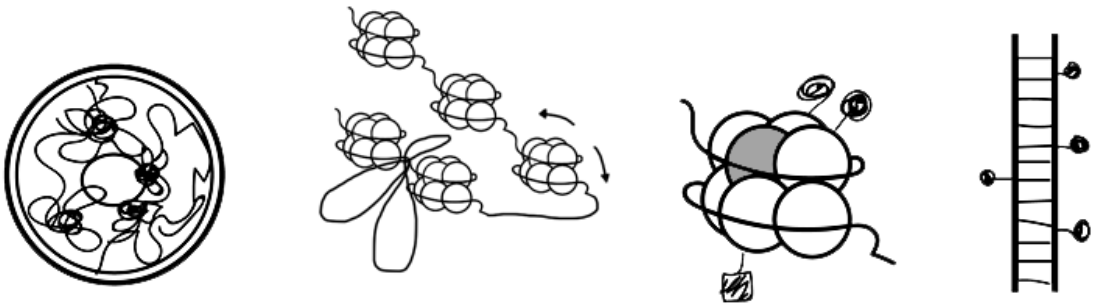

Fig.4. Chromatin-mediated regulation of gene expression occurs on different levels: 1) nuclear architecture, where position relative to the nucleolus and nuclear lamina affect spatial availability of transcription machinery, 2) chromatin density and position of nucleosomes that may block the binding of transcription factors, 3) chemical modification of histones (acetylation, methylation and others) and exchange of histone variants that change the properties of interaction between the nucleosomes and the DNA, 4) chemical modification (methylation) of DNA that affects protein binding abilities

2.1.1 Chromatin conformation

The macromolecular structure chromatin can be divided in two conformations: heterochromatin and euchromatin. Heterochromatin is rich in repetitive DNA sequences and transposable elements, contains few genes, and exhibits little or no transcriptional activity. Furthermore, it displays distinct molecular and biochemical variations according to localization and function. Cytologically, it can be identified as regions exhibiting intense DNA labelling, named chromocenters (del Prete et al., 2014). In contrast, euchromatin occupies uniformly the nucleus and consists of both transcriptionally active and silenced genes. However, even the silenced genes within the euchromatin possess the potential to be easily activated. In interphase nuclei, euchromatic regions emerge as 0.2- to 2-Mbp loops around chromocenters and are enriched in activating histone markers (Fransz et al., 2002).

The more decondensed the chromatin structure, the more active its transcriptional state, and vice versa (Kadauke and Blobel, 2009). Thus, chromatin looping and clustering is a frequent mechanism of gene silencing. The first example in plants of active repositioning of a locus in response to its transcriptional activity was showed in the study of the nuclear organization of transgenic *FLC* alleles tagged with the *lacO* repeat array (*FLC-lacO*) in response to vernalization. In non-vernalized plants where *FLC* is expressed in the endoreduplicated cells of the root differentiation zone, 6 foci of *FLC-lacO* can be seen. After *FLC* silencing by a cold treatment, physical repositioning and clustering of *FLC* in only one or two enlarged *FLC-lacO* foci are present (Rosa et al., 2013; Zhu et al., 2015).

2.1.2 Nucleosome remodeling

Nucleosome-remodeling is achieved by key facilitators of chromatin dynamics that allow the switch between more condensed (and consequently transcriptionally repressed), and less condensed (active) states. At the level of single nucleosomes, these factors are involved in nucleosome-repositioning, altering histone–DNA interactions, disassembly of nucleosomes,

and the exchange of histones with variants of different properties (Becker and Workman, 2013). DNA does not detach from histones on its own, and energy is needed to catalyze this process. ATP-dependent nucleosome remodeling factors are the best described ones. These enzymes weaken the tight wrapping of DNA around the histone octamers, thereby facilitating the sliding of histone octamers to neighboring DNA segments, their displacement to unlinked DNA, and the accumulation of patches of accessible DNA on the surface of nucleosomes (Varga-Weisz and Becker, 2006).

2.1.3 Subnuclear localization and transcriptional status

In yeast, the artificial tethering of a reporter gene to the nuclear envelope via membrane-spanning anchor (Sir proteins) revealed that the nuclear periphery is associated with gene silencing (Andrulis et al. 1998; Gartenberg et al. 2004), while active genes tend to be located within the nuclear matrix. Comparable approaches have been used to study animal nuclei (Finlan et al., 2008). In Arabidopsis, the high compaction of nuclei makes this kind of analysis extremely difficult, but it is believed that the relation between subnuclear localization and gene transcriptional activity in plants is similar to that in other non-plant organisms.

The yeast Sir complex interacts with the membrane-associated protein Esc1 that is excluded from nuclear pores (Andrulis et al. 2002). These pores, on the other hand, were suggested to associate with actively transcribed genes. Indeed, several studies revealed that in yeast, genes associated with nuclear pores are involved in nuclear – cytoplasmic trafficking of tRNA and mRNA (Ishii et al., 2002). Thus, nuclear periphery is a transcription silencing location; nuclear matrix is the location for transcriptionally active genes, and nuclear pores enable the transport of some gene products from nuclear matrix to cytoplasm.

2.1.4 Covalent modification of histones

The covalent modification of histones changes their physico-chemical properties, namely the strength of the interactions between histones and DNA, and histones and other proteins, as well as nucleosome mobility and turnover. This affects chromatin structure, leading to differences in transcriptional ‘on-off’ states, and propagation of higher order chromatin structures. Histone modifications are highly complex due to the large number of residues that can be modified, as well as the large variety of modifications (acetylation, methylation, phosphorylation, ubiquitination and others) that are deposited. Next to the information encoded in DNA sequence, another type of combinatorial information is encoded in histone modifications, being responsible for the creation of different epigenetic states that correlate with transcription. For that reason, the combination of histone modifications is often referred to as ‘histone code’ (Jenuwein and Allis, 2001).

Discovered in 1963, histone acetylation was the first described histone modification (Phillips, 1963). It was associated with highly transcribed genes, so it was suggested that acetylation facilitates transcription (Pogo et al., 1966). Indeed, acetylation neutralizes the positive charge of lysine (K) residues, making the charge of modified histones more negative and weakening the charge-dependent interaction with negatively charged DNA, creating a loose and more accessible chromatin region for TFs (Hong et al., 1993). Histone residues are acetylated by histone acetyltransferases and deacetylated by histone deacetylases (Campos and Reinberg, 2009). Acetylation and deacetylation are highly dynamic, and the high speed of those

reactions is needed to facilitate mobilization and restoration of nucleosomes during polymerase transit (Waterborg, 2002). Histone acetylation is usually associated with histone H3 (in K residues 9 and 14) and histone H4 (in K residues 5, 8, 12, and 16).

K and arginine (R) residues on histone tails can be mono-, di- and (in case of the K) trimethylated. In contrast to acetylation, methylation does not change the physical properties of chromatin *per se*. Methylated histones can be found in both actively transcribed and repressed regions, suggesting that methylation has no direct effect on the transcriptional state of genes. Instead, methylation mediates the regulatory function through the interaction and recruitment of catalytic proteins. For example, H3K27me3 (trimethylated K27 on histone H3) has been proposed to attract Polycomb Repressive Complex (PRC) 1 that ubiquitinates K residues of histone H2A, recruits complementary repressing factors and ensures inhibition of gene expression (reviewed in del Prete et al., 2015). On the other hand, H3K4me3 at the transcription start site recruits PHD finger proteins of the NUCLEOSOME REMODELING FACTOR (NURF) that changes nucleosome occupancy of H3K4me3 regions (Wysocka et al., 2006). The same methylation type also attracts PHD-containing ING proteins that recruit histone deacetylases (Shi et al., 2006) or histone acetyltransferases (Doyon et al., 2006), causing gene expression inhibition or activation, respectively. Histone acetyltransferases can be also recruited by the H3K36me3 mark. This mark is associated with active genes (Li et al., 2015: Yang et al., 2014). MORF RELATED GENE (MRG) proteins bind H3K36me3 and recruits H4-specific acetyltransferases to achieve high expression levels through active histone acetylation at the promoter and 5′ regions of target loci (Xu et al., 2014). On the contrary, the H3K9me2 mark is connected with heterochromatin and recruits CHROMOMETHYLASE 3 (CMT3), an enzyme that methylates DNA, an epigenetic modification associated with gene silencing (Du et al., 2012).

Another covalent modification of histones is ubiquitination. While the abovementioned modifications involve small chemical moieties, ubiquitination adds a 76 amino-acids peptide to a single K residue. Histone ubiquitination occurs mostly in the context of histones H2A and H2B. However, ubiquitination of those histone proteins seems to have opposite effects. While monoubiquitination of H2A (H2Aub1) is associated with transcriptional repression mediated by the PRC1, H2Bub1 is involved in transcription activation (Feng and Shen, 2014).

More detailed information regarding the main localization and function of different histone modifications can be found in Table 1.

Table 1: List of major covalent histone modification in Arabidopsis and their function

Modification	Localization	Function	Source
H3K9/14ac	Transcription start site (TSS) of genes, restricted to euchromatin	Promotes transcription	Zhou et al., 2010
H4K5/8/12/16ac	Cell cycle dependent localization in different nuclear compartments,	Promotes transcription, involved in DNA replication	Jasencakova, 2000 Tian et al., 2005
H3K36ac	Peaks at the 5′ end of genes, mainly on the two nucleosomes close to the TSS	Associated with gene activity, a binary indicator of transcription	Mahrez et al. 2016

H3K4me1	Within transcribed regions, correlated with CG methylation		Zhang et al., 2009
H3K4me2	Promoters and 5' genic regions co-localize with H3K27me3		Zhang et al., 2009
H3K4me3	Promoters and 5' genic regions of highly expressed genes	Involved in transcriptional activation	Zhang et al., 2009
H3K9me2	Mostly in heterochromatin, but also present in euchromatin, TE	Associated with TE silencing, facilitate DNA methylation, gene silencing at constitutive heterochromatin	Roudier et al., 2011, Du et al., 2012
H3K27me1	Both in hetero- and euchromatin, TE	Associated with TE silencing, gene silencing at constitutive heterochromatin	Roudier et al., 2011 Jacob et al., 2009
H3K27me3	Transcribed regions of genes	Promotes transcriptional silencing, independently of other mechanisms	Zhang et al., 2007
H3K36me3	Gene bodies with 5' preference	Promotes gene expression, antagonistic role with H3K27me3	Li et al., 2015 Yang et al., 2014
H2Aub1	Inactive genes	Associated with transcriptional repression by PRC1	Feng and Shen, 2014
H2Bub1	Co-localized with H3K4me3 and H3K36me3	promotes transcriptional activation	Roudier et al., 2011 Feng and Shen, 2014

2.1.5 Histone variants

Histone variants are non-canonical (non-allelic) variants of histones – bearing one or a few amino acid differences that slightly affect histone properties and abilities to undergo chemical modifications. For example, there are two variants of histone H3, H3.1 and H3.3. H3.1 acts as the canonical histone that is incorporated during DNA replication, whereas H3.3 replaces H3.1 outside of S-phase, during chromatin-disrupting processes like transcription (Stroud et al., 2012). A variant of histone H2A, histone H2A.Z is enriched at the TSS of expressed genes, but also at the bodies of genes rapidly regulated during stress or development (Zilberman et al., 2008; Coleman-Derr and Zilberman, 2012). Another H2A variant, H2A.W is associated with inactive chromatin and enhances chromatin condensation by promoting fiber-to-fiber interactions (Yelagandula et al., 2014). A variant of a linker histone H1, H1.3 is undetectable in the genome in standard conditions, except for a pool expressed in guard cells. However, under environmental stress conditions this histone variant rapidly replaces the canonical H1, and this exchange is needed for fast facilitation of chromatin accessibility (Rutowicz et al., 2015).

2.1.6 DNA methylation

DNA methylation (addition of a methyl group to a cytosine base) is associated with gene silencing and is the most evolutionary ancient epigenetic modification (Razin and Riggs, 1980). In plants, this modification occurs in all three possible sequence contexts: **symmetric** (where there is a mirror cytosine on the complementary strand of DNA) context that can take place in **CG** dinucleotides (the most abundant methylation type) and **CHG context** (where H

corresponds to A, T or C), and **asymmetric CHH context**. In Arabidopsis DNA methylation is especially abundant, with more than 14% of all cytosine bases being methylated. It occurs predominantly on transposon regions and other repetitive elements, but it is also present in genes. Depending on the deposition of this mark, two types of DNA methylation can be distinguished: 1) *de novo* methylation, resulting in the generation of new types of methylation marks and 2) maintenance DNA methylation that reproduces methylation patterns on daughter cell DNA strands after replication (Henderson and Jacobsen, 2007; Law and Jacobsen, 2010; Niederhuth and Schmitz, 2017).

Methylation of cytosine at the three contexts described above is carried out in different ways. CG is methylated by METHYLTRANSFERASE 1 (MET1). This enzyme is directed to the hemimethylated sites during replication by VARIANT IN METHYLATION (VIM) 1-5 proteins (Woo et al., 2008). CHG methylation is performed by CMT3 that binds H3K9me2, a histone mark connected with heterochromatin, and methylates CHG around this histone mark. Interestingly, H3K9me2 is equally dependent on DNA methylation, as histone methyltransferases KRYPTONITE (KYP), SU(VAR)3-9 HOMOLOG 5 (SUVH5) and SUVH6 bind methylated DNA and introduce K9me2 on histone H3 in surrounding nucleosomes (Du et al., 2012). CHH doesn't have a mirror cytosine on the opposite strand of DNA and is not connected with maintenance of DNA methylation during replication but with *de novo* methylation. This type of methylation is also connected with H3K9me2 and is introduced by CMT2 (Stroud et al., 2014). However, the crosstalk between all three methylation pathways exists as cytosines at every position can be methylated by *de novo* methylation pathway triggered by 24 nt siRNA (Matzke and Mosher, 2014).

2.1.7. Chromatin states

Regions of the chromatin harboring different combinations of the abovementioned marks have been divided into 9 classes, further grouped into 5 categories with distinct functions: 1) proximal promoters and 5' UTRs (chromatin state 1 and 2) rich in activating marks as H3K4me2/3, H3.3, H2A.Z and H3K36me3 or H2Bub, and DNase I accessible sites; 2) genic regions (chromatin state 3, 6 and 7) with 5' end rich in H3K4me1/2 and poor in H3K27me3; 3) distal regulatory intergenic regions (chromatin state 4) often have properties of bivalent chromatin rich in H3K4me3 and H3K27me3, AT-rich with moderate levels of H3K27me1; 4) Polycomb associated chromatin (chromatin state 5), where Polycomb proteins bind, rich in H3K27me3 and H3.1; and 5) heterochromatin (chromatin state 8 and 9) AT- or GC-rich, with high levels of H3K9me2, H3K27me1, H3.1 and DNA methylation. Chromatin boundaries exist between categories of different functions, for example in transposable elements that flank highly expressed genes and separate them from heterochromatin (Sequeira-Mendes et al., 2014; Vergara and Gutiérrez, 2017).

2.2 Chromatin-mediated regulation of growth phase transitions and reproductive development.

The switch from vegetative to reproductive phase and development of reproductive organs is connected with the suppression of flowering repressors and induction of flowering activators. This broad change in the transcriptional activity of many genes in the genome

demands the reprograming of existing nuclear topology, chromatin conformation, histone landscape and DNA methylation patterns.

2.2.1 Rearrangement of nuclear organization during development

Light and temperature are the main signals determining the developmental fate and growth pattern of plants and act by modulating gene expression. Global changes in nuclear organization in response to these environmental cues and developmental stimuli are relatively poorly characterized. It is broadly accepted that those changes occur, but the exact mechanism is so far unknown. One of the few examples described is the control of nuclear architecture in response to light signals during seedling establishment (Bourbousse et al., 2015; Kaiserli et al. 2018). In the first 3 days after seed germination, nuclei are relatively small with unorganized chromatin structure. During the next 2 days, nuclei expand their size and chromatin differentiates into organized structures that end up with the formation of 8-10 chromocenters. This chromatin organization however, is absent in plants grown in the dark. This observation suggests that during early stages of seedling growth, the dynamic reorganization of nuclear size and heterochromatin condensation depends on photomorphogenesis. During this period, exposure to light causes an increase of RNA Pol II activity, and repositioning of several light-responsive genes toward the nuclear periphery (Kaiserli et al. 2018)

Temperature also triggers changes in chromatin structure and nuclear organization and regulates heterochromatin compaction within the plant nucleus (Pecinka et al., 2010). In particular, heat stress has been shown to lead to a reduction in nucleosome density, which is primarily regulated by the chromocenter-localized protein HEAT-INTOLERANT 4 (HIT4) (Pecinka et al., 2010; Wang et al., 2015).

It is believed that analogous changes in nuclear topology occur with every developmental switch. Recent studies analyzing the DNA sequences connected with nuclear lamina proteins appear to confirm this hypothesis. These results revealed that half of the sequences associated with nuclear envelope in seedlings were absent in flowers, suggesting that chromatin rearrangement occurs throughout development (Rodriguez-Granados et al., 2016). However, full information concerning the mechanisms mediating these processes remains unknown, and only fragmentary data are available, mostly regarding the changes in histone modifications and chromatin organization. Different examples of such information about the involvement of chromatin-mediated regulation are given below.

2.2.2 Chromatin-mediated regulation of the floral transition

Numerous studies have revealed the involvement of chromatin remodeling processes on the regulation of flowering initiation. In fact, many of the mutants affected in chromatin remodelers display defects in the regulation of the floral transition, suggesting the involvement of chromatin remodeling during this developmental process. For instance, mutations in a member of the SWI/SNF subfamily of ATPases, BRAHMA (BRM), delay flowering in SD due to *FLC* overexpression. In contrast, under LDs *brm* mutations cause early flowering, most likely due to the overexpression of *CO, SOC1* and *FT*, and simultaneous reduction in *SVP* transcripts (Farrona et al., 2011). *SVP* expression was shown to be directly activated by BRM, and in *brm* mutants higher occupancy of Polycomb Repressive Complex 2 (PRC2) and H3K27me3 can be observed on the *SVP* locus (Li et al., 2015). Further examples are already available (see He,

2012 and Wagner 2017 for reviews), but by far, the best-known examples of chromatin-mediated regulation of flowering time are related to the transcriptional control of the flowering integrators *FLC* and *FT*. Particularly enlightening are the lessons learnt from a variety of studies that have unveiled numerous epigenetic mechanisms involved in the fine regulation of the floral repressor *FLC*. Below we discuss some of these works related to the transcriptional regulation of these genes with special emphasis on *FLC*.

2.2.2.1 *FLC* - the paradigm of epigenetic control of gene expression in plants

As mentioned above, *FLC* is the main repressor of flowering in Arabidopsis, and different data indicate a close connection of this locus with flowering pathways mediated by temperature. In fact, vernalization (Michaels and Amasino, 1999) and elevated ambient temperature (Gan et al., 2014) repress *FLC* expression whereas short-term cold stress induces its expression (Jung et al. 2013). Interestingly, the same mean temperatures delivered as constant or alternating temperature profiles have different consequences on *FLC* expression (Burghardt et al. 2016). Cold and heat have been proposed to affect *FLC* expression by inducing changes in chromatin conformation (Kaiserli et al. 2018), Screens for mutants in which winter annuals are converted into rapid-flowering types due to attenuation of *FLC* expression identified homologs of yeast RNA Polymerase II Associated Factor 1 (PAF1) complex (He et al., 2004). PAF1-C associates with the RNA polymerase II complex during transcription and simultaneously recruits H3K4 methyltransferases to target genes. This complex links an increase in H3K4 trimethylation with transcription and changes in chromatin structure. This mechanism required for *FLC* activation may serve as a 'transcriptional memory' that establishes a positive feedback loop of gene activity and is involved in *FLC* expression memory (Squazzo et al., 2002; Ng et al., 2003; Krogan et al., 2003).

Another chromatin remodeling mechanism previously described in yeast that is also important for *FLC* expression in Arabidopsis is mediated by PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1), considered a homologue of the ATP hydrolyzing, chromatin-remodeling protein ISW1p. This protein is connected with the modulation of H3K4 methylation levels in the chromatin of certain actively transcribed genes. It is possible that PIE1 binds H3K4me3 on the *FLC* promoter, remodels the chromatin structure and increases *FLC* expression (He and Amasino, 2005). Other reports have proposed that PIE1 might be the catalytic subunit of the **Sw**i2/Snf2-**R**elated 1 (SWR1) complex, mediating the deposition of H2A.Z in the chromatin of the target genes (Jarillo and Piñeiro, 2015).

As described earlier in the 1.1.3 section, FRI acts as an *FLC* activator. The exact mechanism of its function is not fully understood, although it seems to directly activate *FLC* transcription by interacting with the nuclear cap–binding complex, increasing the proportion of *FLC* mRNA that carries a 5′ cap (Geraldo et al. 2009). Additionally, through interactions with some protein partners, FRI facilitates changes in *FLC* chromatin, such as delivery of H2A.Z (by SWR1-C), and deposition of H3K36me3 by the methyltransferase EARLY FLOWERING IN SHORT DAYS (EFS)/SET DOMAIN GROUP8 (SDG8) (Choi et al. 2011) as well as delivery of H3K4me3 by ARABIDOPSIS TRITHORAX-LIKE PROTEIN1 (ATX1) and SDG25 (Pien et al. 2008; Berr et al. 2009). The result is an increased level of 'active' chromatin modifications associated with high levels of transcription. In addition, also the physical structure of *FLC* gene region is changed as a chromatin loop is created between 5' and 3'

flanking regions (Crevillen et al., 2013). In addition, enzymes connected with histone ubiquitylation machinery can be also found among *FLC* activators. Mutants of E2 and E3 ubiquitin-conjugating enzymes show reduced levels of monoubiquitinylated H2B that lead to downregulation of *FLC* expression and early flowering (Xu et al., 2009).

Stable *FLC* expression shutdown is caused by long-term epigenetic silencing, a process involving a series of coordinated chromatin changes at the locus. As described above, prior to vernalization, *FLC* chromatin is enriched in a number of histone modifications associated with actively transcribed genes (Li et al. 2007). During long cold exposure these marks are dynamically removed from the *FLC* chromatin and replaced by the PcG-associated repressive mark H3K27me3 (Yang et al. 2014). Initially, H3K27me3 deposition is limited to the *FLC* gene nucleation region. This localized accumulation of H3K27me3 is mediated by PRC2 complex components, including the Su(z)12 homologue VRN2 and two plant homeodomain (PHD) finger proteins, VRN5 and VIN3 (De Lucia et al., 2008). VRN5 binds the nucleation region and this binding is dependent on the cold-induced PHD protein VIN3. Biochemical purification of the VRN5 complex showed that during prolonged cold, a PHD-PRC2 complex contains core PRC2 components (VRN2, SWINGER, FIE and MSI1), and three related PHD finger proteins, VRN5, VIN3, and VEL1. The PHD-PRC2 activity increases H3K27me3 throughout the *FLC* locus to levels sufficient for stable silencing (De Lucia et al., 2008). In addition, VRN1 and LHP1 proteins are required for stable maintenance of *FLC* repression (Bastow et al., 2004). Recruitment of PHD-PRC2 to the nucleation region has also been proposed to involve the long non-coding RNA (lncRNA) *COLD-ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*). *COLDAIR* is transcribed in the sense direction from intron 1 of *FLC* gene, which harbors a cryptic promoter for this ncRNA. This promoter becomes active when *FLC* is being repressed (Heo and Sung, 2011). Thus, Polycomb-dependent silencing and H3K27me3 spread across the entire *FLC* locus, establishing stable, mitotically heritable silencing upon the return to warmth. Another factor required for the cold temperature-mediated repression of *FLC* is the protein VAL1, which associates with the nucleation zone of the *FLC* locus intron 1. This protein mediates the sequence-specific recruitment of PHD-PRC2 complexes to the genomic region of this floral repressor, triggering *FLC* silencing in response to vernalization. In addition, VAL1 recruits the histone deacetylase HDA19, leading to deacetylation and contributing to reduced *FLC* transcription (Qüesta et al. 2016).

Further evidence has demonstrated that the nucleation region of intron 1 is itself very important for *FLC* expression regulation. In fact, two Arabidopsis accessions, Landsberg *erecta* (L*er*) and *Da(1)-12*, have a functional FRI protein, but they flower earlier due to the lower steady-state mRNA levels of *FLC*. Somehow *FLC* is not effectively activated by FRI in these genetic backgrounds. In *Ler*, there is a 1.2-kb insertion of a Mutator-like transposable element (TE) in the first intron of *FLC*. When this TE was transferred to plants otherwise strongly expressing *FLC*, they gained the L*er* early flowering phenotype. This insertion triggers a silencing mechanism mediated through an siRNA pathway that leads to inactivation of the TE through the creation of islands of heterochromatin occupied by H3K9me2. As a secondary result of TE silencing *FLC* expression is also inhibited (Michaels et al., 2003; Liu et al., 2004).

Stable transcriptional shutdown of *FLC* only occurs after prolonged exposure of plants to cold. However, early mechanisms of *FLC* repression already respond at the first stages of low temperature exposure and is initiated by antisense lncRNAs synthesized just downstream

of the *FLC*-coding sense transcript poly(A) site. A full collection of *FLC* antisense transcripts is collectively named as *COOLAIR*. They are alternatively spliced and polyadenylated, either at a proximal site to give 400 nt class I transcripts or at a distal site within the *FLC* promoter region to give 750 nt class II transcripts (Swiezewski et al., 2009). While sense and antisense transcripts can co-occur in the same cell, they are mutually exclusive at individual loci. Cold and the function of autonomous pathway proteins strongly upregulate *COOLAIR* transcription in an increasing number of cells and through mutually exclusive relationships the shutdown of sense *FLC* transcription in *cis* is facilitated. *COOLAIR* transcripts form dense clouds, acting to influence *FLC* transcription through changed H3K36me3 dynamics (Rosa et al., 2013). The activation of *COOLAIR* expression also causes FLD-dependent reduction in H3K4me2 levels, which suppresses *FLC* expression (Liu et al. 2010).

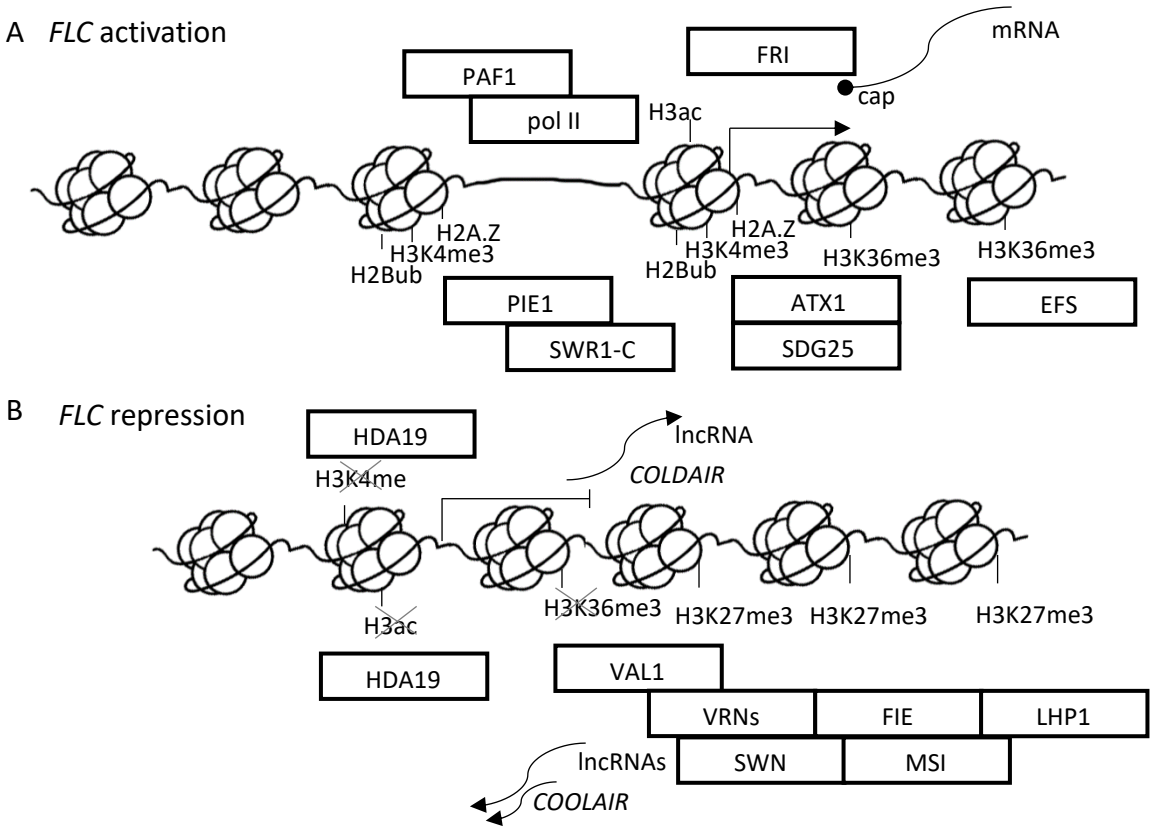

Fig.5 Epigenetic regulation of *FLC* transcription. A) *FLC* transcription takes place through maintenance of an active chromatin state. Several complexes are required to maintain the expression of *FLC*, thus preventing inadequate flowering before spring. B) Silencing of *FLC* transcription is ensured by several protein complexes and lncRNAs that mediate the removal of activating histone modifications and deposition of repressing histone marks.

Remarkable is the fact that reduction in *FLC* expression and deposition of H3K27me3 does not happen gradually as a result of quantitative changes in every locus but is the result of digital switching (simple binary on/off mechanism) in an increasing proportion of cells. These observations suggest that chromatin states are bi-stable, and that there is dynamic switching between states (Angel et al., 2015; Berry et al., 2015).

2.2.2.2 Chromatin-mediated regulation of *FT*

The epigenetic regulation of *FT* expression is not described to such a large extent as in the case of *FLC*, but progress has been achieved concerning the chromatin-mediated control of this floral integrator gene. Phylogenetic shadowing of the *FT* promoter regions between different *Brassicaceae* family members identified three conserved blocks. In the case of the Arabidopsis *FT* homologue these regions are localized at the TSS (block A), a middle region localized ~4kb upstream of the TSS (block B) and a distal region localized ~5.7 k upstream of TSS (block C) (Adrian et al., 2010). Soon it was revealed that each one of them have a different function in the transcription regulation of the gene. Before the reproductive transition, *FT* mRNA is expressed in the phloem companion cells of the minor leaf veins. After the floral transition, the expression level of *FT* dramatically increases in the cauline leaf veins, the vascular bundles of sepals, petals, the funiculi and the septum of developing siliques and in the vasculature of floral stems during silique maturation. Blocks A and C were shown to be very important for the spatiotemporal regulation of *FT* expression. Both regulatory regions are needed for the daylength dependent expression of *FT* in the veins of rosette leaves (Adrian et al., 2010). After the floral transition, block C is needed for *FT* expression in cauline leaves and vascular bundles of sepals and petals, but post-fertilization *FT* expression in the siliques is block C- and day length-independent (Liu et al., 2014).

It was proposed that factors that bind block C and block A interact with each other in the regulation of daylength-dependent *FT* expression. Under inductive LD conditions, CO interacts with Nuclear Factor (NF) transcription factor family members and binds to proximal block A. A complex of other NF family members, named as NF-Y, binds to the distal block C. CO-NF and NF-Y interact with each other, and thus, a loop between block A and C is created. Block C acts as an enhancer, and in consequence the activation of *FT* occurs. The formation of the loop additionally stabilizes *FT* activation by preventing the binding of repressive factors in the promoter region localized inside the loop (Cao et al., 2014; Luo et al., 2018).

Before acquiring the competence to flower, *FT* chromatin is kept in an inactive state. The best-known *FT* repressor is FLC (Searle et al., 2006) but many other factors have a role in *FT* repression, including Polycomb group proteins (PRC1 and 2) (Jiang et al., 2008). For example, PRC1-like complexes containing EMBRYONIC FLOWER 1 (EMF1) and LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1) control the circadian expression of *FT* in the vasculature, preventing *FT* upregulation before dusk and at night by maintaining H3K27me3 levels on *FT* gene body (Wang et al., 2014). LHP1 was shown to be depleted in block A and C, and mediates *FT* transcriptional regulation through the 4.0-kb *FT* promoter region (Adrian et al., 2010). The binding of Polycomb group proteins to *FT* promoter is disturbed by the formation of the loop between block A and C. A different repressive mechanism is conferred by TEMPRANILLO (TEM) proteins 1 and 2. They bind proximal promoter block and competes with CO in *FT* regulation: with low CO level, *FT* expression is being silenced by TEM proteins, but once the level of CO increases, it outcompetes TEM protein and leads to *FT* activation (Castillejo and Pelaz, 2008).

2.2.3 Chromatin-mediated regulation of inflorescence development

For the initiation of the flower primordium at the flanks of the inflorescence meristem, an auxin maximum is required. Information coming from auxins is conveyed to patterns of gene

expression through histone acetylation (Wu et al., 2015). When auxin levels are low, auxin-labile AUX/IAA proteins bind genomic regions of *AUXIN RESPONSIVE FACTORS* (*ARF*), in particular the *ARF5/MONOPTEROS (MP)* gene. AUX/IAA protein interacts with a protein complex containing HDA19 and the TOPLESS (TPL) corepressor. This complex prevents acetylation of the *ARF5* locus and ensures a repressive chromatin environment. In response to hormone stimuli, when auxin levels are high, AUX/IAA proteins are degraded and the HDA19/TPL complex de-attach from the *ARF5* locus (Szemenyei et al., 2008; Wu et al., 2015). This HDA19-containing complex also prevents ARF5 interaction with two members of SWI/SNF complex, BRM and SPLAYED (SYD). Increased auxin levels in the primordium founder cells lead to AUX/IAA protein degradation, loss of TPL/HDA19, and physical interaction of SWI/SNF complexes with transcription factor ARF5/MP, leading to opening up of the chromatin at ARF5/MP target loci such as *LFY* and *FILAMENTOUS FLOWERS* (*FIL*) required for floral development (Wu et al., 2015). Two AINTEGUMENTA-LIKE/PLETHORA (AIL/PLT) transcription factors are also induced by auxin-activated ARF5/MP and jointly with LFY regulate flower primordium initiation (Yamaguchi et al., 2013).

The phyllotaxy of the inflorescence and the size of the internode and pedicels also depend strongly on chromatin remodelers. KNOTTED1-LIKE HOMEOBOX GENE 1 (KNAT1, called also BREVIPEDICELLUS, BP) interacts with the chromatin remodeler BRM and shapes plant architecture through the repression of genes determining meristem maintenance via stabilization of H3K4me3 levels (Zhao et al., 2015). ERECTA (ER), a protein that regulates inflorescence architecture by promoting localized cell proliferation, interacts with the SWR1-C. This complex exchanges histone H2A-H2B dimers with H2A.Z-H2B dimers relaxing the interaction between DNA and histone and activating gene expression, including *PACLOBUTRAZOL RESISTANCE1* (*PRE1*) family genes responsible for shaping inflorescence architecture via regulation of cell proliferation (Cai et al. 2017).

2.2.4 Chromatin-mediated regulation of flower development

Flower development genes are repressed during vegetative growth by Polycomb group (PcG) proteins as revealed by the study that compared the genome-wide distribution of H3K27me3 in PcG mutants and Arabidopsis WT plants (Wang et al., 2016). In this work, distinct sets of loci showed apparent reduction of H3K27me3 in mutant plants deficient in different PcG members, suggesting that PcG subunits have non-redundant roles in H3K27me3 deposition in local regions. Clustering of genes and comparison of expression data led to the selection of two gene groups regulated by single PRC2 components - flower development and seed development. On this way, the expression of flower development genes is repressed in seedlings preferentially via a non-redundant role of CURLY LEAF (CLF), which specifically associates with LHP1 (Wang et al., 2016). EMF proteins also seem to be involved in that process, since the transcriptomic expression profile of seedlings of *emf* mutants is very similar to WT flowers, and the mutants ectopically express flower identity genes (Moon et al., 2003).

Furthermore, genome-wide changes in H3K4me3 and H3K27me3 can be readily observed during flower development, particularly concerning newly expressed genes (Engelhorn et al., 2017). H3K4me3 is concentrated around the TSS and covers the proximal promoter and 5’ region of genes. On the other hand, H3K27me3 is spread all over the transcribed region of genes with a decrease towards the 3’ end. Expression changes during early

flower morphogenesis quantitatively correlate with H3K4me3 levels. A noticeable peak of H3K4me3 at the TSS of expressed genes can be observed. H3K27me3 mark decreases in expressed genes, but it reacts slowly after prolonged expression change, suggesting that the change in H3K27me3 is a response to the already on-going transcriptional transition. Among H3K4me3 marked genes, many targets of homeotic proteins SEP3 and AP1 can be found (Engelhorn et al., 2017). Interestingly, some of the proteins encoded by floral homeotic genes have been associated with the H3K27me3 demethylase RELATIVE OF EARLY FLOWERING 6 (REF6). This protein has a clear opposing function to PcG proteins, and *REF6* overexpression lines resemble *clf* and *lhp1* mutants. It has been proposed that REF6 is part of AG and SEP3 protein complexes (Smaczniak et al., 2012). This observation reinforces the importance of H3K4me3 increase and H3K27me3 decrease in the regulation of expression changes during flower development.

In Drosophila, homeotic genes are repressed by PcG proteins that decorate chromatin with H3K27me3, while they are activated by Trithorax group (trxG) proteins that mark chromatin with H3K4me3. A similar scheme seems to work also for Arabidopsis, as Polycomb and Trithorax proteins seem to have the same targets. *AP3, PI* and *AG*, among some other flowering genes, are repressed by H3K27me3 on their gene body. They are also activated by ATX1, a Trithorax group protein with H3K4 methyltransferase activity that is responsible for H3K4me3 acquisition on those genes (Alvarez-Venegas et al., 2003). More information is available regarding the regulation of the *AG* gene. For this gene, another trxG player, ULTRAPETALA1 (UTL1), is involved (Carles and Fletcher, 2009). This protein directly binds the *AG* locus through the SAND domain. Moreover, UTL1 interacts with ATX1 and together overcome the repressive action of the PcG protein CLF (Carles and Fletcher, 2009). Therefore, the H3K4me3 cascade starts with H3K4 methyltransferases affecting chromatin genome-wide. Among the target regions are MADS-box encoding genes that bind downstream flower development genes. The final effect of the binding is an opening of the chromatin structure around target genes leading to transcriptional activation (Engelhorn et al., 2017). Different chromatin remodelers are also involved in this process. BRM and SYD are recruited by LFY and SEP3 to the B and C class homeotic genes. Their function to overcome Polycomb repression is essential since without the changes mediated by BRM and SYD in chromatin, those genes cannot be properly expressed (Wu et al., 2012). Also, flower homeotic genes themselves can overcome the repressive action of PcG proteins. The genomic region of *KNUCKLESS* (*KNU*) harbors a Polycomb response element (PRE) that is bound by PcG proteins and repressive H3K27me3 is acquired. AG is an activating factor of *KNU* expression. It binds the CArG box-containing region that also harbors the PRE. AG appears to be a competitive inhibitor of Polycomb action, because AG binding prevents the interaction of PcG proteins. As a result, H3K27me3 deposition is blocked and the mark is being diluted through the cell cycle progression to facilitate transcriptional activation (Sun et al., 2014).

Floral homeotic genes are regulated by many other factors. For example, in the *hda19* mutant homeotic transformations from sepal to petal can be observed, as well as defects in carpel development. This is caused by the abnormally high acetylation level at the promoters of B, C and E-class genes and their ectopic expression in broader domains than in WT (Krogan et al., 2012). The H3K36me3 mark, introduced by methyltransferases SDG7 and 8, also seems to be equally important. This mark is enriched in the 5’ region of transcribed genes (Roudier et

al., 2009). The homeotic transformation of floral organs displayed by the *sdg8* mutants is potentially due to spreading of *AG* expression domain into whorls 1 and 2 due to downregulation of *AG* repressors (Grini et al., 2009). Also repressive marks such as H3K9me2 have an impact on the expression of floral homeotic genes. First of all, *AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 21*/*GIANT KILLER* (*GIK*) was identified as an AG target. *GIK* overexpression and *gik* loss of function lines exhibit defects in patterning and differentiation of reproductive organs, supporting a role for GIK as mediator of *AG* action to fine tune expression of genes downstream of this floral homeotic gene. Interestingly, binding of GIK to its target genes is associated with changed levels of H3K9me2 in the promoter region of bound genes (Ng et al., 2009). Another example comes from studies with the rice Jumonji C (jmjC) domain-containing protein JMJ706 that reverses H3K9 di- and trimethylation. This protein is involved in flower development and is localized in heterochromatin. The lack of this protein causes increased H3K9me2/me3 and misregulation of flowering gene expression (Sun and Zhou, 2008). In fact, mutant *jmj706* lines have disturbed flower morphology and organ number. The chromodomain (CHD) family member PICKLE (PKL) promotes flower patterning and upregulation of floral homeotic genes (Aichinger et al., 2011), while BAF60, a component of the SWI/SNF chromatin-remodeling complex, represses them, since in *baf60* mutants genomic regions containing floral homeotic genes displayed altered nucleosomal density and the genes themselves are overexpressed (Sacharowski et al., 2015).

2.3 EARLY BOLTING IN SHORT DAYS, a plant specific histone reader with important roles in reproductive development control

In the light of the recent work of the members of our laboratory, EBS emerged as a very important player in the chromatin-mediated regulation of different aspects of plant development. *ebs* mutants were first identified as early bolting *via* mutant screenings after chemical mutagenesis with ethyl methane-sulfonate (EMS) and transposon tagging strategies. The switch from vegetative to reproductive phase occurs earlier in *ebs* mutants than in WT seedlings as a result of the reduction in the adult vegetative phase of the plant. The early flowering phenotype is especially noticeable under SD conditions (Gómez-Mena et al., 2001). Detailed sequence analysis of EBS revealed that this protein contains three important domains: a nuclear localization domain that directs the protein to the nucleus; a BROMOADJACENT HOMOLOGY DOMAIN (BAH) involved in protein-protein interactions and heterochromatin formation (Yang and Xu, 2013; Zhao et al., 2016); and a PHD finger motif found in many proteins recognizing H3K4me3 and interacting with proteins mediating chromatin remodeling (Wysocka et al., 2006; Matthews et al., 2007; Lee et al., 2009 among others). The induction of the floral transition in the *ebs* mutants is caused by the overexpression of *FT* (Piñeiro et al., 2003), a direct target of EBS that binds regulatory regions close to TSS of this floral integrator. EBS, through the PHD domain, binds specifically di- and trimethylated lysine 4 on H3. EBS also interacts with histone deacetylases, namely HDA6 and HDA19, and modulates the acetylation level on the *FT* locus, preventing a premature activation of this floral promoter. In the *ebs* mutant, the absence of this stabilizing mechanism causes increased histone acetylation and premature expression of *FT* (Fig 6; López-González et al., 2014).

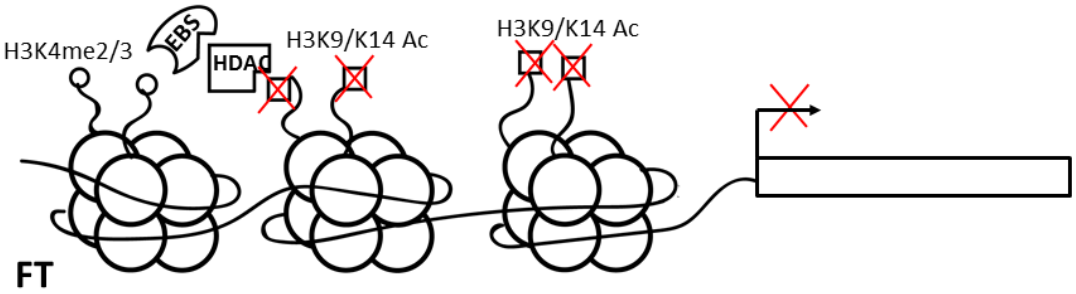

Fig.6. The model of EBS action for the control of *FT* expression. EBS, through the PHD domain binds di- / tri-methylated K 4 of histone H3 in the *FT* promoter, close to its TSS. EBS binding is proposed to recruit histone deacetylases (HDAC) to the locus to remove acetylation from K9/K14 in histone H3, preventing the premature expression of *FT* (adapted from López-González et al., 2014)

Additionally, EBS was shown to be required for the normal expression of some floral organ identity genes during reproductive development, and to partially suppress the floral defects present in strong *lfy* mutants (Gómez-Mena et al., 2001). Moreover, recent results have revealed an additional role for EBS in the regulation of seed dormancy in Arabidopsis (Narro-Diego et al., 2017), suggesting that EBS plays prominent roles in the control of a number of aspects of plant development.

In this work, we pursue the study of EBS involvement in additional aspects of reproductive growth, such as the architecture and phyllotaxy of Arabidopsis inflorescence, flower development, and meristem identity. All these aspects of plant growth are disturbed in plants lacking a functional EBS protein. We present a detailed description of these developmental abnormalities observed in *ebs* mutant, trying to uncover the link between the altered phenotypes with deregulation of genetic pathways and contribute to shed light on the molecular mechanism that mediates the role of EBS in chromatin-dependent regulation of gene expression to modulate reproductive development in plants.

OBJECTIVES

Chromatin remodeling represents a key factor in the establishment and maintenance of spatiotemporal gene expression patterns that govern development in eukaryotic organisms, and particularly in plants. However, and despite recent progress, detailed knowledge on how plants establish and sustain gene expression patterns that characterize specific developmental stages is still largely missing. Our group is interested in understanding how chromatin remodeling mechanisms regulate development, and particularly reproductive development, in the model plant *Arabidopsis thaliana*. The isolation and characterization of mutants for the *EBS* gene have shown that this protein is crucial for different processes connected with reproductive growth. EBS regulates flowering time by participating in chromatin-mediated repression of the floral activator *FT* and is involved in the proper development of the flowers. We decided to gain a deeper insight into the role of EBS during reproductive development of Arabidopsis. To accomplish our goal we proposed the following specific objectives:

1) Characterize the effect of a loss-of-function mutation in *EBS* on reproductive development under different growth conditions.
2) Characterize in detail the effect of the *ebs* mutation on flower development.
3) Analyze the effect of *EBS* on the regulation of genes that control reproductive development in Arabidopsis through transcriptomic studies.
4) Identify candidate genes mediating developmental alterations observed in *ebs* plants.
5) Characterize the effect of EBS on the chromatin features of its target genes.
6) Identify partner proteins mediating EBS function in the control of plant development.
7) Analyze the possible genetic relationships between *EBS* and genes encoding key regulators of reproductive growth.

MATERIALS AND METHODS

1. Plant material

The list of plant material used in this study is given in Table 2.

Table 2: List of plant material used in this study

USE	ACCESSION	SOURCE
tagged protein		
cmyc-EBS	L*er*	already available in the lab (López-González et al., 2014)
INTACT		
SUC2 promoter	L*er*	kindly provided by Franziska Turck
SUC2 promoter in *ebs* background	L*er*	this work
SUC2 promoter in cmyc-EBS background		this work
mutants		
agl24	Col	NASC ID: N854662
agl24 ebs	Col	this work
ft-1	L*er*	already available in the lab (Piñeiro et al., 2003)
ft-1 ebs	L*er*	already available in the lab (Piñeiro et al., 2003)
flc-3	Col	already available in the lab
flc-3 ebs		this work
hda19	Ws-2	Cold Spring Harbor Laboratory
hda19+/- ebs		already available in the lab
lfy-5		already available in the lab
lfy-5 ebs		this work
tfl1-2	L*er*	NASC ID: N3091
tfl1-2 ebs	L*er*	this work
AP1::GUS	Col	kindly provided by Gerco Angenent
FT::GUS	L*er*	already available in the lab
FT::GUS ebs	L*er*	already available in the lab
35S::FT	L*er*	already available in the lab
35S::FT ebs	L*er*	this work

2. Plant growing conditions

2.1 In vitro

Seeds were sterilized in hermetically closed container by the vapors released from the 3% HCl solution in commercial bleach for 4 h at room temperature. After sterilization they were plated on GM Germination Medium (MS medium supplemented with 2% sucrose) and stratified at 4 °C for 2 - 4 days in darkness.

2.2 Plant growth chamber

Seeds were sterilized in -70 °C overnight. After incubation they were sowed on growing substrate containing a 3:1 mix of soil and vermiculate and stratified at 4 °C for 2-4 days in darkness. Short day (SD) conditions refer to 8 hours of light followed by 16 h of darkness in 22 °C. Long day (LD) conditions consists in 16 h of light followed by 8 h of darkness in 22 °C. In both cases light intensity was ~120µE m^{-2} s^{-1} and relative humidity of 65%.

3. Quantification of the frequencies of developmental defects

The numbers of lateral stems and branches were represented as direct numbers. The frequency of phyllotaxy defects and floral reversion were represented as % of plants with defects on a scale 1 - 10 (where 100% = 10). The time of the opening of the first floral bud was

calculated as number of days between bolting and opening of the first flower, the longest time within the experiment was set to 10, and the time for the other background/conditions were normalized over the longest time as a relative fraction (if A is the longest and equals 20 days and B is another condition and equals 5, then after normalization A = 10 and B = 2.5)

4. Scanning electron micrographs

Micrographs of mild flower developmental defects were made in tabletop microscope Hitachi TM3030Plus without fixation. Micrographs of floral reversion were made in JEOL JSM6400 electron microscope. In the latter case samples were first fixed with 4% formaldehyde and 2.5% glutaraldehyde in Milloning buffer (0.1 M NaH_2PO_4, pH = 7.2 adjusted with NaOH) for 12h at 4 °C, washed 3 times x 10 min with distilled water and dehydrated in increasing acetone concentrations (30%, 50%, 70%, 80%, 90%, 100%, 15 min each incubation) and coated with gold.

5. Histochemical GUS Assay

GUS staining was performed as previously described (Lázaro et al., 2008) with the overnight incubation in room temperature instead of 37 °C.

6. Analysis of gene expression

6.1 RNA isolation

Material was collected into 1.5 mL Eppendorf tubes containing 1 mm glass beads, frozen in liquid nitrogen and homogenized in a Ventura Mix 2 vibrating mill for 5 s once, in case of whole seedlings and leaf tissue, or 3 times with refreezing for 5 s in liquid nitrogen in between each shaking cycle, in case of meristematic tissue. RNA was isolated using EZNA Plant RNA Kit (Omega Bio-tek) following manufacturer's instructions. Total RNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

6.2 cDNA synthesis

1 μg of RNA was treated with RQ1 RNase-free DNase (Promega) to remove contamination with genomic DNA. cDNA was synthesized using RevertAid RT reverse transcriptase (ThermoFisher) and random hexamers as primers.

6.3 Quantification of RNA expression

cDNA was diluted 5 times with sterile MilliQ water and 4 μL were taken for the qPCR reaction performed with the Light Cycler FastStart DNA Master Sybr Green I Kit (Roche) following manufacturer's instructions. Relative expression levels were normalized to the expression of housekeeping gene *PP2A* and represented as a fold change of the difference between the expression of a gene and a control. *PP2A* (At1g13320) gene was chosen (Czechowski et al., 2005) as it showed the most stable expression in our conditions (Fig.7). The list of primers used for gene expression analysis can be found in Table 3.

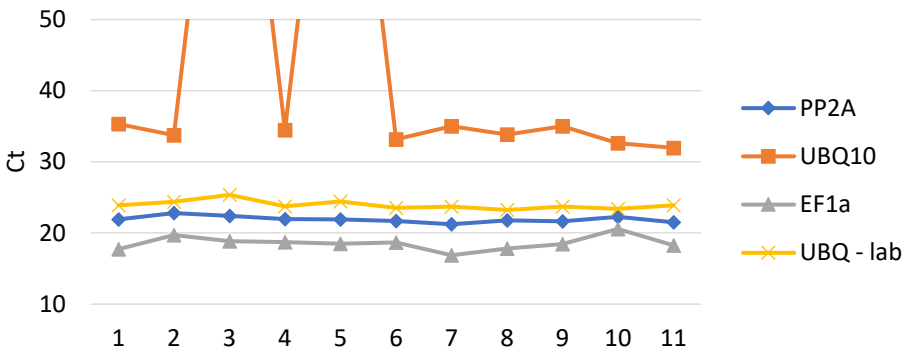

Fig.7 Comparison of different control genes used in qPCR analysis. Seedlings were collected every second day starting 14 days after germination and RNA was prepared. 200 ng of synthesized cDNA were used for qPCR analysis and the Ct- values of chosen housekeeping genes described in Czechowski et al., 2005 and *UBQ* (UBC21, AT5G25760) previously used in the laboratory were compared between samples from different time points. *PP2A* (At1g13320) showed the most stable expression pattern and was chosen for normalization of relative expression in subsequent experiments.

Table 3: List of qPCR primers used in this study

GENE			**SEQUENCE**	
AG	F:	5'-	GGGTCAATGTCCCAAAGA	-3'
	R:	5'-	GCACGAAGAATCTGGTTATCG	-3'
AGL24	F:	5'-	GCGGCTGGAGAAACTACTTG	-3'
	R:	5'-	GCCTCTTTAAGCGTCGTCAG	-3'
AP1	F:	5'-	GCAAGCAATGAGCCCTAAAG	-3'
	R:	5'-	ACTGCTCCTGTTGAGCCCTA	-3'
FT	F:	5'-	CTGGAACAACCTTTGGCAAT	-3'
	R:	5'-	AGCCACTCTCCCTCTGACAA	-3'
FLC	F:	5'-	AGCCAAGAAGACCGAACTCA	-3'
	R:	5'-	TTTGTCCAGCAGGTGACATC	-3'
LFY	F:	5'-	ACGCCGTCATTTGCTACTCT	-3'
	R:	5'-	CTGCGTCCCAGTAACCACTT	-3'
PI	F:	5'-	GGGATGCTAAGCATGAGAACC	-3'
	R:	5'-	ATCTCCATCTGGTGGTCTCG	-3'
PP2A	F:	5'-	AACGTGGCCAAAATGATGC	-3'
	R:	5'-	GTTCTCCACAACCGCTTGGT	-3'
SEP3.	F:	5'-	CATGCTTCGGACACTGGAG	-3'
	R:	5'-	GGGTTCTCTGTAAGGCGTCA	-3'
SHP1	F:	5'-	CGAAGTTGCCCTCGTCATCT	-3'
	R:	5'-	GTGACGGAAGGAGGGTTGAC	-3'
SOC1	F:	5'-	CGTTATCTGAGGCATACTAAGGATCG	-3'
	R:	5'-	CCTTAAACACTTGAGTCTTTCTTGC	-3'
SUC2	F:	5'-	AACAGGTCCACCTGGTAACG	-3'
	R:	5'-	GAGGGACGACAATGGCTAGA	-3'
SVP	F:	5'-	CGAAGTTGCCCTCGTCATCT	-3'
	R:	5'-	GTGACGGAAGGAGGGTTGAC	-3'
TFL1	F:	5'-	CTTCACTTTGGTGATGATAGAC	-3'
	R:	5'-	CTTGGCAATTCATAGCTCAC	-3'

7. Global transcriptomic analysis

Plant material was collected into 1.5 mL Eppendorf tubes, frozen in liquid nitrogen and homogenized in the tube with a plastic pestle. mRNA isolation and cDNA synthesis were performed with Dynabeads® mRNA DIRECT™ Micro Kit (Thermo Fisher Scientific) following manufacturer's instructions. Libraries for RNA-seq analysis were performed with TruSeq RNA library Kit (Illumina).

Bioinformatic analysis was performed with the Galaxy platform (https://usegalaxy.org/), following the standard Tuxedo suit pipeline (Trapnell et al., 2012). The quality of the reads was analyzed by FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). GROOMER was used to convert the reads into Sanger reads format. 15 first nucleotides were trimmed with the FASTQ Quality Trimmer. RNA-seq reads were mapped into TAIR10 reference genome with Tophat, reads were then assembled into transcripts with Cufflinks and datasets were merged with Cuffmerge. Differentially expressed genes between WT and *ebs* samples were identified by Cuffdiff using transcript per kilobase of exon per million fragments mapped (FPKM) normalization. The results were validated by qPCR on separately collected tissues. List of primers used for analysis can be found in Table 4.

Table 4: List of qPCR primers used for the validation RNA-seq results

GENE			SEQUENCE		SOURCE
AG	F:	5'-	GGGTCAATGTCCCAAAGA	-3'	
	R:	5'-	GCACGAAGAATCTGGTTATCG	-3'	
AGL17	F:	5'-	GCAACTAACGGGAGTGGAAT	-3'	Han et al., 2008
	R:	5'-	CTTATATGATCTTAGCTGTTTGAAG	-3'	
AGL24	F:	5'-	GCGGCTGGAGAAACTACTTG	-3'	
	R:	5'-	GCCTCTTTAAGCGTCGTCAG	-3'	
AGO9	F:	5'-	ATCAAGGCGTCCCAACCAAA	-3'	
	R:	5'-	AAGGACTGGCGAACAAGGAG	-3'	
BCA2	F:	5'-	TAAACATGGTCCCCTTTTGG	-3'	
	R:	5'-	TGAGAGGGACGAAGAGAAGG	-3'	
FDM2	F:	5'-	CCACCCTTTGCTCTACATGG	-3'	Xie et al., 2012
	R:	5'-	AAGTCGGGTACCGACCACTT	-3'	
FLC	F:	5'-	AGCCAAGAAGACCGAACTCA	-3'	
	R:	5'-	TTTGTCCAGCAGGTGACATC	-3'	
FLOR1	F:	5'-	TCTACGGGAAGATACCACC	-3'	Torti et al., 2012
	R:	5'-	AAGGGGAGTTCCACAAAGAC	-3'	
FTM	F:	5'-	CCCGATGCTATTCGAACATT	-3'	Torti et al., 2012
	R:	5'-	TCTCTCGTCTGCACGCTCT	-3'	
H1.3	F:	5'-	CGGATCAAGCCCTTATGCTA	-3'	Rutowicz et al., 2015
	R:	5'-	CCTGCTGCCTCGTTATCATC	-3'	
HDA7	F:	5'-	GCTGATTCTTTAGCTGGTGATCCGT	-3'	Cigliano et al., 2013
	R:	5'-	CCAGCAACGAGCAACATTTGGAAG	-3'	
HDA18	F:	5'-	TTTGGCGATGATGGAGACTTT	-3'	Liu et al., 2013
	R:	5'-	CCTTGCTCCCATGGAACGT	-3'	
HEC1	F:	5'-	ATGGATTCTGACATAATGAACATGA	-3'	Schuster et al., 2015
	R:	5'-	GTAAGCTGTGTTGGGAGAGATAGAA	-3'	
LEA7	F:	5'-	GAGGCAAGGCTCAGGAGAA	-3'	Goeres et al., 2007

	R:	5'-	GCAGCTTGAGACGTCTTGTCTT	-3'	
SEP3	F:	5'-	CATGCTTCGGACACTGGAG	-3'	
	R:	5'-	GGGTTCTCTGTAAGGCGTCA	-3'	
TCP1	F:	5'-	GGTACGGTGAAGAAGAAGTGG	-3'	Guo et al., 2010
	R:	5'-	TCCTCTAGCTTTGGCTCCTAG	-3'	
TFL1	F:	5'-	CTTCACTTTGGTGATGATAGAC	-3'	
	R:	5'-	CTTGGCAATTCATAGCTCAC	-3'	

8. Chromatin immunoprecipitation (ChIP)

For ChIP experiments followed by enrichment analysis by quantitative PCR (ChIP-qPCR) chromatin immunoprecipitation on c-myc tagged EBS expressing lines was performed using μMACS Anti-c-myc isolation kit (MACS Miltenyi Biotec). Chromatin extraction was performed as described in detail in Komar et al., 2014, with some modifications. Double fixation with 1% formaldehyde and 5mM dimethyl adipimidate in PBS (0.13M NaCl, 3mM Na_2HPO_4, 3mM NaH_2PO_4, pH = 7) was performed. For immunoprecipitation 50 μL of c-myc coated beads was added. Samples were incubated for 2h at 4ºC on a rotating wheel. Binding of the magnetic beads-protein-DNA complexes was performed on MACS μ Columns (MACS Miltenyi Biotec). 200 μL of each ChIP washing buffer (low salt, high salt, LiCl and TE) was used in every step of the wash. DNA was eluted with 3 portions of 50 μL of boiling TE buffer. Chromatin immunoprecipitation on histone marks was performed as described in detail in Komar et al., 2014. Primers used for quantitative ChIP analysis are given in Table 5. List of antibodies used for the immunoprecipitation is given in Table 6.

Table 5: List of primers used in ChIP-qPCR experiment

GENE	PRIMER			SEQUENCE		SOURCE
AGL24	prom 1	F:	5'-	ACAAGTTCGAAATTTGGGCCA	-3'	Liu et al., 2008
		R:	5'-	TTCACGTTTTACCATTTGCCGT	-3'	
	prom 2	F:	5'-	TGCTGTTCATCAGTTCATCTACC	-3'	Liu et al., 2008
		R:	5'-	CTTATCAGGTGTCGCATCTAG	-3'	
	TSS	F:	5'-	TGGTGTTATGGGTATAGGCAGA	-3'	
		R:	5'-	GAGAAAGTAACTTGTCTCGCTGT	-3'	
	I intron	F:	5'-	ATCCCCAATCATACCAAGTGAC	-3'	Liu et al., 2008
		R:	5'-	GTACTGGGAAATAAGAGAGCAG	-3'	
AP3	prom	F:	5'-	TATCACTTAGTTTTCATCAACTTCTG	-3'	Krogan et al., 2012
		R:	5'-	TATCACTTAGTTTTCATCAACTTCTG	-3'	
	3'UTR	F:	5'-	TTTGCTGGTGCCATCATTGTCTATC	-3'	Krogan et al., 2012
		R:	5'-	GATCACACAATCCATATTTCTTTAGGC	-3'	
FT	1C	F:	5'-	TTCAATCGGAATCAGTTCGAC	-3'	
		R:	5'-	TGTTGGCCAAGATGTCTCAC	-3'	
	64,65	F:	5'-	TCTACATCACATCTTTTGTTTC	-3'	FT-10 from Yan et al., 2014
		R:	5'-	TGCACTTTTTAACGACTAGC	-3'	
	FT+	F:	5'-	TGTATTAGTGTGGTGGGTTTGG	-3'	
		R:	5'-	AACTCTGCTTACTATAAGAGGGT	-3'	
	FT 6	F:	5'-	GTTCTTTCACTTGAACTCCCTTTTG	-3'	
		R:	5'-	GGGGTATACTGAAAATATTTCTTGGG	-3'	
	FT -	F:	5'-	GGGATTTTTCTTTGTTCCTCC	-3'	
		R:	5'-	ATTCCACAACAGAGATTCATCA	-3'	
	76,77	F:	5'-	CAACTGGAACAACCTTTGGTGAG	-3'	

		R:	5'-	CGTAACACACAATCTCATTGCCTG	-3'	FT-20 from Yan et al.., 2014
FLC	prom A	F:	5'-	ACTATGTAGGCACGACTTTGGTAAC	-3'	
		R:	5'-	TGCAGAAAGAACCTCCACTCTAC	-3'	
	prom B	F:	5'-	GCCCGACGAAGAAAAAGTAG	-3'	
		R:	5'-	TCCTCAGGTTTGGGTTCAAG	-3'	
	Exon I	F:	5'-	CGACAAGTCACCTTCTCCAAA	-3'	
		R:	5'-	AGGGGGAACAAATGAAAACC	-3'	
	Intron I	F:	5'-	GGCGGATCTCTTGTTGTTTC	-3'	
		R:	5'-	CTTCTTCACGACATTGTTCTTCC	-3'	
	Exones II-VI	F:	5'-	CTTTTTCATGGGCAGGATCA	-3'	
		R:	5'-	TGACATTTGATCCCACAAGC	-3'	
LFY	prom 1	F:	5'-	GCCAGTATTGCCAACTTTCC	-3'	Pastore et al., 2011
		R:	5'-	GGCCAACCTACGTCTTTTTC	-3'	
	prom 2	F:	5'-	TCACCACAGTGAAAACCCTAA	-3'	Pastore et al., 2011
		R:	5'-	TGTGTCTTGCTGGGAAATTG	-3'	
	TSS	F:	5'-	GGAACCCAACGAGAGCATT	-3'	
		R:	5'-	TCTAAACCACCAAGTCGCATC	-3'	
	4	F:	5'-	CTTTCGTTGGGAGCTTCTTG	-3'	Pastore et al., 2011
		R:	5'-	AGCGTGATGAGTACCGGAAT	-3'	
	5	F:	5'-	ATGGGGTATGGTAGGGGAAC	-3'	Pastore et al., 2011
		R:	5'-	TGAAAACCCTGAGAAATCGTG	-3'	
TFL1	prom 2	F:	5'-	GCTTCAGCAACATTGGCATA	-3'	
		R:	5'-	AGCTTTTTATTCGCCGAGGT	-3'	
	TSS	F:	5'-	CGGTCGTCTCTTTGTCTCCC	-3'	
		R:	5'-	ACATCTCCTACCACTCTCCCC	-3'	
	D	F:	5'-	TGAGAGATGCAAGTGGGAAA	-3'	
		R:	5'-	GTCGGCGTTGAGGATAATTG	-3'	
	E	F:	5'-	TGCCTGTGTTGTGTTTAGCC	-3'	
		R:	5'-	ATCAGGGATGGAATGGAACA	-3'	
	F	F:	5'-	CCCTTGGACCGAGTCTACTG	-3'	
		R:	5'-	GGACCTAGCACGACTCTTCG	-3'	
	6	F:	5'-	TGGATTTCAATCAATCCTCTTTT	-3'	Pérez-Ruiz et al., 2015
		R:	5'-	CCAATCCAAACCAAAAACTGA	-3'	
SEP3.	prom 1	F:	5'-	CATGATTCCCTGAACTCGATTTTATAAG	-3'	Krogan et al., 2012
		R:	5'-	GGTAGGGTCTGATAAATCCACCTGATT	-3'	
	prom 2	F:	5'-	CAAAGCCGTCTGATTCTCATCTCAC	-3'	Krogan et al., 2012
		R:	5'-	CTACACGACAGCTAAGTTGCGGAG	-3'	
	TSS	F:	5'-	TGCTTGCAAGGAAAGAGAGAG	-3'	
		R:	5'-	TTGCAAACGTCACTTGCCTA	-3'	
	3' UTR	F:	5'-	GTTTTCTGTCTTGTGTGCATGTG	-3'	Krogan et al., 2012
		R:	5'-	TGGATCAGGAAGTGTAGGAGTAATGG	-3'	
ACT2		F:	5'-	TGTCGCCATCCAAGCTGTTCTCT	-3'	
		R:	5'-	GTGAGACACCATCACCAGAAT	-3'	

Table 6: List of antibodies used in this work

EXPERIMENT/ AB	MANUFACTURER	PRODUCT NO
CHIP		
α c-myc MicroBeads	MACS Miltenyi Biotec	130-091-123
α H3	Abcam	ab1791
α H3 K9/K14ac	Diagenode	C15410200-10
CoIP		
α HA-HRP	Roche	12013819001
α c-myc-HRP	Millipore	16-213
H2A.W.7	Kindly provided by F. Berger (Yelagandula et al., 2014)	
α Rabbit	Abcam	ab6112
α H3K27me3	Millipore	07-449

For ChIP-seq experiments chromatin immunoprecipitation with Anti-c-myc isolation kit was performed as described above. Quantification of DNA abundance was performed with Ligh Cycler FastStart DNA Master Sybr Green I Kit (Roche) as described in the chapter 5.3 of this section. Libraries for ChIP-seq analysis were performed with MicroPlex Library Preparation Kit v2 x12 (Diagenode) following manufacturer's instruction. Bioinformatic analysis was performed on Galaxy platform (https://usegalaxy.org/). The quality of the reads was analyzed by FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). GROOMER tool was used to convert the reads into Sanger reads format. Adaptors were trimmed with the FASTQ Quality Trimmer tool. Reads were mapped to reference genome TAIR10 with Map with Bowtie tool. Peaks were generated by MACS2 tool. Genomic intervals generated by MACS2 were then annotated to the genome by PAVIS tool (https://manticore.niehs.nih.gov/pavis2/ , Huang et al., 2013). Data of the histone marks occupancy across Arabidopsis genome were downloaded from publicly available data: GSM701923 - H3K4me2, GSM701924 - H3K4me3, GSM701925 - H3K9ac, GSM701928 - H3K27me1, GSM701929 - H3K27me3, GSM701931 - H3K36me3, as a raw bed file and analyzed in MACS2 tool in the same way as EBS data against input file GSM701933. This data set was chosen due to the similarity of the tissue type (aerial part) and developmental stage to our samples (see for more detail Luo et al., 2013). The fact that all histone marks occupancies comes from one analysis limits the bias that could be caused due to different analysis techniques. The overlap between histone mark occupancies and EBS binding site was performed by the intersection of the MACS2 outputs in Intersect tool. For the overlap with H2A.W.7, publicly available data that were used that can be found under GSM2516277 GEO accession.

ChIP-seq results were validated by separately collected tissue. Nucleotide sequences of the peaks were retrieved from genomic intervals with GetFastaBed tool. Primers used for the validation are given in Table 7.

Table 7: List of primers used for ChIP-seq validation

PRIMER			SEQUENCE	
GENOMIC REGIONS				
BAF60_1	F:	5'-	TGCTTCATCACAAGCCTCAA	-3'
	R:	5'-	CCCGATCCAAAGAGATCTGA	-3'
BAF60_2	F:	5'-	GGGTTTTTAAGGGCTTCCTG	-3'
	R:	5'-	GGAGTTTGAGTCTCGGGTTG	-3'

EBS_1	F:	5'-	TCAGAACTGCATTCAGCACA	-3'
	R:	5'-	GCATGACGATTGAAGAAGCA	-3'
EBS_2	F:	5'-	AGCTCTTTGGCTCCATGAAA	-3'
	R:	5'-	GCGAGGAACAATGTGAAGGT	-3'
ING1	F:	5'-	GTTTGGATCCACAGGCAGAT	-3'
	R:	5'-	CTAGCAACAGCAGCGTCAAC	-3'
SCZ	F:	5'-	GCAAGTAACGGCATTGAGTG	-3'
	R:	5'-	CAGGCTTGACATGGTTTTGA	-3'
SCZ_SEEDLINGS	F:	5'-	GCTTTTTGGAGTTTCGTTGC	-3'
	R:	5'-	CGTTTTGCTTGTTTGGTCTG	-3'
TEM2	F:	5'-	AACTCGCTGATGCTTCTCGT	-3'
	R:	5'-	ATAAAGGCGTTGTGCCTCAG	-3'
HETEROCHROMATIC REGIONS				
CHR1_1	F:	5'-	TGGTGTAGCCAAAGTCCGTA	-3'
	R:	5'-	ACCGCAACCGGATCTTAAA	-3'
CHR1_2	F:	5'-	AACCGCAACCCGATGTTAT	-3'
	R:	5'-	CATGGTGTAGCCAAAGTCCA	-3'
CHR2_1	F:	5'-	TCGGGTTGCGATTTAAGTTT	-3'
	R:	5'-	ACGGACTTTGGCTACACCAT	-3'
CHR2_2	F:	5'-	TCGGGTTGCGATTTAAGTTT	-3'
	R:	5'-	ACGGACTTTGGCTACACCAT	-3'
CHR3_1	F:	5'-	TGGTGTAGCCAAAATCCGTA	-3'
	R:	5'-	TAAACCGCAACCGGATCTTA	-3'
CHR3_2	F:	5'-	GGACTTTGGCTACACCATGAA	-3'
	R:	5'-	ATCGGGTTGCGGTTTAAGTT	-3'
CHR4_1	F:	5'-	CATGGTGTAGCCAAAGTCCA	-3'
	R:	5'-	TAAACCGCAACCCGATCTTA	-3'
CHR4_2	F:	5'-	CATGGTGTAGCCAAAGTCCA	-3'
	R:	5'-	TAAACCGCAACCGGATCTTA	-3'
CHR5_1	F:	5'-	TAAGATCGGGTTGCGGTTTA	-3'
	R:	5'-	TCATACGGACTTGGCCTACA	-3'
CHR5_2	F:	5'-	AACAACTTAAACCGCAACTCG	-3'
	R:	5'-	CATGGTGTAGCCAAAGTCCA	-3'

9. FAIRE - formaldehyde-assisted isolation of regulatory elements

Chromatin openness was analyzed with the used of FAIRE method that enables the purification of open chromatin regions (nucleosome-depleted DNA) that preferentially segregate into the aqueous phase during phenol-chloroform extraction. In this method chromatin is crosslinked with formaldehyde, sheared by sonication, and phenol-chloroform extracted as described in Omidbakhshfard et al., 2014. The results were quantified relatively to *ACT2* genomic region and normalized over transposon element region TA3. The list of primers used in FAIRE experiments is given in Table 8.

Table 8: List of primers used in FAIRE experiment

PRIMER			SEQUENCE		SOURCE
AGL24 TSS	F:	5'-	TGGTGTTATGGGTATAGGCAGA	-3'	
	R:	5'-	GAGAAAGTAACTTGTCTCGCTGT	-3'	
FT TSS	F:	5'-	TCAACACAGAGAAACCACCTG	-3'	
	R:	5'-	ACGGATCAAGAACGTCTCCA	-3'	
FLC TSS	F:	5'-	GCCCGACGAAGAAAAAGTAG	-3'	
	R:	5'-	TCCTCAGGTTTGGGTTCAAG	-3'	
LFY TSS	F:	5'-	GGAACCCAACGAGAGCATT	-3'	
	R:	5'-	TCTAAACCACCAAGTCGCATC	-3'	
LFY_CONTROL	F:	5'-	TCACCACAGTGAAAACCCTAA	-3'	
	R:	5'-	TGTGTCTTGCTGGGAAATTG	-3'	
TFL1 TSS	F:	5'-	CGGTCGTCTCTTTGTCTCCC	-3'	
	R:	5'-	ACATCTCCTACCACTCTCCCC	-3'	
D	F:	5'-	TGAGAGATGCAAGTGGGAAA	-3'	
	R:	5'-	GTCGGCGTTGAGGATAATTG	-3'	
E	F:	5'-	TGCCTGTGTTGTGTTTAGCC	-3'	
	R:	5'-	ATCAGGGATGGAATGGAACA	-3'	
F	F:	5'-	CCCTTGGACCGAGTCTACTG	-3'	
	R:	5'-	GGACCTAGCACGACTCTTCG	-3'	
6	F:	5'-	TGGATTTCAATCAATCCTCTTTT	-3'	Perez-Ruiz et al., 2015
	R:	5'-	CCAATCCAAACCAAAAACTGA	-3'	
SEP3 TSS	F:	5'-	TGCTTGCAAGGAAAGAGAGAG	-3'	
	R:	5'-	TTGCAAACGTCACTTGCCTA	-3'	
ACT2	F:	5'-	TGTCGCCATCCAAGCTGTTCTCT	-3'	
	R:	5'-	GTGAGACACCATCACCAGAAT	-3'	
TA3	F:	5'-	TAGGGTTCTTAGTTGATCTTGTATTGAGCTC	-3'	
	R:	5'-	TTTGCTCTCAAACTCTCAATTGAAGTTT	-3'	

10. INTACT - isolation of nuclei tagged in specific cell types

INTACT is a method for purification of nuclei from individual cell types. The surface of the nuclei is labelled by nuclear targeting fusion protein (NTF) that consists of three parts: the WPP domain of Arabidopsis thaliana RAN GTPase activating protein 1 (RanGAP1, locus At3g63130; amino acids 1–111, inclusive), which is necessary and sufficient for nuclear envelope association in plants; green fluorescent protein (GFP) for visualization; and the biotin ligase recognition peptide, which serves as a substrate for the *E. coli* biotin ligase BirA19. Expression of NTF driven by cell type specific promoter results in biotin-labeled nuclei exclusively in those cells that can then be then isolated by affinity biotin – streptavidin interaction (Deal and Henikoff, 2011).

Plant cross-linked tissue was grinded in liquid nitrogen and resuspended in 30 mL of Nuclear Purification Buffer (NPB, 20 mM MOPS pH = 7, 40 mM NaCl, 90 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, 1X complete protease inhibitor ROCHE), filtered through miracloth and centrifuged at 1000 g at 4 °C for 10 min. The pellet was resuspended in 15 mL of NPB with 0.1% vol of Triton X-100 to remove photosynthetic

pigments and secondary metabolites, and centrifuged at 1000 g in 4 °C for 10 min. Later on, the pellet was resuspended in 1 mL of NPB. 100 µL of streptavidin coated magnetic beads (Invitrogen) was added and the solution was incubated for 1h. After this step, efficiency of the nuclei isolation was monitored under fluorescent microscope. Nuclei were isolated on a magnetic rack and supernatant (negative nuclei) was kept as a negative control. We then proceeded with the standard ChIP protocol.

11. Co immunoprecipitation (Co-IP) of nuclear proteins from Arabidopsis

5 g of plant tissue were grinded in liquid nitrogen and resuspended in 30 mL of Nuclear Extraction Buffer 1 (NEB1, 10 mM HEPES, pH = 7.4, 0.4 M sucrose, 2 mM EDTA, 2.5 mM DTT, 5 mM 2-mercaptoethanol, 1X complete proteinase inhibitors ROCHE), filtered through miracloth and centrifuged at 1000 g and 4ºC for 10 min. The pellet was resuspended in 5 mL of Nuclear Extraction Buffer 2 (NEB2, 10 mM HEPES pH = 7.4, 0.25 M sucrose, 10 mM $MgCl_2$, 0.5% vol Triton X-100, 5 mM 2-mercaptoethanol, 1X complete proteinase inhibitors ROCHE) and centrifuged at 1000 g and 4ºC for 10 min. The cleaned pellets were resuspended in 300 µL Nuclear Extraction Buffer 3 (NEB3, 10 mM HEPES pH = 7.4, 1.7 M sucrose, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1X complete proteinase inhibitors ROCHE). In the 1.5 mL tube 500 µL of NEB3 were added, and resuspended material was gently pipetted on the top of NEB3 and centrifuged at 16 000 g and 4ºC for 45 min. Supernatant was removed and pellet stored at -80 ºC overnight. The pellet was then thawed on ice and resuspended in Immunoprecipitation Buffer (IP-B, 100 mM Tris-HCl pH = 7.4, 10% vol glycerol, 1 mM EDTA, 75 mM NaCl, 0.1% vol of Triton X-100, 0.05% SDS, 1X complete proteinase inhibitors ROCHE) and sonicated 20 X for 30 s with 30 s break between each sonification cycle in the Bioruptor (Diagenode) set to high power. The sample was sonicated at 12000 g and 4ºC for 30 min. 10% of the supernatant was taken as an input and frozen in -20 ºC. The rest of supernatant was taken for further processing. 30 µL of magnetic beads coated with protein G were incubated for 4 hours at 4ºC with 5 µg of the appropriate antibody. Beads were added to the sample and incubated overnight at 4ºC. After the immunoprecipitation beads were washed 3x with IP-B on a magnetic rack and resuspended in 40 µL with 5X protein loading buffer, and then incubated for 10 min at 95 ºC. 5 µL of input and 20 µL of the Co-IPed sample were used in Western Blot. Antibodies used are given in Table 6.

12. Western Blot

Samples were loaded onto 10% SDS-PAGE gels and the electrophoresis was made in Tris-Glycine running buffer. The proteins were then transferred to PCV membrane by wet transfer method. Membrane was washed for 5 min in 1X PBS buffer and blocked with 3% BSA in 1X PBS overnight. Primary Ab was added at a 1:5000 dilution and the secondary Ab fused with horseradish peroxidase (HRP) with a 1:50000 dilution preceded by 2X 5 min washes in 1X PBS with 0.01% of Tween-20 and 1x 5 min wash in 1X PBS. HRP reaction was activated by Clarity™ Western ECL Blotting Substrate (BIO-RAD).

13. DAPI staining of the nuclei

Young seedlings were harvested, fixed in Carnoy's solution (ethanol/acetic acid 3:1), and stored at −20°C. Spread preparations were made as described by Schubert et al., 2001, with

a modified enzymatic cell wall-degrading mixture, as described by Tessadori et al., 2009. Nuclei slides were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 2 μg ml^{-1}; Roche) in Vectashield (Vector Laboratories).

RESULTS

1. The *ebs* mutant displays a wide array of phenotypic alterations during reproductive development

As described in detail in the Introduction (section: 1.3 Arabidopsis inflorescence architecture), plant architecture is strictly controlled and depends on the timing of floral transition, fate of inflorescence meristem, number of branches and rosette stems and the time and the scheme of flower development. The *ebs* mutant exhibits multiple defects during the reproductive phase of development, resulting in an inflorescence architecture that differs significantly from the one described for Arabidopsis WT plants (Alvarez et al., 1992, Fig.8.A). The shoot apical meristem of the main stem of *ebs* is frequently arrested, causing growth inhibition of the main inflorescence and formation of an increased number of both rosette stems and secondary, or higher order branches. The inflorescence of the *ebs* mutant displays a more vegetative character since in early stages of its growth leaves are formed instead of flowers, and in later stages flowers develop abnormally: the phyllotaxy is disturbed (several floral nodes cluster together in a single point of the main stem) and floral reversion events occur (Fig.8.B).

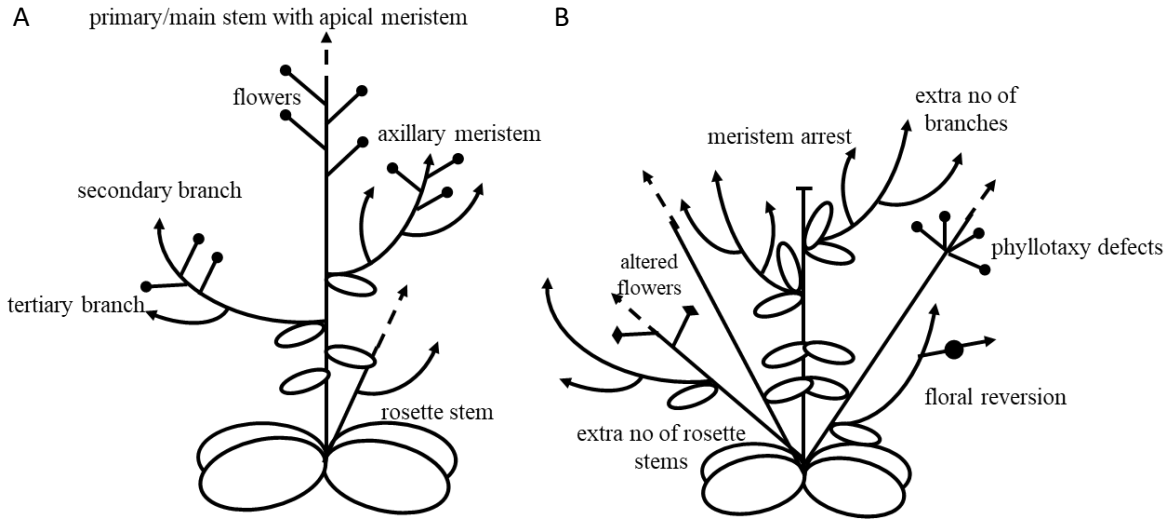

Fig.8 The comparison between WT and *ebs* inflorescence structure: A) Schematic inflorescence structure of a representative WT Landsberg *erecta* plant. Indeterminate inflorescence meristems enable potential indefinite growth. Axillary meristems developed at the axils of cauline leaves (leaves formed on the main flowering stem) produce branches. Additional rosette stems emerge from rosette leaves axillary meristems. Adapted from Alvarez et al., 1992; B) Schematic inflorescence structure of a representative *ebs* (L*er*) plant. The shoot apical meristem on the main stem is arrested, and excessive number of rosette stems and secondary and higher order branches is formed. Altered flowers, phyllotaxy abnormalities and floral reversion can be observed.

1.1 Effect of photoperiod on the developmental defects present in inflorescences and flowers of the *ebs* mutant

Environmental stimuli such as light, temperature and nutrient availability can influence the shape and identity of inflorescence structures. Plants grown under non-inductive flowering conditions that are able to create bigger rosettes with higher leaf number, often develop more secondary branches and rosette stems. In our conditions, L*er* plants grown in LD tend not to

create any rosette stems and only 2-3 secondary branches. When grown in SD, these plants usually generate an extra rosette stem and 3-4 secondary branches on average. Interestingly, *ebs* plants grown in SD conditions showed increased frequency of the growth abnormalities compared with LD photoperiods. In order to understand the impact of growth conditions in the *ebs* phenotypic alterations, we measured the frequency of developmental abnormalities in *ebs* plants grown under different environmental conditions such as photoperiod and ambient temperature.

Regarding photoperiod, and as shown in Figure 9 and Table 9, the defects described for the *ebs* mutant appear at a significantly lower frequency in plants grown in flowering promoting conditions of LD than in plants grown in non-inductive SD conditions. Under LD the apical meristem of the main stem is not arrested, and apical dominance is maintained throughout reproductive development in the mutant. *ebs* plants tend to create one rosette stem and 2 - 3 secondary branches. Developmental abnormalities of the flowers also occur less frequently under LD. Under these conditions, floral reversion is observed in only 8% of the plants. The inflorescence of the *ebs* mutants retains some vegetative features and displays a delay in the development of flowers. This lag measured as the time between the formation of the flowering stem and the opening of the first flower is not long, as first flowers open on average only 2 days after the flowers of the WT. In contrast, phyllotaxy defects occur frequently, as more than 90% of the mutant plants exhibit some alteration in LD (Fig.9). Under SD, apical dominance is lost in *ebs* plants that create on average 2 rosette stems and 8 branches on a main stem. All analyzed plants exhibit signs of floral reversion and phyllotaxy defects. Moreover, the first floral bud opened around 18 days after the formation of a flowering stem, two weeks later than in WT. These observations confirm that the alterations observed in the *ebs* mutant during reproductive development are enhanced under SDs.

Table 9: Detailed quantification of the developmental defects of *ebs* mutants grown under LD and SD photoperiodic conditions

	Meristem arrest [%]	*Lateral stems*	*Branches/main stem*	*Phyllotaxy defects [%]*	*Floral reversion [%]*	*opening of first floral bud [days]*
						LD, 22 ºC
WT	0	1.4	2.3	8.3	0	1.2 +/-1
ebs	0	1.6	2.4	91.7	8.3	3 +/- 1.5
						SD, 22 ºC
WT	0	1.8	3.6	9.2	28.6	3.3 +/- 2.1
ebs	100	2.8	8.3	100	100	17.8 +/- 3.5

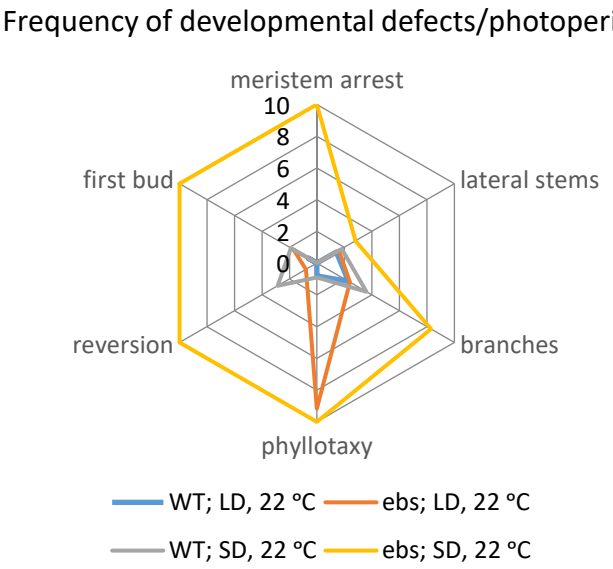

Fig.9 Frequency of reproductive developmental defects observed in the *ebs* mutant as compared to WT plants grown in LD versus SD conditions. Lateral stems and branches are represented as direct numbers; meristem arrest, phyllotaxy defects and floral reversion represented as % of all the plants (10 = 100%); floral bud opening represented as the number of days between bolting and opening of the first flowers, normalized to longest time period (longest time = 10, other values = relative fractions; see material and methods for more detailed description).

1.2 Effect of temperature on the developmental defects present in inflorescences and flowers of the *ebs* mutant

Another factor that directly influences the time of flowering of Arabidopsis plants is ambient temperature. While lower ambient temperature (16-18ºC) results in a delay in the initiation of flowering, warmer environments (26-28ºC) promote an earlier floral transition in most Arabidopsis accessions analyzed (Balasubramanian et al., 2006). For that reason, we also explored a possible influence of this environmental cue on additional stages of reproductive development of the *ebs* mutant. We observed an increase in meristem arrest in *ebs* plants grown at low temperature (18ºC) as compared with the standard 22ºC. But for the rest of traits analyzed during inflorescence and flower development, *ebs* mutant plants grown in LD and 18ºC conditions behave similarly to mutant plants grown in LD and 22ºC. Apical dominance is reduced and 2 or 3 extra rosette stems are formed, however not that many secondary branches are created and overall architecture of the *ebs* mutant is not as severely disturbed by growth in lower ambient temperature as in SD-grown plants (Table 10, Fig.10; Fig.11.A, G).

Finally, a combined effect of non-inductive SD conditions and low ambient temperature was analyzed. Further limitation of flowering promoting signals resulted in a notable increase of the frequency of developmental alterations in reproductive development in the *ebs* mutant (Table 10, Fig.10). In fact, developmental defects as reduced apical dominance caused by the inhibition of growth in the main apex as well as floral reversion can be observed also for the WT plants in this environment. These conditions mainly affect the development of the flowers in *ebs* mutant. The inflorescences of most of the plants keep on producing leaves, and flowers were never formed during one-month screening process starting from the time of bolting (Table 10, Fig.11.L). The percentage of the plants that exhibit phyllotaxy defects in the distribution of

the flowers seems to be lower than in other conditions. However, this observation is likely a consequence of the fact that flowers were not formed in the majority of the plants, and therefore, floral phyllotaxy defects could not be readily observed.

Table 10: Detailed quantification of the developmental defects of *ebs* mutants grown under LD and standard ambient temperature (22 °C), LD and low ambient temperature (18 °C), SD photoperiodic conditions and standard ambient temperature (22 °C), and combined SD photoperiodic conditions and low ambient temperature (18 °C)

	Meristem arrest [%]	*Lateral stems*	*Branches/main stem*	*Phyllotaxy defects [%]*	*Floral reversion [%]*	*opening of first real bud [days]*
LD, 22 °C						
WT	*0*	1,3	2,3	7	0	1.8 +/-1
ebs	*0*	1,5	2,5	87	6,3	3.5 +/- 1.5
LD, 18 °C						
WT	*0*	2,1	3,5	0	0	2.2 +/-1
ebs	*73*	3,7	5,2	73	0	2.9 +/- 1.2
SD, 22 °C						
WT	*0*	1	1,13	12,5	0	3.7 +/- 1.5
ebs	*100*	2	2,88	87,5	100	17.2 +/- 4.2
SD, 18 °C						
WT	*60*	1	9,7	0	60	9.2 +/- 3
ebs	*100*	7,4	8,6	40	100	28+

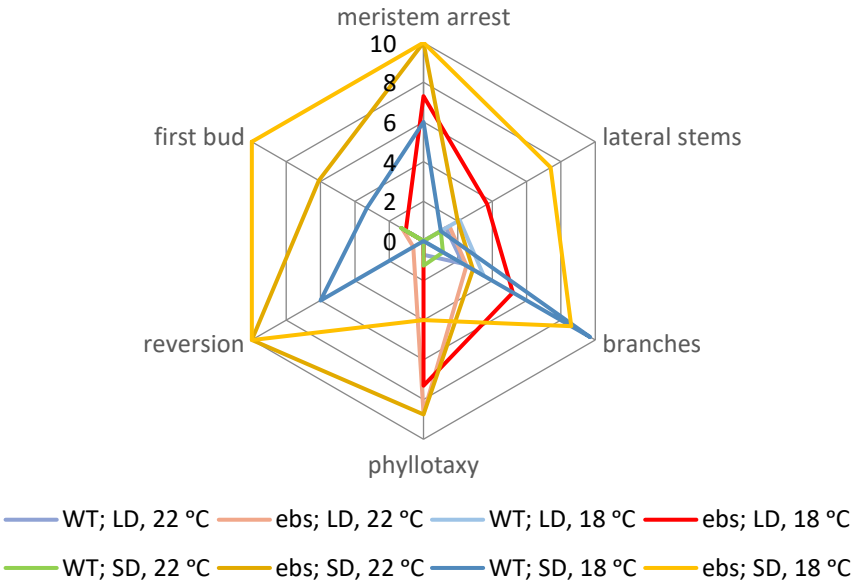

Fig.10 Frequency of reproductive developmental defects observed under different growth conditions in the *ebs* mutant as compared to WT plants. The combined impact of photoperiod (LD versus SD) and variable ambient temperature (standard, 22°C versus lower temperature, 18°C) was analyzed. Lateral stems and branches are represented as direct numbers; meristem arrest, phyllotaxy defects and floral reversion represented as % of all the plants (10 = 100%); floral bud opening represented as the number of days between bolting and opening of the first flowers, normalized to longest time period (longest time = 10, other values = relative fractions; see material and methods for a more detailed description).

In summary, growth conditions have a significant impact in phenotypic alterations of the *ebs* mutant during reproductive development. Conditions delaying the initiation of flowering increase the frequency of the observed defects. Photoperiod seems to have a stronger effect than changes in ambient temperature, but the concurrence of less promoting flowering conditions of SD photoperiod and low ambient temperature result in the strongest defects.

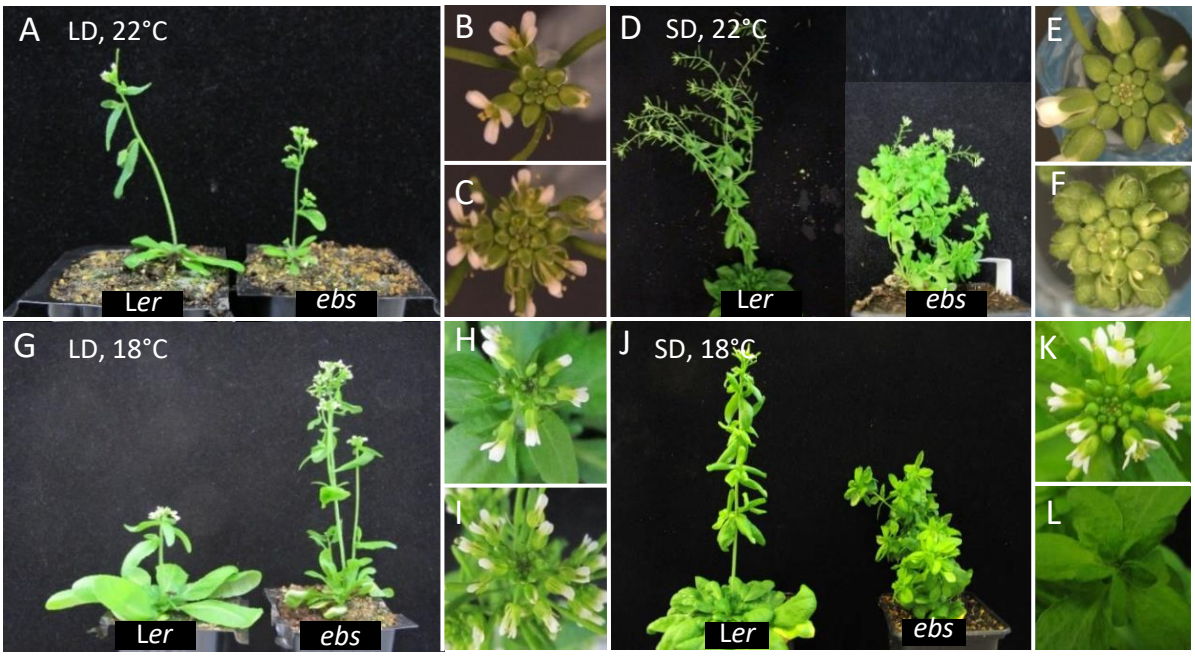

Fig.11 Phenotypes of WT L*er* and *ebs* mutant plants grown under different environmental controlled conditions. A), D), G), J) – Photographs of plant inflorescences; B), C), E), F), H), I), K), L) - Photographs of shoot apices. B), E), H), K) – WT plants; C), F), I), L) – *ebs* mutant.

1.3 Detailed description of the impact that SD photoperiods have on developmental defects observed in *ebs* mutant plants

To analyze in depth the developmental defects of *ebs* mutant plants during reproductive growth, we focused on the comparison of WT and mutant plants grown in SD conditions. These conditions provide a more conspicuous difference between WT and mutant plants that LD only or combinations of LD and low ambient temperature cues, but not as drastic as combined SD and low ambient temperature conditions. A detailed phenotypic analysis performed with the *ebs* mutant both in L*er* (Fig.12) and Columbia (Col, Fig.13) genetic backgrounds revealed that under SD, apical dominance in the inflorescence is indeed drastically reduced making the main stem difficult to distinguish from rosette stems and secondary branches. Rosette stems are not only formed in higher number, but they also often elongate to the same height as the main stem (Fig.12.C, Fig.13.C). Every one of these stems give rise to secondary and higher order branches, providing a bushy appearance to these mutant plants. In fact, the number of secondary branches emerging from the longest stem doubles the average branch number in the WT, and often secondary branches outgrow stem's apices (Fig.12.B, Fig.13.B). In the most extreme cases, the apical part of the primary stem is not only inhibited in growth or completely arrested, but it undergoes senescence and dies. Overall, under non-inductive photoperiodic conditions for flowering, the *ebs* mutant produces dwarf inflorescences with abnormal bushy architecture.

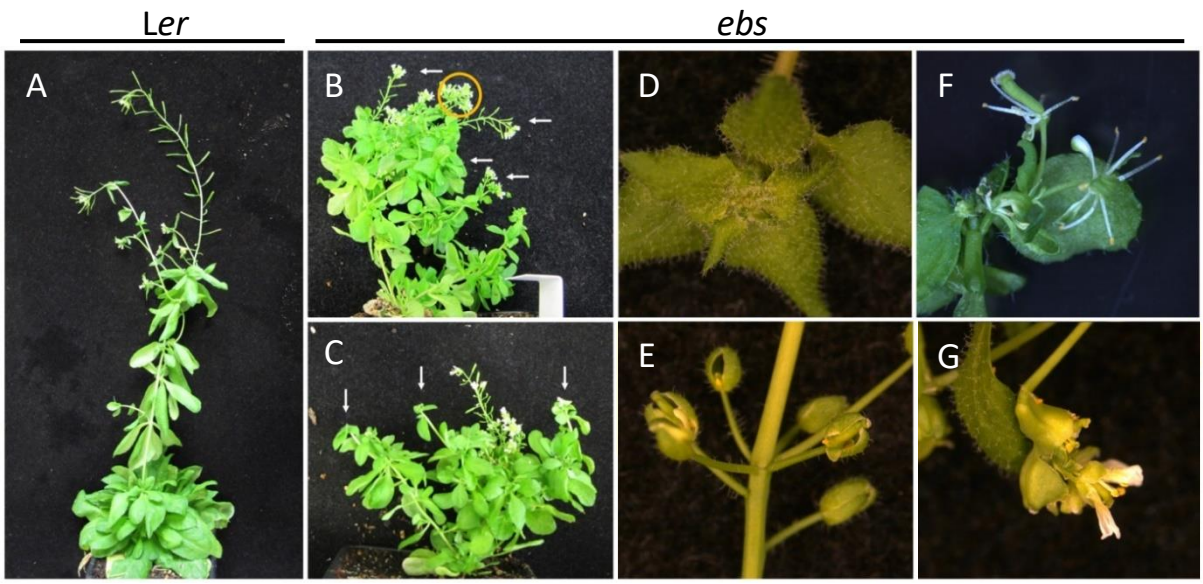

Fig.12 Developmental defects in reproductive traits of the *ebs* mutant in L*er* background grown under SD conditions, non-inductive for flowering: A) WT L*er* plants, B) - G) *ebs* mutant plants in L*er* background showing the loss of apical dominance characteristic of this mutant: B) Extra branches created on every stem. Arrested apical meristem in the main shoot is marked by an orange circle, C) Additional rosette stems. D) Leaves, instead of flower meristems created from the inflorescence meristem, E) Phyllotaxy defects, F) Alterations in flower development, G) Floral reversion

As mentioned above, the observed defects in *ebs* also affect the development and position of flowers. Although the *ebs* mutant bolts prematurely, during early stages of development the inflorescence does not produce flowers. Instead, an abnormal vegetative structure is created in which floral organs are replaced by leaves (Fig.12.D, Fig.13.D). The time between the formation of the first of these inflorescence structures to the opening of the first flower with visible floral organs usually takes two and a half weeks, but can take more than one month in most extreme cases as compared to a maximum of six days that are needed from the formation to the opening of first floral bud in WT (see Table 9, 10). When flowers are eventually formed they often do not develop properly and exhibit morphological and homeotic transformations of floral organs (Fig.12.F, Fig.13.F), where deformed perianth organs, sepals and petals, are fused, together with disturbed floral phyllotaxy (Fig.13.F). The ‘golden angle’ (137.5°; Mirabet et al., 2012) defined by two consecutive flower buds in the SAM is lower in *ebs* than in WT plants. In addition, internodes are shirked, resulting in the clustering of flowers/siliques and the formation of an umbrella-like structure (Fig.12.E, Fig.13.E). The angle between the flowers in these umbrella-like structures depends on the number of flowers present in them, with higher number of flowers resulting in narrower angles, and less flowers in structures with wider angles.

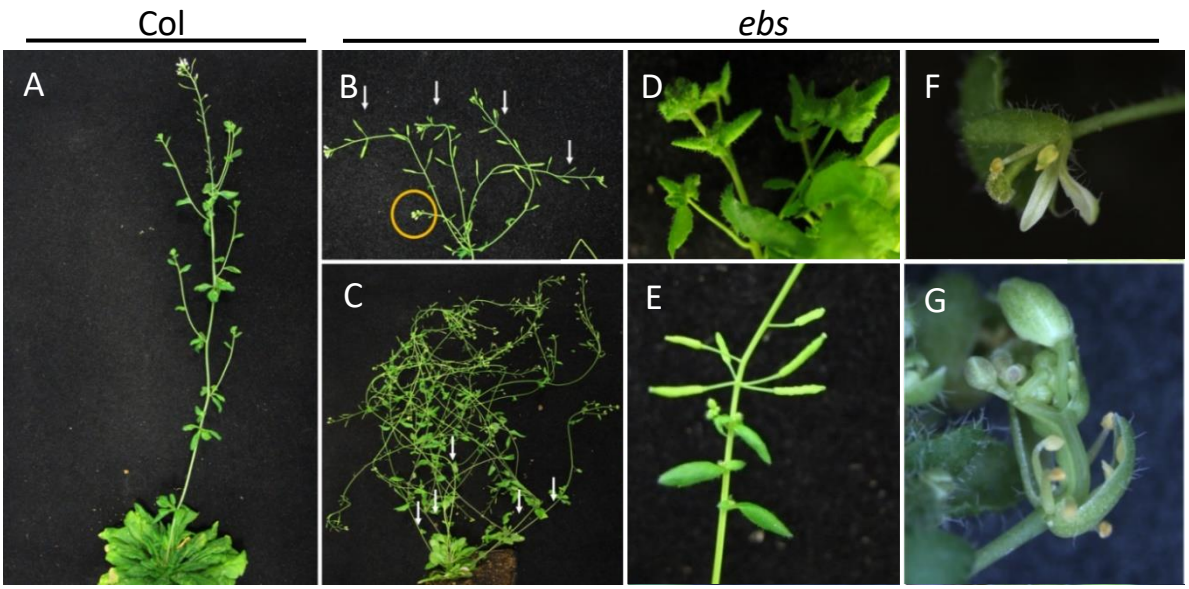

Fig.13 Developmental defects in reproductive traits on the *ebs* mutant in Col background grown under short day conditions, non-inductive for flowering: A) WT Col plants, B) - G) *ebs* mutant plants in Col background showing the loss of apical dominance characteristic of this mutant: B) Extra branches created on every stem. Arrested apical meristem in the main shoot is marked by an orange circle, C) Additional rosette stems; D) Leaves, instead of flower meristems created from the inflorescence meristem; E) Phyllotaxy defects; F) Alterations in flower development; G) Floral reversion

A particularly remarkable case of flower developmental abnormality observed in the *ebs* mutant is floral reversion. Floral reversion consists in the return of a flower to previous developmental stages acquiring inflorescence features in the flower (Battey and Lyndon, 1990). In WT, the segment of the inflorescence giving rise to flowers (the apical zone) is separated from the region generating cauline leaves and branches (basal zone). In some Arabidopsis mutants including *ebs*, floral reversion can be observed as cauline leaves or bracts formed after appearance of individual flowers in the apical region of the main stem. However, the most extreme examples of floral reversion in *ebs* are the formation of an entire inflorescence branch inside a flower, whereby the determinacy of the floral meristem is lost, and an indeterminate inflorescence is generated within the flower (Fig.12.G, Fig.13.G). This type of floral reversion was observed at many different developmental stages of the flower: in floral buds, mature flowers or siliques (Fig.14). Interestingly, flowers produced in emerging inflorescences formed as a consequence of the floral reversion seem to maintain more robustly floral organ identity and determinacy, and to display developmental defects less frequently. Often floral reversion precedes the plant ability to produce fertile flowers, and vegetative buds keep on being formed until floral reversion occurs (Fig.14.D).

Fig.14 Floral reversion defects in the *ebs* mutant during different stages of flower and fruit development: A) At the stage of the floral bud, B) At the stage of the flower, C) At the stage of the fruit, D) Increased fertility of flowers formed after a floral reversion event in the *ebs* mutant. Secondary inflorescence is not fully committed to flowering as leaves instead of flowers are created. The new inflorescence produces only flowers, without any leaf structures. The arrowhead marks a position where floral reversion occurred.

Altogether, this detailed analysis shows that the loss of function of the *EBS* gene causes dramatic alterations in reproductive development in the two genetic backgrounds studied, indicating a central role for EBS in the control of different aspects of this developmental phase, ranging from inflorescence architecture to flower development.

1.4 Microscopic analysis of developmental abnormalities observed in *ebs* mutant inflorescences

In order to gain a deeper insight into the phenotypic alterations observed in the *ebs* mutant at the cellular level, we used Scanning Electron Microscopy (SEM) on detached flowers and apical parts of inflorescence stems. This microscopic analysis revealed that flower development defects in the *ebs* mutant display different levels of expressivity ranging from mild alterations in some floral organs, to extremely severe abnormalities with dramatic consequences in the arrangement and identity of floral-like structures. Subtle phenotypic alterations include flowers that open prematurely and are unfertile due to the senescence of petals and stamens (Fig.15.C-F). Another example of mild defects is represented by flowers affected by partial homeotic transformations of floral organs like sepals bearing branched trichomes and epidermis cells normally found in leaves, or sepals with sectors of conical shaped cells, characteristic of petals (Fig. 15.I, J).

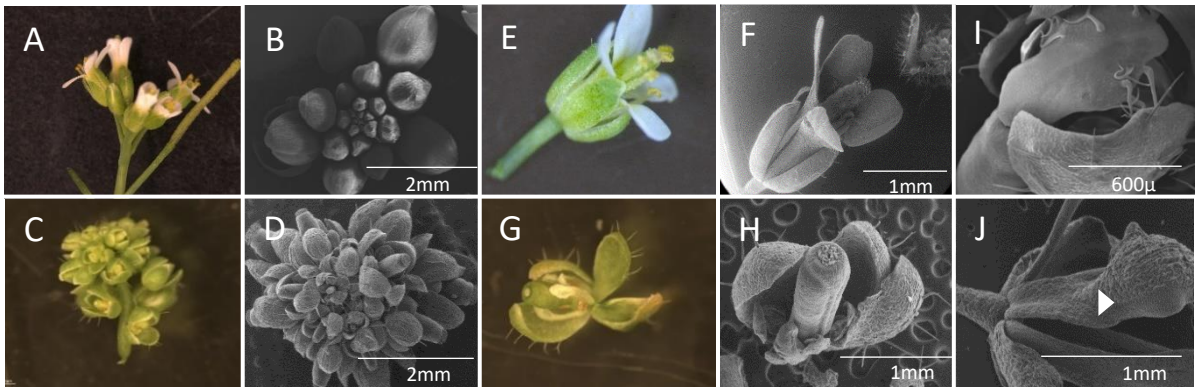

Fig.15 Subtle morphological and homeotic alterations observed in the flowers of *ebs* mutant. A), C), E) and G) – Photographs of inflorescences and flowers; B), D), F) and H-J) – Scanning electron micrographs. A), B), E), F) – Phenotypes of L*er* flowers. C), D) – Shoot apex of *ebs* mutant with prematurely opened flowers. G), H) – An example of prematurely opened flower of *ebs* mutant where growth inhibition and senescence of petals and stamens can be observed. I), J) – Examples of partial homeotic transformation of floral organs in the *ebs* mutant: I) Sepal with leaf-like edges, J) Sepal-to-petal conversion, where clear separation of elongated cells characteristic of sepals and conical cells characteristic of petals can be observed. Arrowhead marks the sepal-to-petal cell conversion line.

In addition, organs with severe abnormalities that result in flower-like structures where canonical floral organs are hardly distinguishable are also frequently observed in *ebs* mutant plants grown under SD, and are likely a consequence of floral reversion events. Some of these alterations can result in the complete conversion of all floral organs into vegetative structures. The presence in these structures of irregular, puzzle-like cells typical for leaf epidermis seen on the surface of all organs, and trichomes grown from both abaxial and adaxial surfaces suggest that floral organs are partially transformed into leaves (Fig.16.B). In some cases, cadastral information concerning the distribution of whorls in the flower is missing so that floral organs emerge from undefined places and in much higher number than in normal flowers causing overproliferation of the flower domain, loss of strictly defined whorls, conversion between floral organs (petal↔stamen; stamen↔carpel) and conversion of gynoecium into structures resembling floral buds (Fig.16.C).

Frequently, the floral reversion events observed in the *ebs* mutant result in an inflorescence emerging from a young silique, suggesting that floral reversion is initiated within the fourth whorl of the flower. Figure 16.D shows deformed gynoecia at different stages of floral reversion. Due to the loss of determinacy in this fourth whorl and abnormal proliferation of cells with altered identity, the inner part of the gynoecium is swollen and deformed. Often carpels are not fully fused and an interstice can be seen through stigma and style. Gynoecium bursts open alongside the replum under pressure of new structures growing out from its inside leading to severe malformations in the flowers.

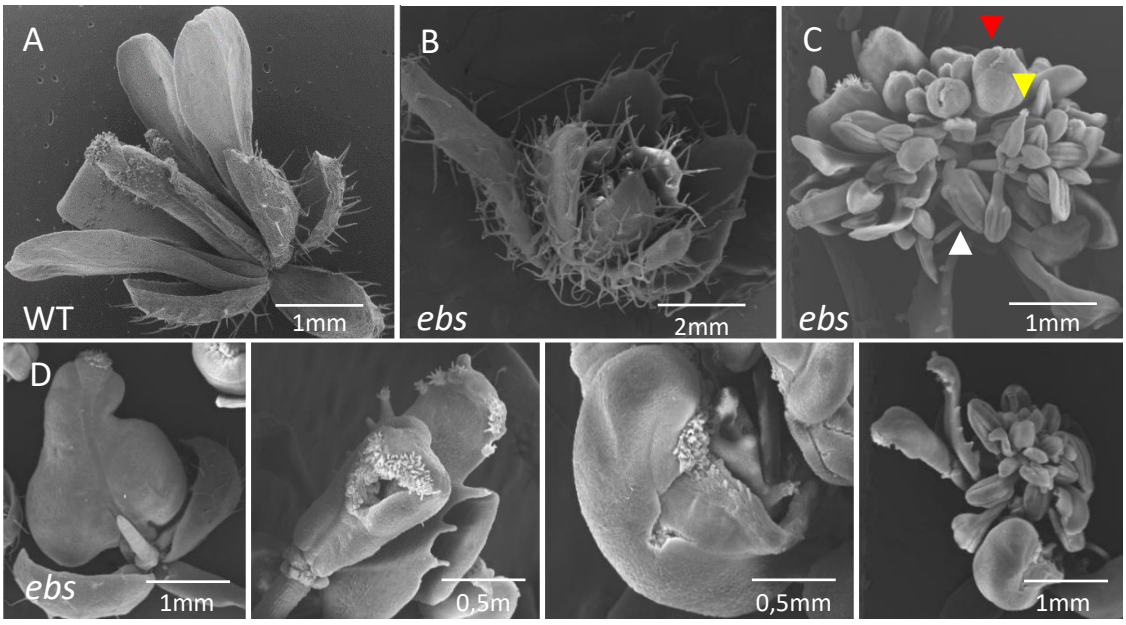

Fig.16 Scanning electron micrographs of strong developmental alterations of *ebs* flowers: A) Typical fully-mature WT flower, B) Strong alterations resulting in complete conversion of all floral organs into leaves, C) Floral reversion. Overproliferation of the flower domain, loss of strictly defined whorls, conversion between floral organs (petal↔stamen, white arrowhead; stamen↔carpel, yellow arrowhead) and conversion of gynoecium into structures resembling floral buds (red arrowhead), D) Floral reversion initiates in the fourth whorl. Different stages of gynoecia deformation caused by inflorescence developing in the ovary.

Some less severe events of floral reversion can also be observed in (seemingly) unaltered SAMs of *ebs* mutant. In WT plants, flowers emerge one after another at the flanks of inflorescence. In *ebs* mutant, single flowers are replaced by floral clusters at the end of floral pedicels that lack subtending cauline leaves or bracts. This structure is created outside the basal region of the inflorescence where production of secondary branches is expected (Fig.17). In those cases, the altered structure of the *ebs* mutant inflorescence resembles a panicle more than a typical Arabidopsis raceme (Prusinkiewicz et al., 2007).

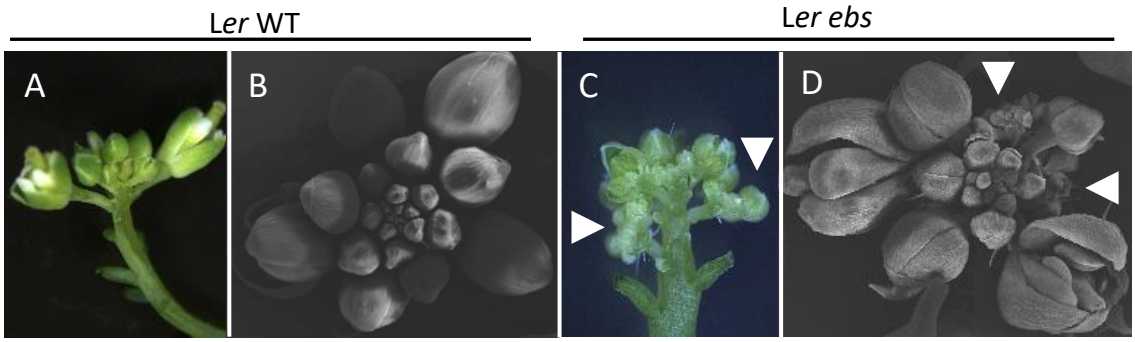

Fig.17 Inflorescence-like structures occupy positions in the apical region of the *ebs* mutant inflorescence where single flowers are expected to be formed. Places of the additional floral clusters indicated by arrowheads. A), C) – Lateral view of SAM. Some flowers were removed to increase the clarity of inflorescence-like structures; B), D) – Scanning electron micrographs of WT and *ebs* SAMs respectively.

Altogether, these observations support a central role for EBS in the control of different aspects of inflorescence and flower development, including cell identity within floral organs. The results obtained show that EBS function is particularly necessary to maintain proper development of reproductive organs under non-inductive flowering conditions.

2. Molecular analysis of EBS function in reproductive development

2.1 Analysis of the genes misregulated during flower development

After characterizing the different types of developmental abnormalities that occur when Arabidopsis plants are deficient in EBS function, we pursued to understand the molecular mechanisms responsible for the observed defects in the *ebs* mutant. Initially, we focused our analysis on abnormalities during flower development, and particularly floral reversion, as this phenotypic alternation is very interesting and the molecular basis for this trait remains largely unknown. We analyzed the events of floral reversion when inflorescence branches emerge within the flower buds. For that, we compared changes in gene expression comparing floral buds and flowers of *ebs* mutant and WT plants with the reverting floral bud of the mutant. As described in the Introduction (section: 1.4.1 Reproductive reversion) many examples of floral reversion are connected with misregulation of flowering integrator genes (McCullough et al., 2010; Asbe et al., 2015). For that reason, we decided to assess in our samples the expression levels of different flowering genes that were previously linked with floral reversion phenomena.

We firstly focused on flowering integrator genes such as *FT*, one of main activators of the floral transition (Wigge et al., 2005) and *FLC*, a central repressor of the floral transition (Sheldon et al., 2000; Choi et al., 2009), as their role in mediating reproductive reversion was previously reported (Müller-Xing et al., 2014; Satake et al., 2016). We also included two other flowering time genes, *SOC1* and *SVP* that were misregulated during floral reversion episodes in *Arabidopsis suecica* (McCullough et al., 2010). *SOC1* promotes flowering and integrates signals from the photoperiod, vernalization and autonomous floral induction pathways (Lee et al., 2000). SVP acts inhibiting the floral transition and is involved in the control of the determination of floral meristems and the expression of floral homeotic genes (Gregis et al., 2009).

Complications with the determination of floral meristem identity due to the ectopic expression of vegetative and inflorescence meristem identity genes were reported to cause floral reversion (Pérez-Ruiz et al., 2015). We included in the analysis meristem identity genes, marking different developmental stages of the meristem. *TFL1* is connected with the vegetative state of meristem and inhibits expression of floral meristem identity genes (Liljegren et al., 1999). *AGL24* is a transition meristem identity gene that promotes the transition of the meristem from vegetative to inflorescence state and needs to be silenced to allow the acquisition of floral meristem identity (Yu et al., 2004). *LFY* is an early floral meristem identity gene that is required for the transition of an inflorescence meristem into a floral meristem (Bowman et al., 1993).

A similar effect is caused by the lack of functional floral meristem identity genes as it can also influence flower determination. Mutations in *AP1*, a main floral meristem identity gene, were reported to cause floral reversion and reproductive de-differentiation (Irish and Sussex, 1990; Bowman et al., 1993), so we also included *AP1* in the screening.

New inflorescences of reverting buds in *ebs* mutant plants were observed to emerge from the forth whorl, from the inside of the gynoecium. Some events of floral reversion

emerging from the siliques, where the three outer whorls are absent, were also observed. This advocates for a role of genes involved in female reproductive organ development in floral reversion. In fact, mutants deficient in the function of *AG*, a floral homeotic gene, responsible for the regulation of stamens and carpels (Yanofsky et al, 1990; Mizukami and Ma 1992, Mizukami and Ma 1995) or downstream genes, also display floral reversion phenotype (Grbić and Bleecker, 1996; Jung et al., 2014). In fact, *AG* was shown to be required for the determinacy of the floral meristem (Lenhard et al., 2001). For that reason, we included in our analysis *SEP3*, required for the acquisition of floral organ identity (Pelaz et al., 2000), *AG* and *SHP1*, needed for the proper development of ovules (Ehlers et al., 2016), and *PI*, required for the development of petals and stamens (Krizek and Meyerowitz, 1996).

The flowering time genes *SOC1* and *SVP* as well as genes involved in the regulation of floral organ development such as *SEP3, PI, AG,* and *SHP1*, showed no statistically significant change in expression between the analyzed samples of WT and *ebs*. Weak downregulation of *FT* expression was observed between flower buds and flowers of *ebs* mutant and WT plants; however, the change was not consistent within replicates. In contrast, *FLC* was significantly upregulated in the flowers of the *ebs* mutant at all investigated stages. Also, the expression of meristem identity genes differed between the WT and *ebs* mutant samples. *AGL24* and *LFY* were found upregulated in the flower buds and flowers of *ebs* mutant. Their levels are also quite high in the buds undergoing floral reversion in *ebs*. Another meristem identity gene, *TFL1,* was not upregulated in normally developing flower buds and flowers of *ebs* mutant, but high expression level was observed in the reverting buds. On the other hand, no expression change was observed for the floral meristem identity gene *AP1* (Fig.18).

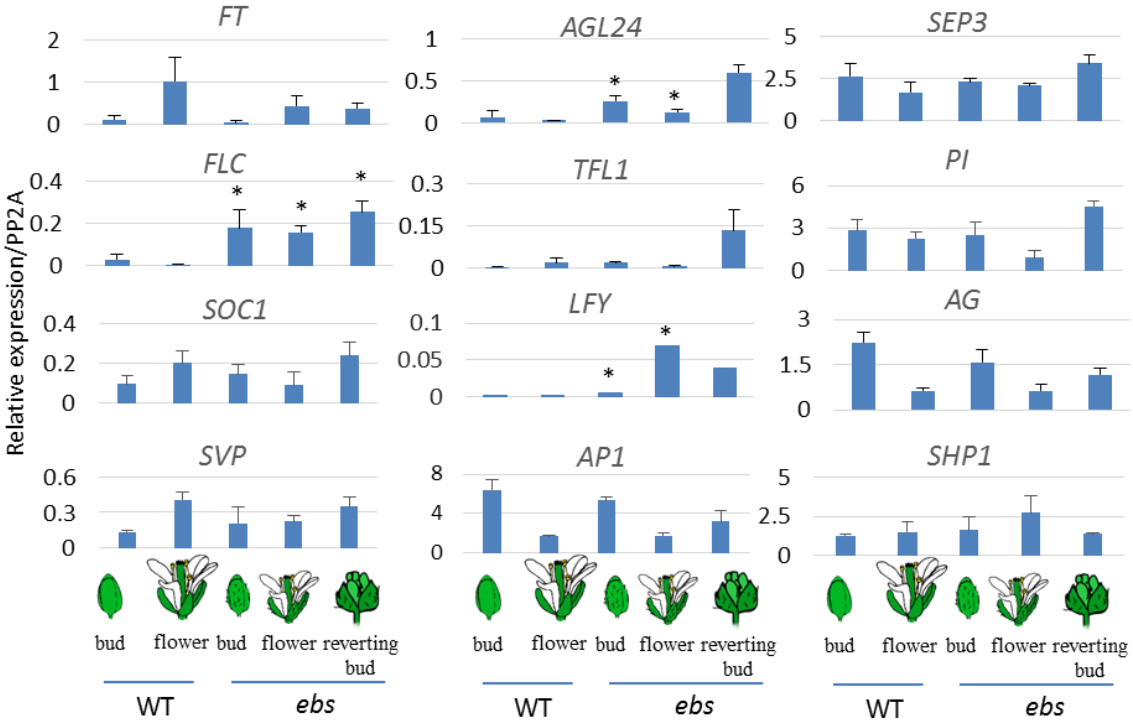

Fig.18 Analysis of the expression levels of flowering genes linked with floral reversion in the flower buds and flowers of WT and *ebs* plants; flower buds undergoing floral reversion in the *ebs* mutant were also included in the analysis. Asterisks represent statistical significance by Student t-test.

This gene expression study enabled us to pinpoint the upregulation of the meristem identity genes *AGL24, TFL1* and *LFY* and the flowering repressor gene *FLC* as potential candidates to cause the alterations during flower development observed in *ebs* mutant. Moreover, these comparative analyses of gene expression in developing flowers between WT and *ebs* samples revealed that in the absence of functional EBS protein, misregulation of central loci involved in the regulation of reproductive growth occurs. Based on these observations, we decided to perform a genome-wide transcriptome profiling, to unveil at the global level the genes whose expression was affected by the *ebs* mutation.

2.2 Transcriptomic approach to characterize the role of EBS in the regulation of reproductive development

The functional domains present in EBS suggest that this protein is a transcriptional regulator involved in chromatin remodeling processes. In fact, previous data obtained in our laboratory demonstrated that the expression of the floral integrator gene *FT* is upregulated in the *ebs* mutants before the floral transition, and that EBS binds regulatory regions of this locus. These observations indicate that EBS acts as a transcriptional repressor of *FT*. In addition, previous global expression analyses performed with the *ebs* mutant revealed that a number of genes are misregulated in fresh seeds (Narro-Diego et al., 2017) and seedlings (López-González et al., 2014) of these plants, consistent again with a role for EBS in the regulation of gene expression. Also, some genes with important roles in the control of reproductive growth were misregulated during flower development in the *ebs* mutant (Section 2.1). Based on these results,

our working hypothesis was that the phenotypic defects observed in the *ebs* mutant during reproductive development are likely due to alterations in the expression of genes that are required for proper development of different reproductive organs and structures such as inflorescences or flowers. For that reason, to better understand the molecular basis for the phenotypic abnormalities observed in the *ebs* mutant during reproductive development, transcriptome analysis approaches were pursued.

2.2.1 Optimization of the biological material for the transcriptomic analysis

The lack of the functional EBS protein accelerates bolting, indicating that EBS acts as a repressor of the transition to reproductive phase in Arabidopsis plants. However, EBS is required for proper inflorescence architecture and to promote the acquisition and maintenance of floral organ identity in later stages of inflorescence development. To differentiate between these two independent functions, we decided to collect the material for the transcriptomic analyses before the floral transition and after the production of inflorescences, and perform RNA-seq analysis in order to unveil the genes that are misregulated by the *ebs* mutation and might explain the defects observed in the mutant. To make sure that the samples we collected for the stage previous to the initiation of flowering were truly vegetative, we performed an expression analysis of genes that flag the beginning of the floral transition. First, we measured the expression of the floral integrator genes: *FT*, known to be upregulated in the *ebs* mutant (Piñeiro et al., 2003), and *SOC1*, another floral integrator that is sharply upregulated upon flowering initiation (Samach et al., 2000). SD-grown WT and *ebs* plants were harvested every 2 days, between 18 and 24 days after germination to assess the expression of these floral integrator genes. At day 24 no morphological signs of floral transition could be observed in those plants (not shown). qRT-PCR experiments allowed us to establish that, in agreement with previous observations, *FT* induction is accelerated in the *ebs* mutant samples as compared with WT and increases between days 20 – 22 (Fig.19.A). Consistently, the expression of *SOC1* remains constant at days 18 - 20 and shows a sharp increase between days 20 - 22 (Fig.19.B), indicating that before day 20 there is no sign of upregulation of the floral integrator genes investigated neither in *ebs* nor in WT plants.

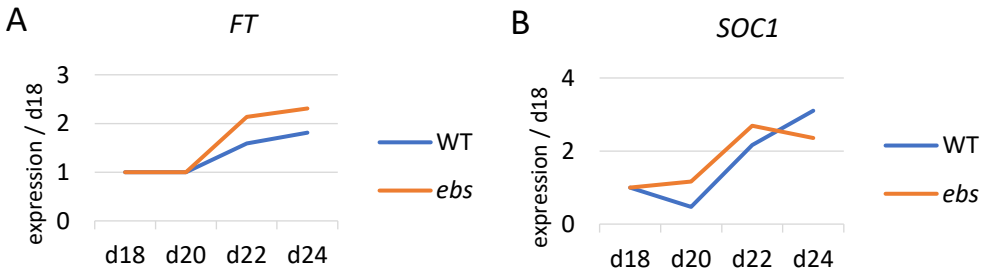

Fig.19 Changes in *FT* (A) and *SOC1* (B) expression levels during the transition to reproductive growth in *ebs* and WT plantlets. No noticeable changes were observed between day 18 and 20. At days 20 - 22 upregulation of both genes can be observed. Data from a representative experiment are shown.

In order to further corroborate that plants at the selected developmental stage remain in vegetative phase, we investigated the expression of *AP1*, a flower meristem identity gene that is expressed downstream of the floral integrator genes and has been considered a marker of reproductive development (Mandel et al.,1992; Liljegren et al., 1999). When grown under SD

conditions, no expression of *AP1* could be detected in WT plants at day 21 (Fig 20.A, B). We then transferred these plants to LD conditions to induce the floral transition. *AP1* expression was monitored for 9 days after the shift to inductive photoperiods. *AP1* expression in the WT plants and GUS staining in *pAP1*::GUS line (kindly provided by Dr. Gerco Angenent from Wageningen University and Research Centre, WUR, Wageningen, the Netherlands) could be detected only 7 days after the shift (Fig.20). The results confirm that at day 19, at least WT plants are still growing vegetatively.

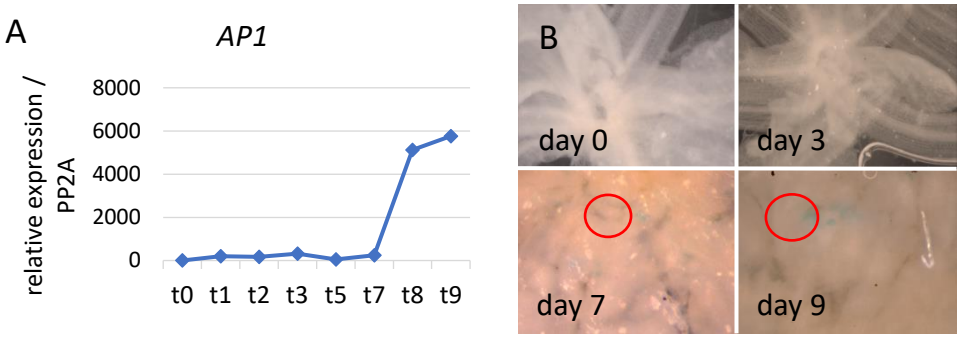

Fig.20 *AP1* expression after transfer of WT plants from SD to LD conditions. Upregulation of *AP1* can be detected at 7 days after this transfer. A) Quantification of *AP1* expression by qRT-PCR, B) *AP1* expression shown as blue staining resulting from GUS activity in the *pAP1*::GUS line used. Day 0 corresponds to the day of the transfer from SD to LD conditions.

In view of these results we selected day 19 for the collection of two types of vegetative samples, manually dissected meristematic regions and rosette leaves, to carry out the transcriptomic analysis (Fig. 21).

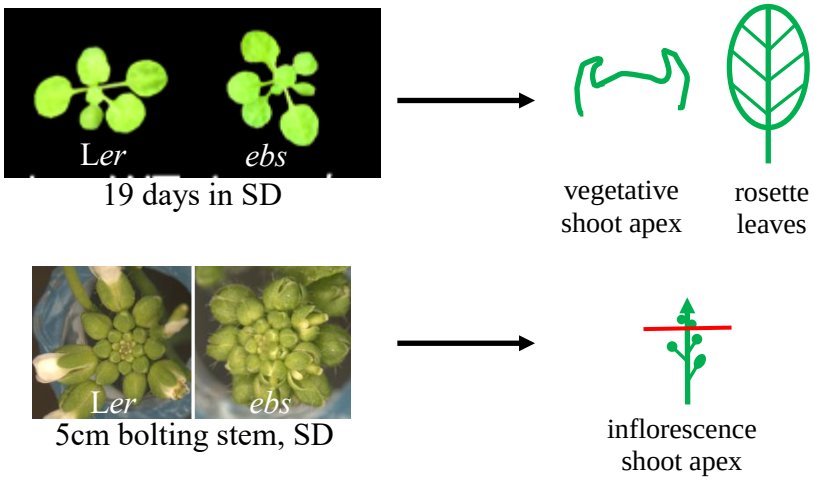

Fig.21 Schematic representation of the tissues collected for the RNA-seq analysis of WT and *ebs* mutant plants in L*er* background: shoot apex and rosette leaves at vegetative state (upper panel); manually dissected inflorescence shoot apices (lower panel). Flowers older than stage 8 were removed from sample tissue

In addition to the two vegetative samples described above (leaves and meristematic region), we also collected shoot apices after the production of the inflorescence from the WT

and *ebs* mutant plants. In both cases, reproductive shoot apical samples were harvested when the main inflorescence stem was 5 cm long, but only the top part was included in the analysis (Fig. 21). To enrich the sample in the inflorescence meristem tissue, flowers and flower buds older than stage 8 (Smyth et al., 1990) were manually removed from the harvested apices. RNAs prepared from these three types of tissues in duplicates were used for RNA-seq experiments, carried out in collaboration with the group of Dr. Gerco Angenent (WUR, The Netherlands).

2.2.2 Identification of differentially expressed genes in *ebs* mutant through RNA-seq analysis

In order to identify genes differentially expressed in the *ebs* mutant we performed an RNA-seq analysis with 50 bp single end sequencing using the Illumina HiSeq2000 platform, and 20 M reads per sample. We used two biological replicates of each sample. On average, 88% of reads mapped to the Arabidopsis reference genome, TAIR10 (Table 11).

Table 11: Read mapping rate represented as % of mapped reads to total reads of every RNA-seq library

L*er*						*ebs*					
VM		L		IM		VM		L		IM	
88.5	88.7	89.3	89.2	88.4	88.7	88.8	85.5	85.7	89.4	88.9	88.3

Among the genes that showed differential expression between the *ebs* mutant and WT samples with a p-value ≤ 0.05, we selected those displaying at least a 2-fold change in expression. The analysis revealed 327 and 516 upregulated genes and 208 and 196 downregulated genes in vegetative shoot apex and leaves respectively, consistent with a role for EBS as a transcriptional repressor in early developmental stages. Besides, 505 genes were upregulated and 600 genes were downregulated in the apices at the inflorescence stage (see Annex I for a full list of differentially expressed genes). A heat map with the 25 most upregulated and downregulated genes in each of tissue types collected is shown in Figure 22.

Fig.22 Heat map representation of the 25 most upregulated and 25 most downregulated genes in the different tissues analyzed for the *ebs* mutant. The results are expressed as fold ratio of gene expression for *ebs* plants compared to WT plants. VM – vegetative shoot apex; L – rosette leaves; IM – inflorescence shoot apex; p – protein; f – family; sf - superfamily

Comparisons of these data with those obtained from microarray hybridizations where we analyzed the transcriptome of *ebs* mutant seedlings in ATH1 arrays using RNA from 18-day old whole seedlings grown in SD and that were carried out previously in our laboratory revealed a high degree of overlap between them (Fig.23). The plant material collected for the microarray experiment differs from the material collected for RNA-seq in the tissue type (entire seedlings versus dissected vegetative shoot apex and leaves, respectively) and developmental stage (vegetative in both cases, but at different DAG) and in the robustness of the analysis, as the number of differentially expressed genes identified in the deep RNA sequencing experiment is much higher than the one identified in microarrays. Still, one third of the genes identified as misregulated (with 2-fold change and p-value ≤ 0.05) in the microarray experiment overlaps with genes identified as misregulated (with 2 fold change and p-value ≤ 0.05) in the RNA-seq experiment, which is a highly significant over-representation as revealed by the hypergeometric test (representation factor = 9.4; p-val < 2.015e-39) using population size of 33159 (all the

genes, pseudogenes and transposon elements included in our RNA-seq data). These results reinforce the accuracy of the analysis performed.

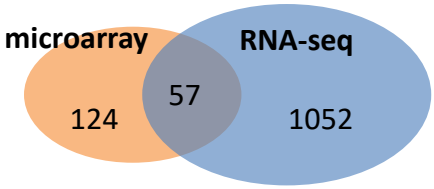

Fig.23 Venn diagrams showing the overlap between the genes misregulated in *ebs* mutant revealed by a microarray experiment on whole seedlings performed previously in our lab and genes misregulated in *ebs* mutant in two vegetative state samples (leaves and shoot apex) revealed by the new RNA-seq experiment.

2.2.3 Analysis of the signature of expression profiles in the samples analyzed

To confirm that the collected samples show the expected gene expression pattern, the 400 most expressed genes of the WT samples were analyzed by the Genevestigator signature tool (https://genevestigator.com/, Hruz et al., 2008) that matches the provided expression data with the expression pattern of different anatomical structures of Arabidopsis. The results are described as a relative similarity score, where 0 stands for a similarity between the input signature and an average over all anatomical structures, and 2.5 stands for the ideal similarity. Expression patterns of all WT RNA-seq samples matched the signature of anatomical structures they were collected from. On this manner, vegetative shoot apex sample showed highest relative similarity with shoot apex, whereas rosette leaves showed the highest relative similarity with shoot and rosette. Regarding inflorescence shoot apex samples, they showed the highest relative similarity with the expression patterns of flower, pistil, and receptacle (anatomical structures that were expected to be present in the sample due to the sample isolation technique), but also with inflorescence what corroborates high enrichment of inflorescence meristem tissue in the sample (Table 12).

We then performed the same analysis on *ebs* samples. While the expression profiles obtained still matched the tissue they were collected from, some differences could be observed. The most striking one was the expression profile of vegetative shoot apex that was associated with the expression signature of the flower, suggesting the premature expression of reproductive organ genes in the *ebs* apex previous to the floral transition (Table 12). Ectopic expression of floral genes has been described in other mutants deficient in genes encoding chromatin remodelers (Yang et al., 2010; Mozgova et al., 2015; Sacharowski et al., 2015), consistent with a key role of epigenetic mechanisms in maintaining gene expression patterns associated with specific developmental phases.

We then analyzed the expression profile of the genes with differential expression between *ebs* and WT. Genes that are abnormally abundant can change the identity of the cell, but so can genes that are missing from the transcription profile. For that reason, we compared 200 most upregulated and 200 most downregulated genes from each sample. None of the groups of misregulated genes analyzed matched well the signature of the anatomical structure they were collected from, suggesting that some genes are ectopically expressed in the *ebs* mutant. The most interesting deviation could be observed for an inflorescence sample, where misregulated genes conferred upon inflorescence the signature of vegetative tissues: leaves and

shoots (Table 12). This observation is consistent with a defective reproductive organ identity determination in the *ebs* mutant.

Table 12: Relative similarity of expression profile for RNA-seq samples to the profiles of anatomical structures of Arabidopsis obtained from Genevestigator signature tool. VM – vegetative shoot apex, L –rosette leaves, IM – inflorescence shoot apex

	WT		***ebs***		***ebs* vs WT**	
VM	shoot apex	1.849	flower	1.880	embryo	1.157
	seedlings	1.772	seedling	1.707	radicle tip	1.122
	shoot	1.601	shoot apex	1.690	endosperm	1.116
L	shoot	1.138	shoot apex	2.028	leaf	1.206
	rosette	1.097	seedling	1.899	blade (lamina)	1.113
	shoot apex	1.077	rosette	1.834	lateral root cap and columella cell	1.096
IM	flower	2.438	flower	1.809	leaf	1.123
	pistil	1.654	receptacle	1.515	shoot apex	1.089
	receptacle	1.452	pistil	1.460	rosette	1.082
	inflorescence	1.320	inflorescence	1.324	shoot	1.082

2.2.4 Overlaps between the genes misregulated in the different samples analyzed

We then compared the genes misregulated in different samples with each other in order to gain an insight on the specificity that EBS displays in the regulation of gene expression in the different tissues explored in our transcriptomic analysis. We analyzed the overlap of the upregulated (Fig.24.A) and downregulated (Fig.24.B) genes in *ebs* vs WT between the different tissues collected. 83 of the upregulated genes were shared between shoot apex and rosette leaves at the vegetative state. This overlap is higher than expected by chance with statistical significance according to a hypergeometric test (representation factor = 1.1; p-val < 0.082, hypergeometric test using the sum of misregulated genes from all investigated tissues, 2351, as a population size). Gene Ontology (GO) analysis performed in the publicly available AgriGO biological process toolkit (http://bioinfo.cau.edu.cn/agriGO/; Du et al., 2010) revealed that within the genes upregulated in both vegetative tissues there is a group of genes involved in flower development. 87 genes were upregulated in both apical tissues (vegetative and inflorescence). This overlap is statistically significant (representation factor = 1.2; p-val < 0.014, hypergeometric test), although the GO analysis showed no enriched categories. Inflorescence apices and rosette leaves samples shared 91 of the upregulated genes. This overlap is not statistically significant (representation factor = 0.8; p-val < 0.009, hypergeometric test), however, the GO analysis revealed that some of these 91 genes are involved in photosynthesis. 38 genes were upregulated in all collected samples, but no significant GO categories could be found. The enrichment of shared loci between downregulated genes is not statistically significant with the exception of the two vegetative tissues, apices and leaves that display 21 genes in common (representation factor = 1.2; p-val < 0.201, hypergeometric test). These genes are involved in the response to abiotic stimulus. 34 genes were shared between both apical samples (representation factor = 0.6; p-val < 6.518e-04, hypergeometric test) and 11 genes were shared between inflorescence sample and rosette leaves (representation factor = 0.2; p-val < 3.79e-14, hypergeometric test) with no enriched GO categories. Finally, only 4 genes were shared between genes downregulated in all three tissue types tested. These results

confirm previous observations that *ebs* mutant plants express reproductive phase genes prematurely at vegetative state, but the inflorescence produced by this mutant displays vegetative features. Interestingly, the overlap of misregulated genes between apical tissues at different developmental stages (vegetative and inflorescence) despite being statistically significant (at least in case of upregulated genes) does not include the enrichment of any GO categories. On the contrary, upregulated and downregulated genes shared between the samples from the same developmental stage (vegetative shoot apex and leaves) are both statistically significant and belong to shared GO categories. Lack of the functional similarity between genes affected by the *ebs* mutation coming from the same tissue at different developmental stages suggest that EBS-mediated regulation is developmental state-specific and advocates for the role of EBS in the developmental control.

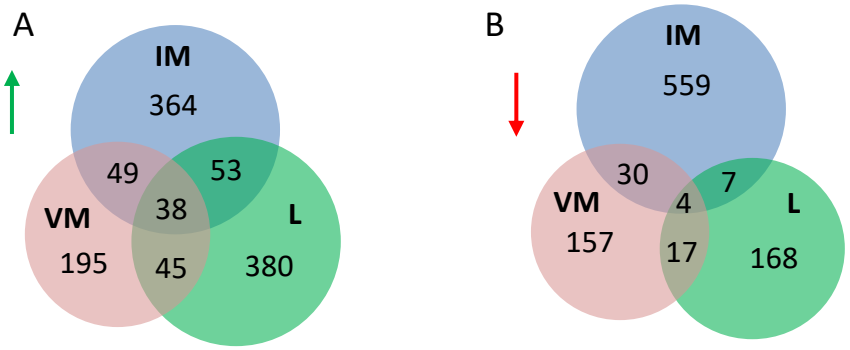

Fig.24 Venn diagrams showing the overlap of the genes misregulated in *ebs* with the same direction of change between the different tissues collected. A) upregulated genes, B) downregulated genes. VM, pink – vegetative shoot apex; L, green – rosette leaves; IM, blue – inflorescence shoot apex

EBS plays distinct roles in vegetative and inflorescence development. This behavior might be caused by a group of differentially regulated genes during vegetative and inflorescence growth. Those genes could show opposite changes in expression in tissues collected at the two developmental stages in our analysis. We assessed the overlap between genes upregulated in the vegetative tissues and genes downregulated in the inflorescence shoot apex in *ebs* vs WT plants (Fig.25.A), and the overlap between genes downregulated in the vegetative tissues and upregulated in the inflorescence shoot apex in *ebs* vs WT plants (Fig.25.B). The number of overlapping genes in this analysis was rather limited as only 34 genes were upregulated during the vegetative state (from which 15 came from vegetative shoot apex) but downregulated at inflorescence state. Similarly, only 23 genes were downregulated at vegetative state (from which 19 came from vegetative shoot apex) but upregulated at inflorescence state. None of the overlaps fulfil the statistical significance by hypergeometric test (representation factor = 0.2; p-val<1.64e-86 and representation factor = 0.3; p-val < 2.411e-21, respectively). The overlaps did not include any enriched GO terms. This observation suggests that in general terms *EBS* does not have opposite effects in the regulation of the same genes during vegetative and reproductive development.

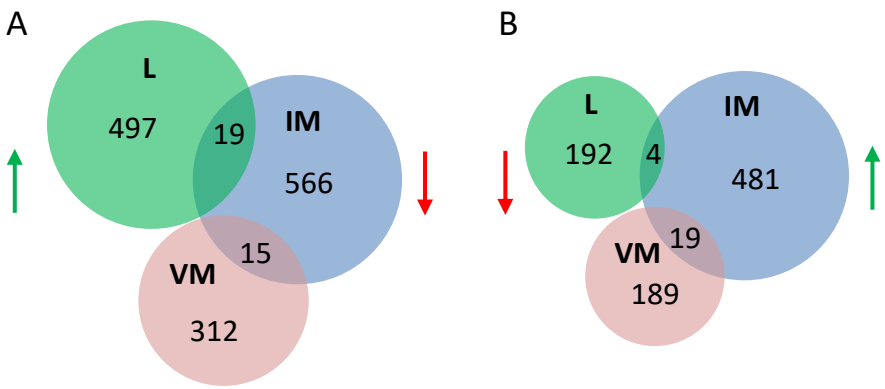

Fig.25 Venn diagrams showing the overlap of the genes misregulated in *ebs* with opposite direction of change between the different tissues collected. A) Overlap between genes upregulated in vegetative tissues and genes downregulated in inflorescence shoot apex. B) Overlap between genes downregulated in vegetative tissues and upregulated in inflorescence shoot apex. VM, pink – vegetative shoot apex; L, green – rosette leaves; IM, blue – inflorescence shoot apex

2.2.5 GO analysis of genes differentially expressed in the *ebs* mutant

In order to better understand the molecular and biological processes regulated by EBS, we decided to perform a GO analysis using the publicly available AgriGO biological process toolkit. Redundant categories were removed with REVIGO toolkit (http://revigo.irb.hr/; Supek et al., 2011). The functional categories overrepresented among the genes upregulated in vegetative apices of *ebs* were linked with normal meristem maintenance and development, but also an ectopic expression of genes involved in flower development could be observed, consistent with the observation described above that the vegetative apex of *ebs* plants is enriched in genes associated with reproductive development as compared to WT plants (Fig.26). Genes downregulated in vegetative meristematic apices of *ebs* belonged to gene groups responsible for the response to endogenous (hormones, stress) and exogenous (cold, water deprivation, abiotic signals) stimuli. Interestingly, genes within the category of lipid localization are significantly overrepresented in this group of misregulated genes. The biological significance of this observation will have to be explored in future works. Genes belonging to a wide range of genetic categories were upregulated in rosette leaves of *ebs*, particularly those related to plant responses to environmental cues. The categories included response to biotic, abiotic, endogenous and exogenous stimuli, photosynthesis, and cell death, among others. The category of floral whorl development is also overrepresented, consistent again with ectopic gene expression in the *ebs* mutant. Genes downregulated in rosette leaves of *ebs* were linked with cell cycle and cytoskeleton. Genes upregulated in inflorescence tissue of *ebs* were connected with secondary metabolism processes, biotic and abiotic stimuli, photosynthesis, and DNA and RNA metabolism. Finally, genes downregulated in inflorescence tissue of *ebs* were linked with lipid localization, morphogenesis, pollen and gametophyte development and meiotic cell cycle, and secondary metabolic processes (Fig.26).

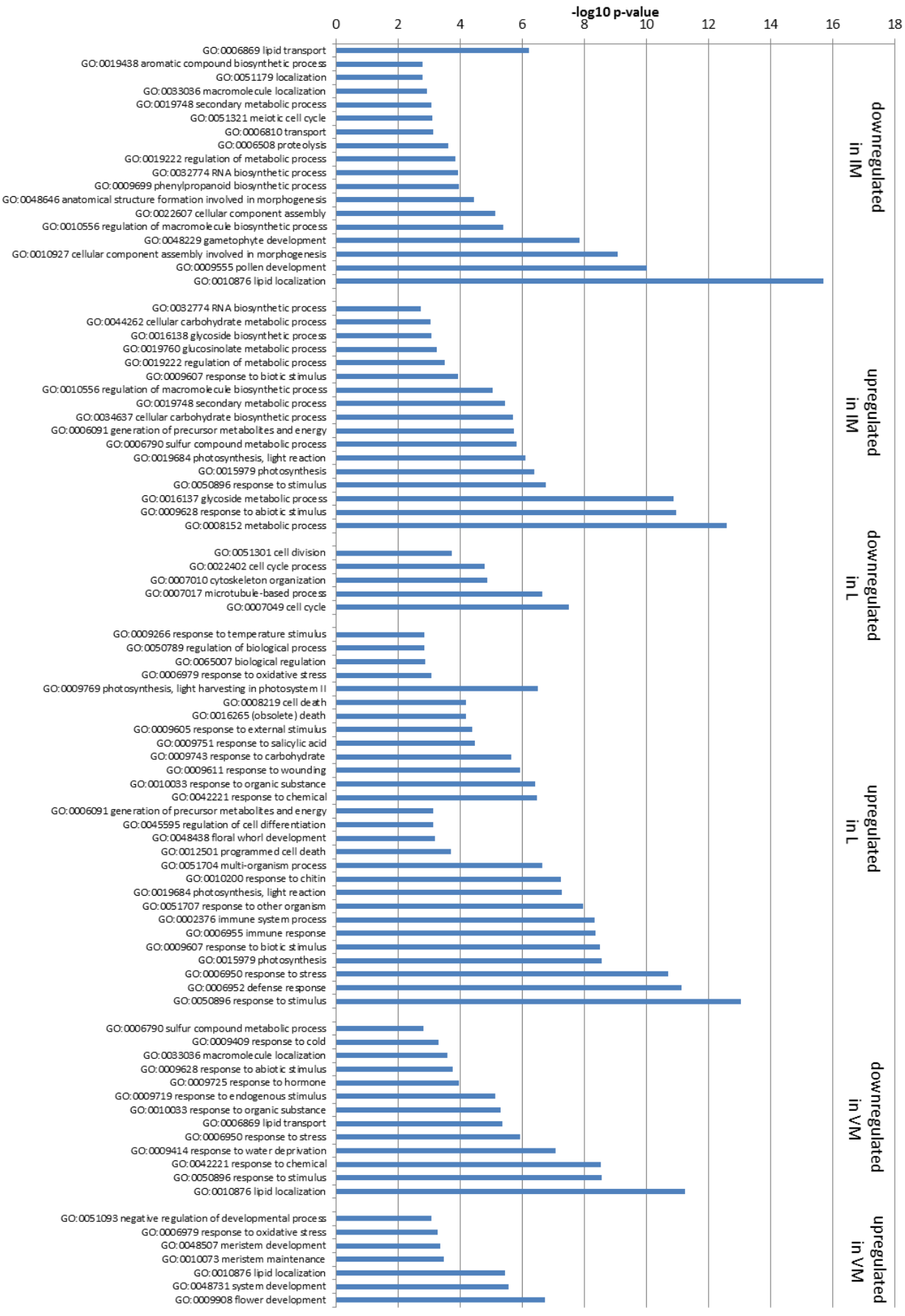

Fig.26 GO categories overrepresented in different tissue types. Graphics display separately up- and downregulated genes. GO analysis performed with the AgriGO toolkit.

2.2.6 Reproductive development genes differentially expressed in the *ebs* mutant

Since the main focus of this work was to understand the role of *EBS* in the control of different aspects of reproductive development, we next centered our attention on those genes that could mediate the effect of this chromatin protein on different aspects of reproductive growth, namely the floral transition, meristem identity or function, and flower development. A detailed description of the expression changes observed in the different tissues in the *ebs* mutant for the genes belonging to those categories is shown in Fig.27.

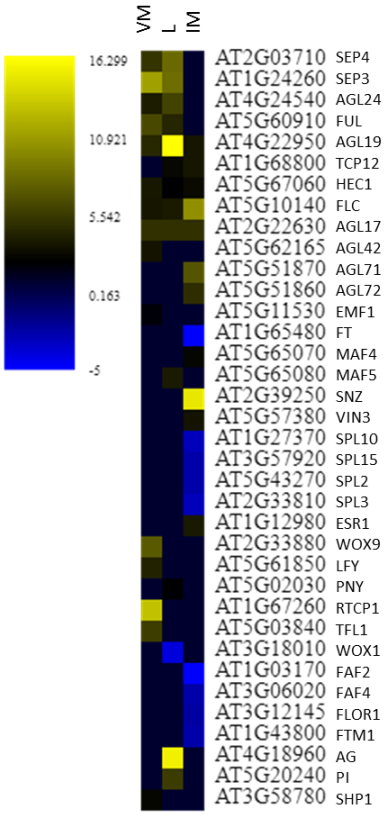

Fig.27 Heat map plot of genes regulating different aspects of reproductive development, and differentially expressed in the tissues of the *ebs* mutant. The results are expressed as fold change of gene expression for *ebs* plants compared to WT plants. No significant change in gene expression was marked as 1. VM – vegetative shoot apex; L – rosette leaves; IM – inflorescence shoot apex

Floral transition genes

Considering all the tissues analyzed, we identified 16 genes differentially expressed in *ebs* and related with the regulation of the floral transition. Some of them function as activators of flowering in Arabidopsis. For example, *AGL17* is a flowering promoter gene that acts in the so-called *FT*-independent photoperiod pathway (Han et al., 2008). The expression of this gene was detected in all *ebs* analyzed samples, but not in WT samples. On the other hand, *AGL19* promotes flowering in response to an *FLC*-independent vernalization pathway (Schönrock et al., 2006) and acts as an activator of *FT* in SD conditions. This MADS-box gene was found to be overexpressed in all *ebs* samples analyzed. Regarding *AGL42, 71* and *72*, they are paralogues phylogenetically related to *SOC1*. They promote flowering in shoot apical and axillary meristems in a gibberellin-dependent manner (Dorca-Fornell et al., 2011). *AGL42* is

additionally involved in the senescence of lateral organs (Chen et al., 2011). *AGL42* was overexpressed in the vegetative shoot apex of *ebs*, while *AGL71* and *72* were upregulated in the inflorescence shoot apex. *FT* could be detected in inflorescence shoot apices, but only in the WT plants. *FT* is a major activator of the floral transition (Wigge et al., 2005), but a role for *FT* in other aspects of reproductive development, as inflorescence architecture (Huang et al., 2013), flowering in axillary meristems (Niwa et al., 2013), and maintenance of flower identity (Müller-Xing et al., 2014) has been described in recent years.

Beside the floral activators described above, a number of floral inhibitors were also differentially expressed between WT and *ebs* in the samples analyzed. *FLC* acts as a major repressor of the floral transition and responds to vernalization (Sheldon et al., 2000; Choi et al., 2009). This gene was found to be overexpressed in all *ebs* samples analyzed. *MAF4* is a paralog of *FLC* that also responds to vernalization. Overexpression of this gene delays flower development and was found upregulated in the reproductive shoot apices of *ebs*. Another *FLC* homologue, *MAF5*, also negatively regulates flowering in SD, probably through the photoperiodic and vernalization pathways (Ratcliffe et al., 2003), and was found upregulated in *ebs*. *VIN3* is a central player in the vernalization pathway, needed for *FLC* repression by deacetylation of the histones in the promoter of this floral repressor gene (Greb et al., 2007). This locus was found to be overexpressed in the inflorescence shoot apex and in rosette leaves of *ebs*. *SCHNARCHZAPFEN (SNZ)* is a repressor of flowering that belongs to the AP2 family of transcription factors (Schmid et al., 2003). This gene was also overexpressed in the inflorescence shoot apex of *ebs.* Finally, several members of the *SPL* family of genes that positively influence flowering in the leaf and in the shoot apical meristem and are responsible for lateral organ maturation in association with shoot maturation in the reproductive phase (Shikata et al., 2009), were downregulated in inflorescence shoot apex of *ebs*.

Genes involved in meristem identity or function

At least 10 genes linked with meristem identity or function were found to be misregulated in *ebs* samples. As in case of genes involved in the regulation of the floral transition, also several members of the MADS-box family of transcriptional regulators were identified in this category. One of those MADS-box TFs, *AGL24* is a transition meristem identity gene that promotes the transition of Arabidopsis meristems from vegetative to inflorescence state, and needs to be silenced to allow the acquisition of floral meristem identity (Yu et al., 2004). *AGL24* is upregulated in vegetative shoot apex and rosette leaves of *ebs.* Another MADS box gene, *AGL8* (also known as *FRUITFULL*, *FUL*) promotes early floral meristem identity and is required for the transition of an inflorescence meristem into a floral meristem (Ferrándiz et al., 2000). This gene was upregulated in vegetative shoot apex and rosette leaves of *ebs*. Additional families of transcription factors were identified among the reproductive meristem identity genes misregulated in the *ebs* mutant. *LFY* promotes early floral meristem identity (Bowman et al., 1993) and was found to be upregulated in the vegetative shoot apex of *ebs*. Some of the genes differentially expressed in the *ebs* mutant belong to families of transcription factors needed for meristem development and formation of lateral organs. *HOMEOBOX-3 (HB-3)* is required for meristem growth and early development (Wu et al., 2005) and was upregulated in the vegetative shoot apex of *ebs. WUSCHEL RELATED HOMEOBOX 1* (*WOX1)*, also needed for proper meristem development (Zhang et al, 2011),

was found to be downregulated in rosette leaves of *ebs*. On the other hand, *ENHANCER OF SHOOT REGENERATION 1 (ESR1)* regulates gene expression patterns in meristems and thus modulates organ development (Kirch et al., 2003), and was found to be overexpressed in inflorescences of the *ebs* mutants. Another transcription factor involved in lateral organ development, PNY is involved in the preservation of the spiral phyllotaxy arrangement leading to a regular pattern of organ initiation. It is required for maintenance of stem cell fate in the shoot apical meristem, and is essential for specifying floral primordia and establishing early internode patterning events during inflorescence development (Smith and Hake, 2003). *PNY* was upregulated in the rosette leaves of *ebs*. *TCP DOMAIN PROTEIN 1* (*TCP1*) and *TCP12* are the axillary meristem identity genes that prevent axillary bud outgrowth and delay axillary bud development, having therefore considerable impact on apical dominance (Aguilar-Martínez et al., 2007). *TCP12* was upregulated in rosette leaves and the inflorescence shoot apex of *ebs*, while *TCP1* was upregulated in the vegetative shoot apex of *ebs.*

TFL1 on the other hand is a member of the FT florigen protein family (Wickland and Hanzawa, 2015), and controls meristem identity throughout development. TFL1 is required for maintenance of an indeterminate state of the inflorescence (Liljegren et al., 1999). This gene was found to be upregulated in the vegetative shoot apex of *ebs.*

An additional group of meristem identity genes, including *FANTASTIC FOUR 2* (*FAF2*), *FAF4, FLOR1* (*FLR1*) and *FLORAL TRANSITION AT THE MERISTEM1* (*FTM1*) was found downregulated in the inflorescence apex of *ebs*. They were previously identified as floral transition factors expressed specifically in the meristem in response to the induction of flowering (Torti et al, 2012).

Floral development genes

Six genes involved in floral organ identity determination were found to be misregulated in *ebs*. *SEP3* and *4* play an important role in the determination of flower meristem identity and are needed to ensure proper floral organ development (Pelaz et al., 2000; Ditta et al., 2004). They are both upregulated in vegetative shoot apex and rosette leaves of *ebs*. Floral homeotic genes *PI,* required for the development of petals and stamens (Krizek and Meyerowitz, 1996) and *AG,* required for the development of stamens and carpels, repression of A-class floral organogenesis genes and maintenance of the determinacy of the floral meristem (Sun et al., 2009), were upregulated in rosette leaves of *ebs*. Besides *AG*, two other genes misregulated in *ebs* mutant, are responsible for the proper development of female reproductive organs. The SHP1 protein interacts with SEP3 and is essential for the coordination of cell divisions in the ovule (Ehlers et al., 2016). *SHP1* was upregulated in the vegetative shoot apex of *ebs*. *HECATE 1 (HEC1)* is required for the female reproductive tract development and fertility (Gremski et al., 2007). This gene was found to be overexpressed in all *ebs* samples.

This detailed analysis concerning the function of the genes differentially expressed in the *ebs* mutant and involved in floral transition, meristem identity or function, and flower development revealed some clear trends. First of all, genes connected with inflorescence meristem (*AGL24, TFL1*), flower meristem (*AGL8, LFY, TCP1*) and floral organ determination (*SEP3, SEP4, SHP1, HEC1*) are prematurely expressed in vegetative shoot apex of *ebs*. Some of the genes connected with floral meristem (*AGL8, PNY, TCP12*) and floral organ determination (*AG, PI, SEP3, SEP4, HEC1*) are also ectopically expressed in rosette leaves.

These results are consistent with the observed early flowering phenotype of *ebs* mutant and confirm that EBS is involved in the regulation of many levels of reproductive growth. Secondly, some of the repressors of floral transition (*FLC, MAF4, SNZ*) are upregulated in the inflorescence shoot apex of *ebs.* Similarly, some activators of the floral transition (*FT, SPL2, 3, 10, 15*) are downregulated in the inflorescence shoot apex of *ebs*. Also, genes that mark the induction of flowering in the meristem (*FAF2, FAF4, FLR1, FTM1*) are downregulated in the inflorescence shoot apex of *ebs*. These results confirm that despite early bolting, the maintenance of inflorescence identity is compromised in the *ebs* mutant.

2.2.7 Validation of RNA-seq results by qRT-PCR

In order to confirm the gene expression changes observed in the RNA-seq analyses we performed qRT-PCR experiments with a number of selected genes on independently collected samples (Fig.28). We analyzed some of the abovementioned differentially expressed genes involved in the regulation of the floral transition, meristem identity and function, floral transition at the meristem and flower development, showing the biggest difference in the expression level between WT and *ebs*. We assessed the expression of genes like *AG, AGL17, AGL24, FLC, FLOR1, FTM1, HEC1, SEP3, TCP1,* and *TFL1* in the tissue they were misregulated in. Additionally, we analyzed some of the highly misregulated genes connected with epigenetic regulation like *ARGONAUTE 9* (*AGO9*), involved in RNA-mediated post-transcriptional gene silencing (Durán-Figueroa and Vielle-Calzada, 2010), *Factor of DNA methylation 2* (*FDM2*), involved in RNA-directed DNA methylation (Ausin et al., 2012), *HDA7* and *18*, responsible for deacetylation of lysine residues of histones (Xu et al., 2005; Fong et al., 2006), and one histone H1 variant, *H1.3*, connected with rapid regulation of gene expression (Rutowicz et al., 2015), as well as β-CARBONIC ANHYDRASE 2 (BCA2), important for the photosynthesis processes (Dąbrowska-Bronk et al., 2016) and LATE EMBRYOGENESIS ABUNDANT 7 (LEA7), involved in abiotic stress response (Popova et al., 2015). In most cases, the results obtained corroborate the robustness of the transcriptomic analyses and further confirm that EBS is a key regulator for the expression of a number of genes that play central roles in reproductive development of Arabidopsis.

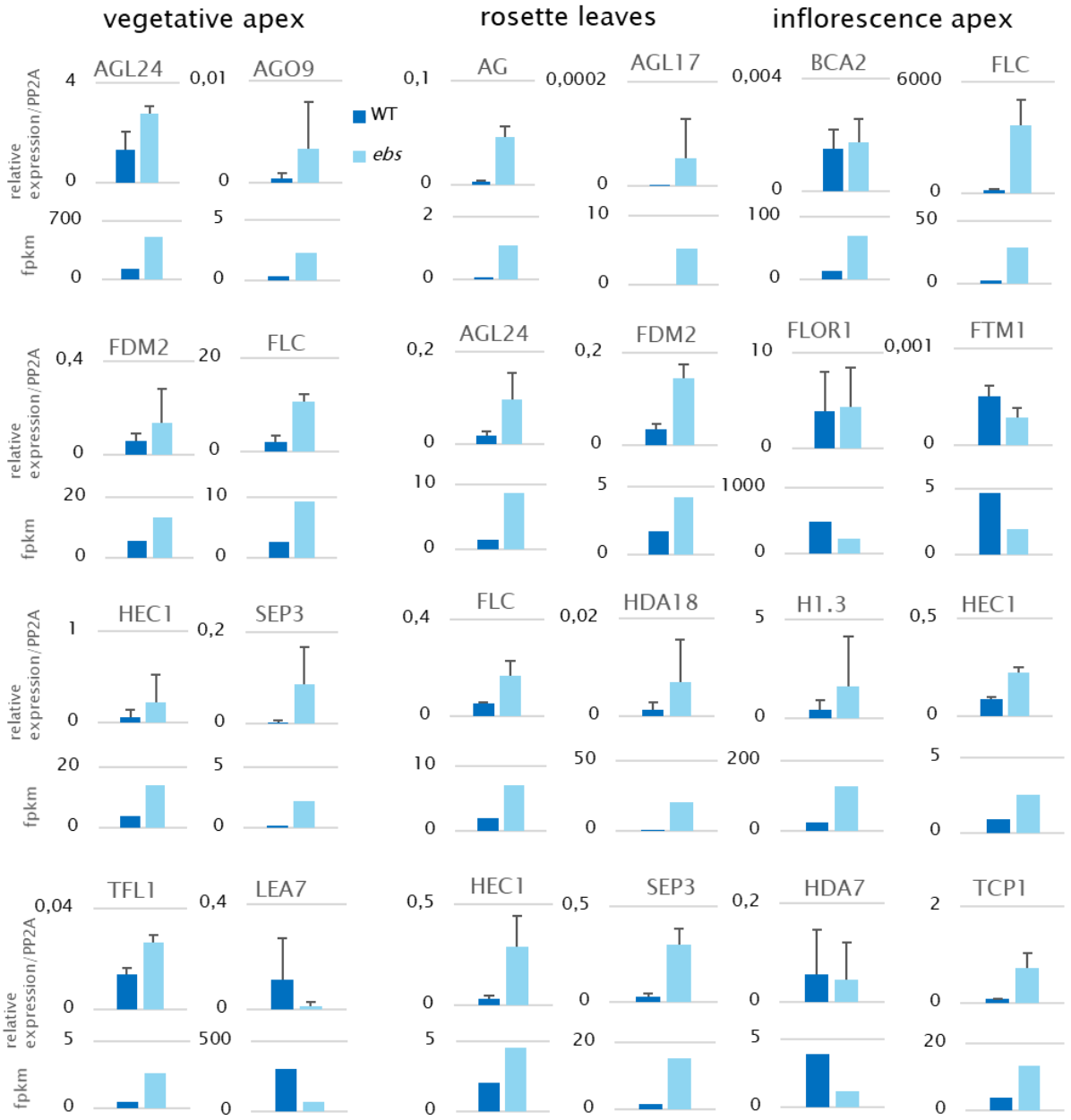

Fig.28 RNA-seq validation by qPCR in independently collected tissues of WT and *ebs* plants. For each gene, graphs in the upper row represent gene relative expression; graphs in the lower row represent fpkm transcript abundance from the RNA-seq analysis. First column represents the results for vegetative shoot apex; second column denotes the results from rosette leaves; and third column represents the results from inflorescence shoot apex.

3. Genome-wide analysis of EBS binding sites

3.1 Chromatin immunoprecipitation followed by next generation DNA sequencing (ChIP-seq)

To gain a deeper insight into the mechanism of transcriptional regulation mediated by EBS, we attempted to obtain genome-wide binding sites of the EBS protein through Chromatin Immunoprecipitation followed by next generation DNA sequencing (ChIP-seq). For that, we used manually dissected vegetative and inflorescence meristematic tissues, as well as rosette leaves of the *ebs* complementing line expressing a c-myc tagged version of EBS under the control of the *EBS* promoter (*EBS::c-myc-EBS*) (López-González et al., 2014). However, EBS contains no DNA binding domain and the binding to the genomic regions of target genes is likely indirect, via histone proteins. Presumably as a consequence of that, the amount of DNA isolated during the immunoprecipitation was not high, resulting in low signal-to-noise ratio and only a few binding sites for EBS identified. Since this approach did not render conclusive results, we decided to modify the initial experimental scheme and use whole vegetative seedlings at the same developmental stage that was used for RNA-seq, and apical parts of the inflorescence, without dissecting flowers. This alternative approach, with immunoprecipitated DNA sequenced in an Illumina platform with 50 bp single reads, enabled us to increase the strength of the signal and to identify 735 potential binding sites for EBS that mapped to 75 genes in seedlings and 1490 potential binding sites for EBS that annotated to 163 genes in inflorescence apices (detailed information about the potential EBS binding sites can be found in Annex 2).

3.1.2 Overlap between EBS binding sites and chromatin regions marked by different histone modifications

Previous results showed that EBS contains a PHD motif that binds di- and trimethylated lysine K4 on the histone H3 (H3K4me2/3) (López-González et al., 2014). We compared the distribution of the potential EBS binding sites identified with available online data regarding the distribution of seven well-described covalent modifications of histones that decorate genomic regions with diverse transcriptomic activity (Fig.29). As some of these modifications span long genomic fragments exceeding the length of sequencing reads, we clustered together peaks located very close to each other. As expected, high overlap was found between EBS binding sites and genomic regions marked with H3K4me2/3, and also H3K36me3, connected with transcriptionally active genes. Among these marks the highest overlap with EBS binding sites corresponds to H3K4me3, consistent with being the mark recognized and bound by EBS. In contrast, nucleosomes with histone H3 marked with lysine methylation that is connected with transcriptionally inactive genes, such as H3K27me1 and H3K27me3, were present at much lower level among the EBS binding sites. Interestingly, the potential binding sites of EBS strongly overlapped with genomic regions occupied by nucleosomes bearing dimethylated lysine K9 on histone H3, H3K9me2. This mark is enriched in heterochromatic regions and is required for the silencing of transposable elements and other repetitive DNA sequences (Bernatavichute et al., 2008).

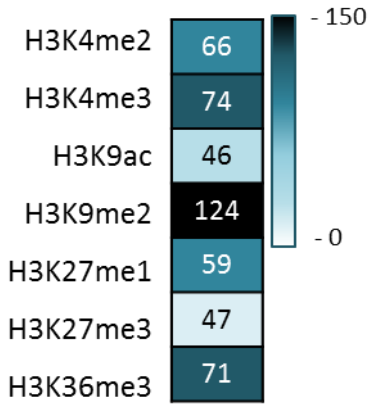

Fig.29 A scheme representing the overlap between potential genomic regions occupied by EBS and seven well-described covalent modifications of histones that affect transcription.

One of the H2A histone variants, H2A.W.7 is known to be strongly associated with H3K9me2 (Yelagandula et al., 2014). H2A.W.7 creates two pools across the genome: one connected with pericentromeric regions and another, associated with transient heterochromatin formed in the chromosome arms (Lorković et al., 2017). We decided to analyze separately the overlap between these two pools of H2A.W.7 and potential EBS occupancy. Only 31 out of 735 regions bound by EBS overlap with H2A.W.7 located in chromosome arms. However, a prominent overlap was identified between the potential genomic regions occupied by EBS and regions bound by H2A.W.7 that were annotated to pericentromeric regions (558/735 sites which represents 76%), localized very close to each other. Indeed, when we visualized the location of the potential regions occupied by EBS across chromosomes with close-by peaks grouped together and represented as one line, we could observe that many of those binding sites are localized in the areas connected with constitutive heterochromatin as pericentromers, centromers or 45 rDNA (Fig.30.A). These EBS binding sites appear clustered (instead of 558 sites, just a few red lines in the plot concentrate the sequences of EBS binding), indicating that many separate binding sites were grouped together because of close proximity. Thus, this analysis of the ChIP-seq data suggest that clusters of EBS protein occupy heterochromatic regions. A similar distribution can be observed in the data from inflorescence tissue samples (Fig.30.B).

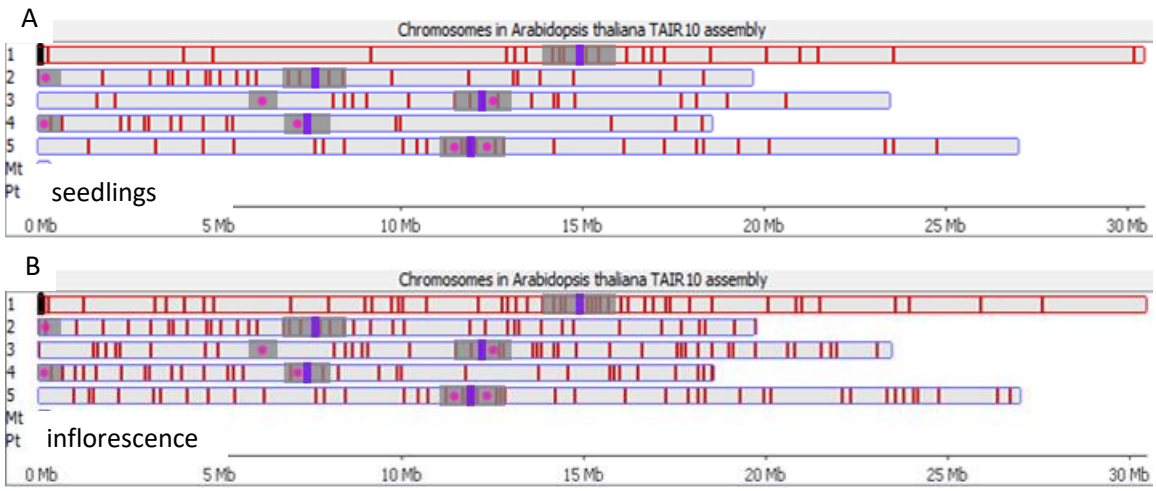

Fig.30 Schematic representation of potential EBS binding sites (red) over chromosomes generated by SeqMonk, where close-by peaks are grouped together and represented as one red line. A) Seedlings, B) Inflorescence apex. Dark gray corresponds to heterochromatin around centromeres, dark violet corresponds to centromeres and bright violet corresponds to 45 rDNA clusters in L*er* Arabidopsis (Zapata et al., 2016)

3.1.3 Overlap between EBS binding sites and transcriptomic results

Next, we assessed the overlap between genes differentially expressed in vegetative tissues (apical region and rosette leaves) or inflorescence tissue in our RNA-seq of *ebs* and genes identified as potential binding sites for EBS in seedlings or inflorescence. In this comparison we incorporated all significantly misregulated genes found in the transcriptomic analysis. In this way we obtained a list of 23 genes that included *AT3G41768*, encoding an 18S rRNA, *AT5G49120*, encoding a putative DUF581 family protein; *AT2G42380*, encoding the BZIP3 transcription factor; *ATCG01080*, encoding a chloroplast NADH dehydrogenase ND6; *AT5G40330*, encoding MYB DOMAIN PROTEIN 23 (MYB23), involved in the ectopic trichomes formation, and *AT1G68840*, encoding the TEMPRANILLO 2 (TEM2) transcription factor; *AT5G14170*, encoding the chromatin remodeler BAF60; *ATMG01390*, a gene coding mitochondrial ribosomal RNA; *AT4G34390*, encoding a GTP-binding protein; *AT3G10050*, encoding a chloroplast OMR1 protein; *AT1G64400*, encoding a LONG-CHAIN ACYL-COA SYNTHETASE 3 (LACS3); *AT4G38770*, encoding a PROLINE-RICH PROTEIN 4 (PRP4); *AT4G00880* encoding a SMALL AUXIN UPREGULATED RNA 31 (SAUR31); *AT3G23890* encoding TOPOISOMERASE II (TOPII), and *AT5G10100*, encoding TREHALOSE-6-PHOSPHATE PHOSPHATASE I (TPPI). From those genes only the binding site localized in the genomic region of *BAF60* overlapped with H3K4me2/3, the histone marks recognized and bound by EBS (Fig.31).

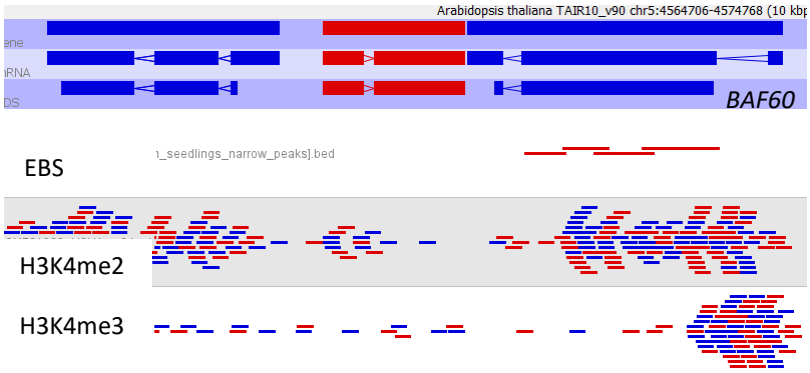

Fig.31 Representation of a potential EBS binding region generated in SeqMonk. DNA reads from *EBS::c-myc-EBS* seedlings sample were mapped to the genomic region of *BAF60*, the binding site overlaps with the regions occupied by histone marks recognized and bound by EBS: H3K4me2 and 3.

3.1.4 Validation of ChIP-seq data

In the data described above we revealed an unexpected correlation of EBS putative binding sites with heterochromatin features. In addition, we identified a low number of euchromatinic genes encompassing EBS binding sites as well as a limited overlap between these loci and genes differentially expressed in the *ebs* mutant. In view of these observations we decided to analyze by ChIP-qPCR some of the regions that were selected as putative EBS binding sites in the ChIP-seq experiment and mapped to euchromatic regions in seedlings (Fig.32.A) and inflorescence (Fig.32.B) samples, as well as regions that were annotated to constitutive heterochromatin, the pericentromeric regions, shared between seedlings and inflorescence samples (Fig.32.C, D). We investigated four genomic regions in seedlings: a fragment localized in the genomic region of *AT1G46264*, encoding the heat shock transcription factor SCHIZORRIZA (SCZ); two fragments within the chromatin remodeler *BAF60* locus, and one fragment localized within *AT3G24010*, encoding the PHD-domain containing transcriptional regulator *ING1*. A weak but reproducible binding signal was confirmed in all four fragments tested (Fig.32.A), confirming ChIP-seq data obtained in vegetative tissues. Besides, we investigated four genomic regions in inflorescence sample: two fragments localized within the genomic region of *EBS* itself, and two fragments localized within the genomic regions of *TEM2* and *SCZ* genes, respectively. In this case, binding of EBS was only confirmed in case of the second fragment analyzed of *EBS* genomic region, 'EBS 2' (Fig.32.B). We also analyzed the potential binding of EBS to heterochromatic regions by ChIP-qPCR. Our ChIP-seq data revealed very high values of binding to heterochromatin when normalized over input DNA; however, similar, or in some cases even higher values could be detected for the ChIP-qPCR negative control, WT sample (data not shown), compromising the reliability of EBS binding to these regions. In fact, when we normalized the results from DNA immunoprecipited with c-myc-EBS line over WT (the normalization used in all ChIP-qPCR experiments in this study, including validation of EBS binding to genomic regions in seedlings and inflorescence), the results for all the analyzed fragments were close to 1, suggesting that the binding of EBS to heterochromatic regions was somehow unspecific.

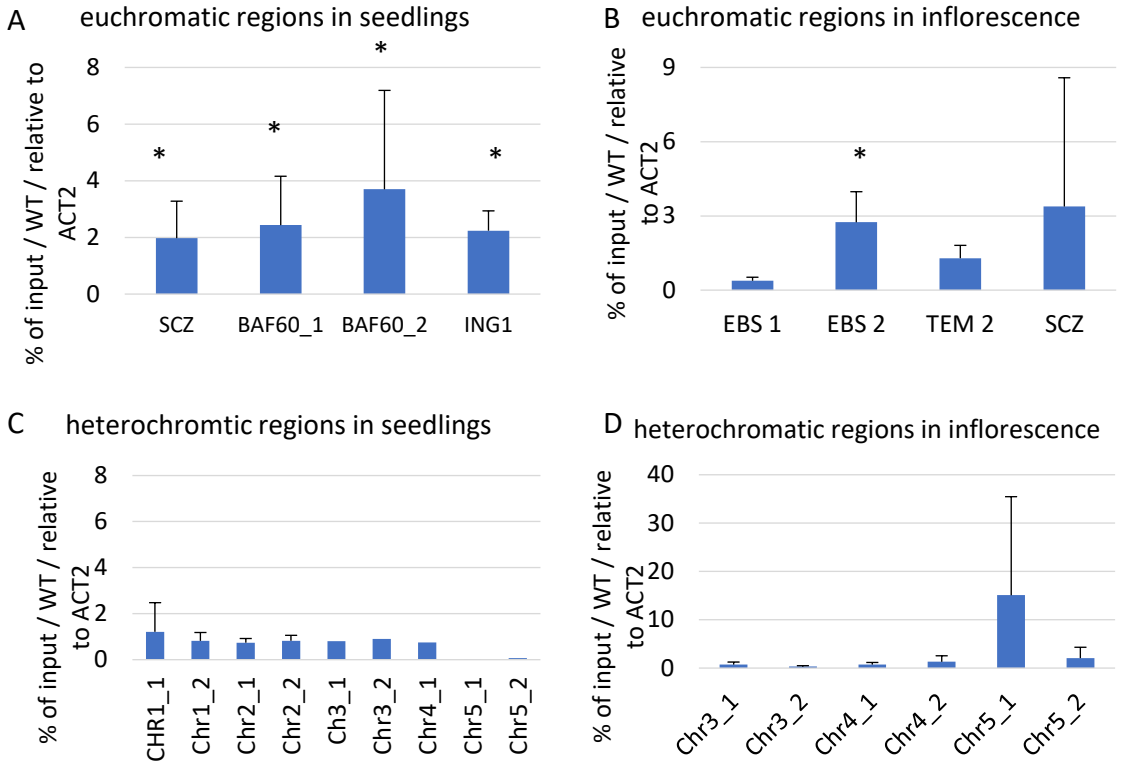

Fig.32 Validation of selected EBS binding sites by ChIP-qPCR method. A) Binding of EBS to euchromatic regions in seedlings; B) Binding of EBS to euchromatic regions in inflorescence, C) Binding of EBS to heterochromatic regions in seedlings; D) Binding of EBS to heterochromatic regions in inflorescence.

Since the heterochromatic EBS binding sites could not be confirmed by ChIP-PCR, it is possible that immunoprecipitated DNA fragments that map to heterochromatin regions in the ChIP-seq experiments result from the densely packed arrangement of this type of chromatin. In contrast, a number of euchromatic potential binding sites for EBS could be validated through ChIP-qPCR approaches, suggesting that at least some of them represent genuine targets of EBS in the Arabidopsis genome. However, the few binding sites identified are unlikely to represent genome-wide targets of this protein. The tagged version of EBS expressed in these experiments is driven by the *EBS* promoter, and it is possible that the amount of DNA immunoprecipitated by the chimeric myc-EBS protein is limiting to obtain robust results with this technique. Alternatively, EBS binding sites could be highly dynamic and/or tissue/cell specific and the DNA immunoprecipitated in each cell type is diluted in the mix of tissues present in the whole seedling. Further experiments to optimize the immunoprecipitation procedure will be needed to acquire a global view of EBS binding sites. For this reason, we decided to carry out ChIP-qPCR to analyze the potential binding of EBS to genomic regions of genes that were identified as differentially expressed in the *ebs* mutant and are putative candidates to mediate the effect of EBS in the regulation of reproductive development in Arabidopsis. These analyses corresponding to each one of the candidate genes assessed are shown in the following sections.

4. Candidate genes to mediate the effect of EBS in the regulation of reproductive development in Arabidopsis

To understand the molecular mechanism of EBS function in the control of reproductive development, we decided to focus on some candidate genes that appear as misregulated in the transcriptome analysis and could mediate the reproductive developmental defects observed in the *ebs* mutant. We analyzed the binding of EBS protein to genomic region of those genes to assess whether they are directly regulated by EBS. A physical interaction between EBS and histone deacetylases such as HDA6 and HDA19 was previously shown in the context of the regulation of flowering time, and particularly in relation to the repression of *FT* by EBS (López-González et al., 2014). These data led to the hypothesis that EBS directs histone deacetylases to the TSS of *FT*, modulating the acetylation state of *FT* chromatin and preventing the early activation of *FT* expression, particularly under non-inductive photoperiodic conditions. In this scenario, the absence of EBS signposts leads to increased H3 acetylation levels that in turn cause premature expression of *FT* (López-González et al., 2014). We decided to assess whether this repressive mechanism mediated by the EBS protein could be also operating in the regulation of other genes. H3K9/14 acetylation state at the TSS of candidate genes was analyzed. Increased acetylation is usually connected to less compacted chromatin structure around TSS that allows attachment of transcriptional machinery and gene expression. We performed Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) assays in which nucleosome depleted regions are amplified in qPCR analysis and the compaction level of chromatin can be assessed (Giresi et al., 2007). Finally, to investigate possible genetic interactions between *EBS* and candidate genes acting in genetic pathways controlling processes disturbed in *ebs* mutant, double mutants between them were generated.

4.1 Analysis of the possible genetic interaction between *EBS* and the floral integrator *FT* in the control of reproductive development

4.1.1 Analysis of the *FT* expression in the inflorescence of *ebs* mutant

During recent years a role for *FT* in other aspects of reproductive development beside the regulation of the floral transition has been described, including the impact on inflorescence architecture (Huang et al., 2013), flowering in axillary meristems (Niwa et al., 2013) and flower development. *FT* is necessary to prevent floral reversion, since low expression levels of *FT* have been proposed to disturb flowering memory and the commitment to flower production (see below for details, Müller-Xing et al., 2014).

In our RNA-seq data, *FT* expression was detected in the meristematic region of WT inflorescence, but not in the *ebs* mutant (Fig.27). This limited *FT* expression in the inflorescence shoot apex of the *ebs* mutant would be consistent with the floral reversion observed on the mutant. We validated this result on separately collected inflorescence tissue and confirmed a significant downregulation of *FT* in the inflorescence of the *ebs* mutant (Fig.33.A). This result was additionally supported by the FAIRE analysis of chromatin openness in the TSS of the *FT* gene. The dynamic alterations of openness in chromatin play important roles in many biological processes, including transcription, replication and differentiation (Cockerill, 2011; Anderson and Widom, 2000; Li et al., 2011). Higher chromatin openness at TSS of genes increases chromatin accessibility by transcription factors and is usually linked with increased expression (Cockerill, 2011). In the *ebs* mutant, the chromatin conformation at *FT* TSS region is more

compacted as compared with the control region (a promoter region of *LFY* that showed no difference in compaction between WT and *ebs* with the lowest variability between the repeats), suggesting a low transcriptional potential of the locus in the mutant (Fig.33.B).

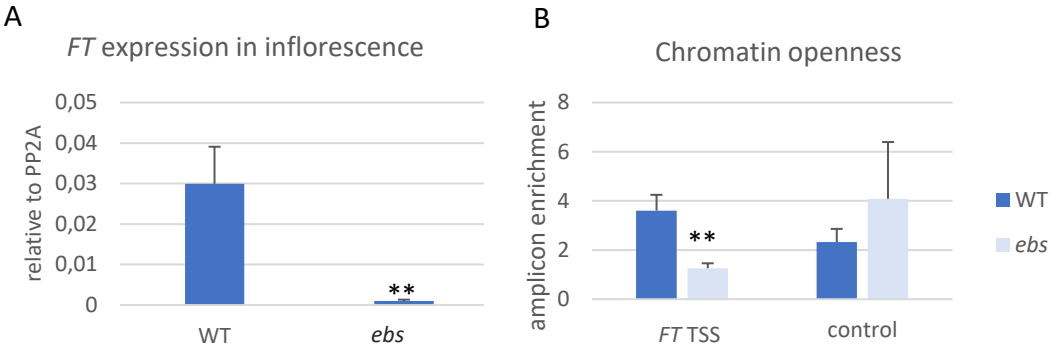

Fig.33 Expression of *FT* is downregulated in the inflorescence apex of the *ebs* mutant: A) Expression of *FT* measured by RT-qPCR in the inflorescence of WT and *ebs* mutant; B) Level of the openness of the chromatin at TSS region of *FT* as quantified by qPCR in the inflorescence of WT and *ebs* mutant. Asterisks represent statistical significance by Student's t-test.

4.1.2 Analysis of the influence of EBS on *FT* expression in distinct cell types

Low expression of *FT* in the inflorescence of *ebs* mutant suggests that *ebs* mutation has opposing consequences on *FT* expression - activating in vegetative tissues and repressing in inflorescence. To better understand this diverse effect, we compared the mechanism of regulation of *FT* by EBS in distinct cell types. It is known that *FT* is not uniformly expressed in all cell types of rosette leaves, but the expression of this gene is limited to the phloem companion cells (Takada and Goto, 2003; Chen et al., 2018). We used INTACT (ISOLATION OF NUCLEI TAGGED IN SPECIFIC CELL TYPES) method that enables selective isolation of nuclei from individual cell types (Deal and Henikoff, 2011) to differentiate between the cells expressing and not expressing *FT*. For the generation of INTACT lines, plants need to constitutively express biotin ligase BirA from *Escherichia coli* and a nuclear targeting fusion protein (NTF) under the control of a tissue specific promoter. The NTF chimeric peptide consists of a domain necessary and sufficient for nuclear envelope association in plants, green fluorescent protein (GFP) for visualization, and the biotin ligase recognition peptide, which serves as a substrate for BirA. This mechanism enables selective biotinylation of nuclei and isolation of biotin-labeled nuclei via affinity purification with streptavidin-coated magnetic beads. To address if *FT* is regulated by EBS by the same mechanism in all cell types, or the regulation is limited to a certain cell type, we analyzed if known traits of *FT* regulation by EBS can be detected in all the cells, or is limited to phloem companion cells, where *FT* is natively expressed. For that, we used a transgenic INTACT Arabidopsis line kindly provided by Franziska Turck's group (Max Planck Institute for Plant Breeding Research, MPIPZ, Cologne, Germany), expressing NTF protein under control of *SUC2* promoter. *SUC2* encodes a sucrose-H+ symporter that directs the expression of reporter genes, such as the *uidA* gene of *Escherichia coli* that encodes the enzyme β-glucuronidase (GUS), to the phloem (Truernit and Sauer, 1995) and is routinely used for companion cells studies (Cayla et al., 2015).

We validated and optimized the method by analyzing the expression of GFP protein through fluorescence microscopy (not shown) and *SUC2* gene expression (Fig.34.A). For that,

we assessed the following variants of the protocol: use or absence of fixation (tissue crosslinking with formaldehyde); different volume of the magnetic beads coated with streptavidin that are used in the isolation step; and different number of washes. From the conditions we tested, the best results were obtained without the use of fixation agent, increased volume of magnetic beads (100 µL) and extensive washes (at least 3 washing steps). We then measured the expression of *SUC2* and *FT* in INTACT SUC2 positive (+) and negative (-) nuclei and observed a noticeable increase in the expression level of these genes in SUC2 positive nuclei (Fig.34.B), confirming the enriched isolation of nuclei from companion cells through INTACT approaches.

Fig.34 Optimization and validation of INTACT method: A) Optimization with the combination of different fixation conditions and beads volume, B) Measurement of *SUC2* and *FT* expression in nuclei isolated with the optimized method.

We then analyzed the binding of EBS to *FT* regulatory regions specifically in the phloem companion cells where this floral integrator gene is expressed. For that we crossed SUC2 INTACT lines with *ebs* mutant plants expressing the complementing construct of EBS protein tagged with c-myc peptide under the control of the *EBS* promoter, *EBS::c-myc-EBS*. We analyzed 6 regions, spanning the entire *FT* genomic region: a distant region of the promoter important for the post-fertilization expression of *FT* (1C, Liu et al., 2014), a middle region of the promoter (64-65), a promoter region in the vicinity of TSS, known to be bound by EBS (FT+, López-González et al., 2014), two regions in the first intron (6, FT-) and a region in the 3' UTR (76,77). ChIP experiments performed in this line using the α-c-myc antibody and followed by the qPCR analysis on the nuclei isolated with INTACT method allowed us to show that previously reported binding of EBS to *FT* promoter (López-González et al., 2014) was present in SUC2 positive nuclei and absent from SUC2 negative nuclei (pooled nuclei from the rest of the plant) (Fig.35). This result indicates that EBS binds the *FT* promoter preferentially in the vascular tissue, where *FT* is normally expressed.

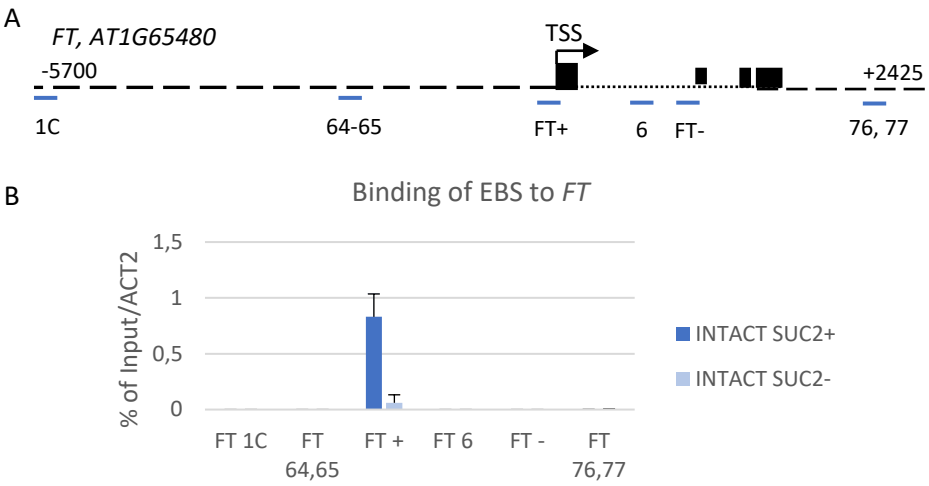

Fig.35 EBS binding to *FT* chromatin in the distinct cell types. A) Schematic representation of the genomic region of *FT*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR; B) Analysis of EBS binding to *FT* chromatin in nuclei isolated with the use of INTACT technique followed by ChIP-qPCR. Binding can only be observed in SUC2 positive nuclei.

Since *ebs* mutations cause increased levels of histone H3ac in the genomic region of *FT* (López-González, 2014), we performed chromatin profiling in nuclei isolated by INTACT in WT and *ebs* mutant background. This experiment showed that H3K9/14 acetylation changes are also limited to cells natively expressing *FT* (Fig.36.B, C). Additionally, we compared the distribution of H3K27me3, a repressive mark that has not been reported to have a role in the regulation of *FT* by EBS, in the SUC2 positive and SUC2 negative nuclei of WT and *ebs* mutant plants. We did not observe significant changes in H3K27me3 distribution in *FT* chromatin between WT and *ebs*, nor between SUC2 positive and SUC2 negative nuclei (Fig.36.D, E). These findings suggest that *FT* expression regulation by EBS is very selective and spatially limited to cells natively expressing *FT* in the vascular tissue of the leaves, where *FT* is expressed. This is consistent with our previous observations that demonstrated that in the *ebs* mutant before the floral transition *FT* is expressed at higher levels but only in the same tissues where this flowering gene is normally expressed in WT plants (not shown).

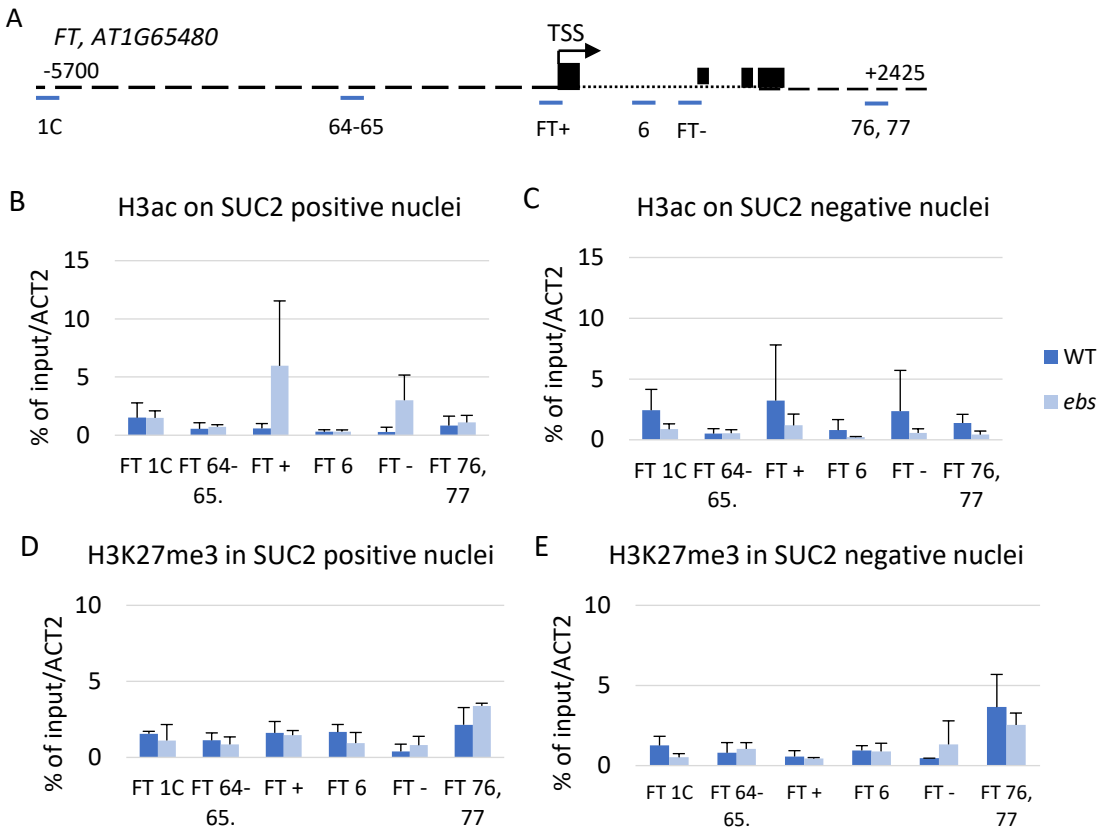

Fig.36 Analysis of chromatin features on the *FT* locus in nuclei isolated from phloem companion cells with the use of INTACT technique: A) Schematic representation of the genomic region of *FT*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR, B) H3ac levels in SUC2 positive nuclei, C) H3ac levels in SUC2 negative nuclei, D) H3K27me3 level in SUC2 positive nuclei, E) H3K27me3 level in SUC2 negative nuclei.

We then assessed the binding of EBS to *FT* in the shoot apical parts of the plants after flowering initiation. We analyzed a promoter region in the vicinity of TSS where EBS binds in phloem companion cells, and the distant promoter region, so called block C, needed for post-fertilization *FT* expression (Liu et al., 2014). No binding for EBS was observed in any of the regions analyzed (Fig.37).

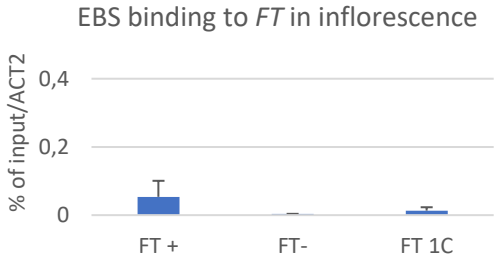

Fig.37 Analysis of EBS binding to *FT* chromatin in inflorescence tissues. No binding to the investigated regions was observed. The regions analyzed correspond to some of those described in Fig. 35.A

The results described above suggest that the regulation of *FT* expression by EBS is spatio-temporally controlled, and is consistent with a repressive role of EBS on *FT* expression

specifically in the phloem companion cells of vegetative leaves but not in other tissues of the leaf or the inflorescence apex.

4.1.3 Analysis of the *FT* expression patterns in the inflorescence

FT is needed to prevent reproductive reversion connected with the loss of flowering memory (Müller-Xing et al., 2014), a phenotype similar to the floral reversion observed in *ebs*. On the other hand, *FT* expression in rosette leaves for several days is sufficient to trigger commitment to flowering. Because FT activity in leaves is not maintained after transient photoperiodic induction, the molecular basis for stable floral commitment is unclear. However, decreased expression of *FT* in different parts of inflorescence causes reproductive reversion (Liu et al., 2014). This phenomenon consists in reversion from flower formation to the development of vegetative structures. Morphologically, it is manifested by cauline leaves or paraclades being formed after inflorescence nodes where fully developed flowers are generated.

Since the levels of *FT* were lower in the inflorescence apex of the *ebs* mutant (Fig.27; Fig.33) and decreased *FT* expression is associated with floral reversion, we decided to further investigate the potential role for *FT* in mediating the floral reversion events observed in the *ebs* mutant by analyzing the effects of this mutation on the spatial pattern of *FT* expression in the inflorescence. For that, we monitored the expression of *FT* with the use of GUS staining assay. We used WT and *ebs* mutant plants expressing the GUS activity under the control of the *FT* promoter (*pFT::GUS*). As expected, in WT plants GUS staining is clearly observed in the pedicel of flowers, consistent with previous results (Liu et al., 2014). Moreover, there was no significant difference in GUS signal in the pedicel of mature flowers between WT and *ebs* mutant line (Fig.38.A, B). In the siliques, slightly stronger coloration of GUS could be observed in the pedicel of *ebs* mutant although the spatial pattern is similar to WT (Fig.38.C, D). Interestingly, abnormally strong GUS signal could be observed in the pedicel of the flowers of *ebs* mutant undergoing floral reversion. Figure 38.F shows four flowers formed consecutively in the inflorescence of the *ebs* mutant. The second flower is undergoing floral reversion. GUS coloration in the pedicel of this flower is much stronger than in the pedicels of the previous flower or the following one, which has already lost the floral organs except for the carpels. Also, in the pedicel of the WT flower at a comparable developmental stage no signs of GUS overexpression could be observed (Fig.38.E). Further studies will be necessary to establish the possible relationship between this increased *FT* expression and floral reversion observed in the *ebs* mutant.

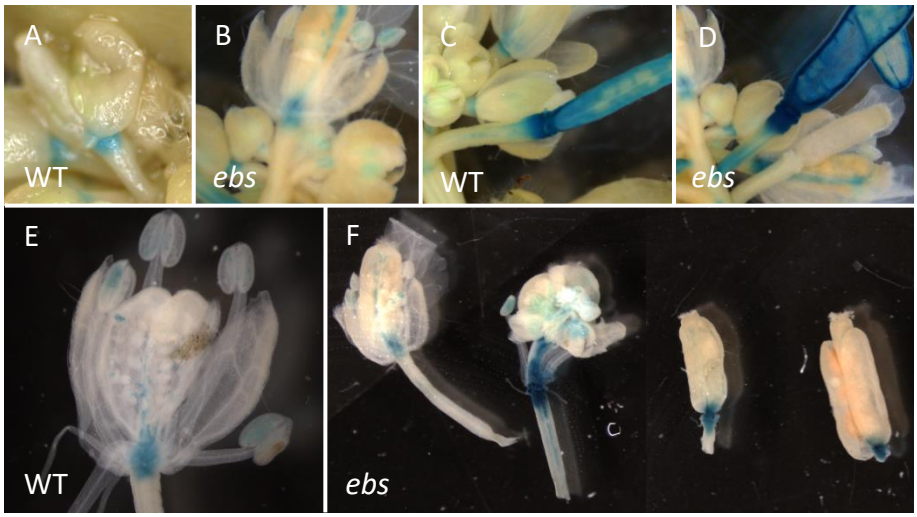

Fig.38 GUS staining in WT and *ebs* mutant plants expressing GUS under the control of the *FT* promoter: A, B) Mature flowers; C, D) Siliques, E) WT flower; F) *ebs* mutant flower undergoing floral reversion as compared with one plastochron younger flower, and one plastochron and two plastochron older siliques.

4.1.4 Analysis of genetic interactions between *EBS* and *FT* during inflorescence development

In order to further understand the possible relationship between *EBS* and *FT* in the regulation of different aspects of inflorescence development, we undertook a genetic approach that could reveal genetic interactions between both loci in the control of this developmental process. First, we attempted to monitor inflorescence development and episodes of floral reversion in *35S::FT* plants with constitutive *FT* expression. However, even under SD *35S::FT ebs* plants exhibit an extreme early flowering phenotype (after formation of 3 rosette leaves) (Teper-Bamnolker and Samach, 2005) with premature termination of the inflorescence and development of only a few flowers that precluded the observation of any floral reversion phenomena. Alternatively, high levels of *FT* expression could prevent floral reversion even in a genetic background deficient in EBS activity (Fig.39).

Fig.39 Comparison of the phenotype of WT, *ebs*, *35S::FT* and *35S::FT ebs* in L*er* background grown for 2 weeks in SD conditions.

We then analyzed the phenotype of *ft ebs* double mutant, to establish whether a complete lack of *FT* function enhances the frequency or severity of floral reversion events in the *ebs* mutant. However, as shown in Figure 40.E, under SD conditions the double mutant *ft ebs*

continues to produce leaves instead of flowers for a longer period of time, making it impossible to assess the combined effect of these two mutations on floral reversion in this experimental setting. Growth conditions that favor an extended reproductive development of the double mutant will be required to address in detail this question. Nevertheless, these observations suggest that the vegetative features in the inflorescence of the double mutant are enhanced as compared to each single mutant, supporting the hypothesis that *EBS* and *FT* act in independent pathways to control floral identity in the inflorescence under SD conditions.

Fig.40 Comparison of the phenotype of WT, *ft, ebs* and *ft ebs* in L*er* background: A) Comparison of the inflorescence architecture; B-E) Comparison of shoot apices of the genotypes analyzed. Plants were grown for 8 weeks in SD conditions.

Interestingly, the characterization of the inflorescences present in *ft ebs* plants grown in LD revealed that this double mutant produces a significantly higher number of rosette stems than the *ebs* and *ft* single mutants, whose phenotypes are close to WT (Fig.41.A, B). Under SD the *ft ebs* double mutant also displays an increased number of rosette stems as compared to WT and *ft* mutant plants, but the number of rosette stems is significantly lower than in the *ebs* single mutant (Fig.41.C). Given that the *ft* mutation on its own does not affect significantly the number of rosette stems under SD, this observation suggests that *FT* function is required for full expressivity of the alteration in the number of rosette stems of the *ebs* mutant. Moreover, apical dominance is severely altered in the *ft ebs* double mutant grown in LD but is partially rescued in SD-grown *ft ebs* double mutant plants. This finding confirms a role for *FT* in the regulation of inflorescence architecture and suggests that *FT* interacts with *EBS* and environmental stimuli to modulate branching and consequently inflorescence architecture.

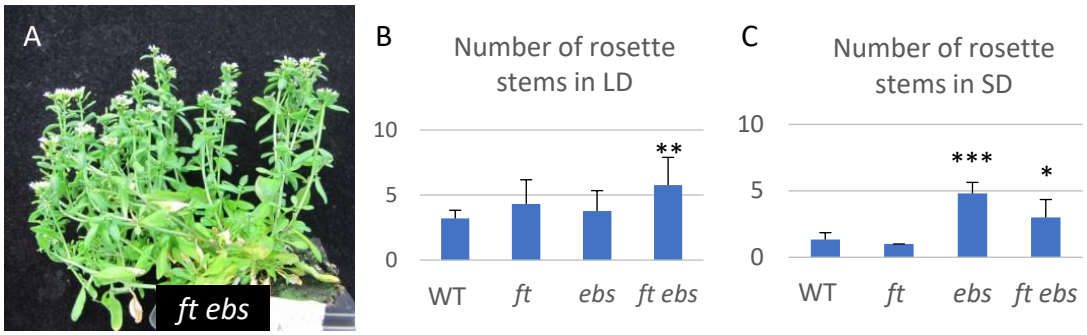

Fig.41 Inflorescence architecture alterations observed in the *ft ebs* double mutant: A) Phenotype of *ft ebs* inflorescence under LD; B), C) Quantification of the number of rosette stems formed by WT, *ft*, *ebs* and *ft ebs* in L*er* background grown in LD (B) and SD (C) Conditions. Asterisks represent statistical significance by Student's t-test.

Altogether, the data discussed above indicate that EBS is necessary for proper control of *FT* expression during inflorescence development, although this regulation might depend on a different regulatory mechanism than during the vegetative growth of the plant, where *FT* is epistatic over *EBS* on flowering time control. Furthermore, the genetic analyses performed suggest that these two loci interact differentially to regulate various aspects of reproductive development, displaying synergistic interactions for floral identity determination under SD and for rosette stem development in LD. Besides, FT is also required for extra rosette stem development in *ebs* mutant grown under SD. Further studies are needed to unveil the intricate nature of the observed interactions between these two loci to modulate the inflorescence response to photoperiod.

4.2 Analysis of the possible interaction between *EBS* and the floral repressor gene *FLC* in the control of reproductive development

FLC is one of 38 genes found to be overexpressed in all three samples collected for RNA-seq analysis and further confirmed by qPCR on separately collected tissue. Also, timeline experiments measuring expression of *FLC* at different developmental stages of WT and *ebs* plantlets have confirmed overexpression of *FLC* in the *ebs* mutant at different developmental stages (Fig.42).

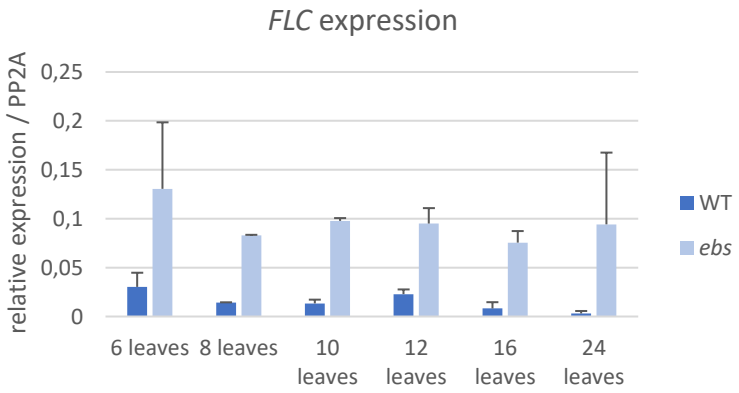

Fig.42 Analysis of *FLC* expression by RT-qPCR in plantlets of WT and *ebs* mutant collected over a period of 18 plastochrons.

4.2.1 Influence of EBS on chromatin organization of the *FLC* locus

We performed ChIP-qPCR analyses in *EBS::c-myc-EBS ebs* seedlings to check the binding of EBS to five *FLC* genomic regions important for the regulation of this gene. The results obtained show that the enrichment of EBS in the regulatory regions of *FLC* is similar to that found in WT negative controls, indicating that EBS does not bind to the genomic fragments analyzed (Fig.43.B).

We then analyzed chromatin features that might be associated with the transcriptional induction found in *ebs* background by assessing the levels of histone H3 acetylation at *FLC* TSS and the chromatin accessibility at the TSS and other locations in the genomic region of this locus. We observed a slightly increased H3K9/14 acetylation level (Fig.43.C) at the TSS of *FLC* in the *ebs* mutant, consistent with the upregulation of this gene in the mutant plants described above. However, no significant changes in the chromatin compaction levels were detected (Fig.43.D). *FLC* allele of L*er* has an insertion of a Mutator-like element (MULE) transposable element into the first intron. This insertion acts in cis and causes *FLC*-L*er* to be expressed at low levels due to the higher chromatin compaction (Michaels et al., 2003). We analyzed chromatin openness at the retrotransposon insertion site, but again no difference between WT and *ebs* was observed.

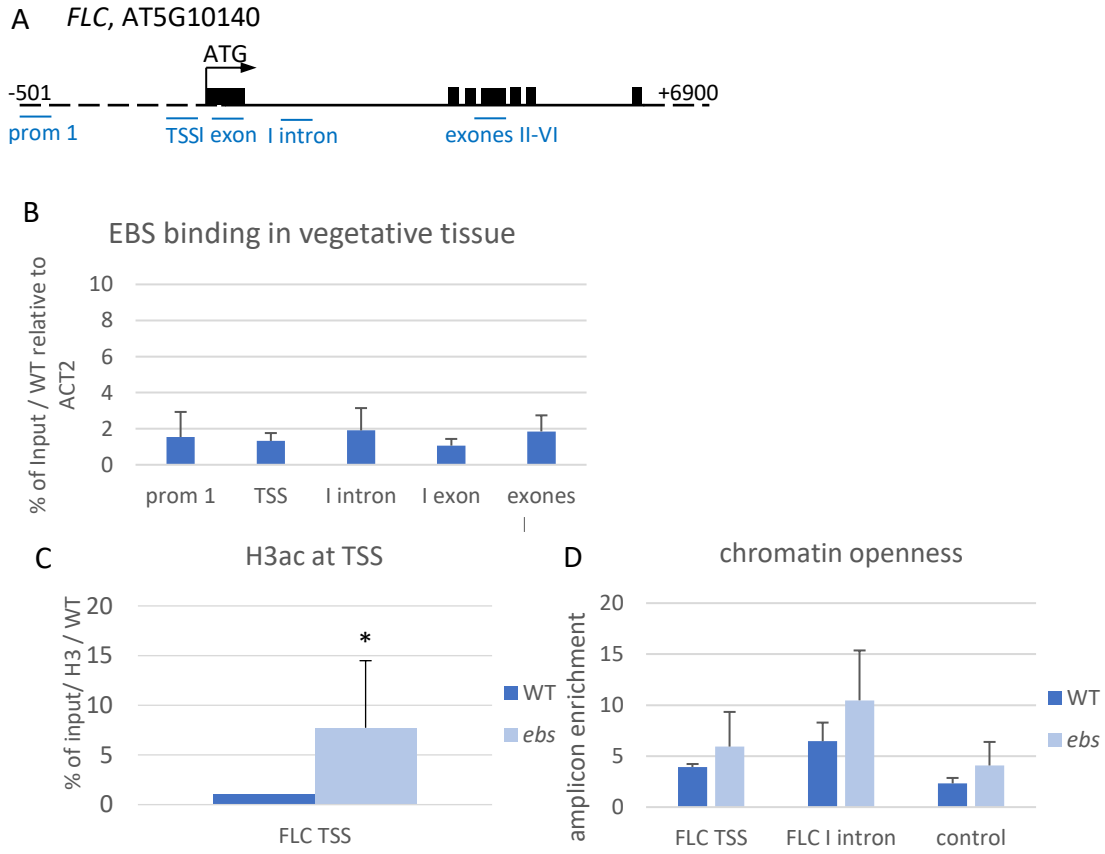

Fig.43 Effect of EBS on chromatin features of the *FLC* locus: A) Schematic representation of the genomic region of *FLC*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR. B) Binding of EBS to *FLC* chromatin in seedlings; C) Comparison of H3 acetylation state at the TSS of *FLC* in WT and *ebs* mutant; D) Levels of chromatin openness at the TSS and the first intron of *FLC* in WT and *ebs* mutant. Asterisk represents statistical significance by Student's t-test.

4.2.2 Analysis of genetic interactions between *EBS* and *FLC*

When overexpressed in the mutants of PcG genes, *FLC* causes reproductive reversion through downregulation of *FT* expression in the inflorescence (Müller-Xing et al., 2014). Also, floral reversion episodes, very similar to those described for *ebs* where an inflorescence is created inside the silique, were reported for the lines overexpressing *FLC* (*35S::FLC*, Hepworth et al., 2002). However, we observed no changes in inflorescence architecture or flower development in the *flc* mutant (data not shown) and neither differences between the phenotypes of *ebs* single and *flc ebs* double mutant were observed under SD (Fig.44). Although these observations may exclude a role for *FLC* in mediating the effect of EBS on reproductive development, further experiments will be needed to explore the possible relationship of both loci in the control of inflorescence architecture and flower development. The analysis of the genetic interaction between the *ebs* mutant and *35S::FLC* plants is underway to explore the possible interplay of these genes to modulate these developmental processes.

Fig.44 Comparison of the phenotype of WT, *flc*, *ebs* and *flc ebs* plants in Col background grown for 7 weeks under SD conditions.

4.3 Analysis of the possible interaction between *EBS* and the transition meristem gene *AGL24* in the control of reproductive development

In our RNA-seq study *AGL24* was overexpressed in both vegetative tissues of *ebs*: meristems and leaves. The expression of this gene was also upregulated in later stages of *ebs* inflorescence development: overexpression was observed in floral buds, flowers and during floral reversion (Fig.18). Additionally, AGL24 was proven to be involved in floral reversion since in *AGL24* overexpressing lines (*35S::AGL24*), secondary flowers are created in the axils of sepals (Yu et al., 2004; Liu et al., 2007). Later in development, a new inflorescence can emerge from the ovary in these *35S::AGL24* plants. Moreover, floral organs undergo homeotic transformations toward structures normally found in inflorescence shoots (Yu et al., 2004). Gene regulatory network analysis revealed that overexpression of *AGL24* can change the identity of the floral meristem into inflorescence/floral meristem mixed identity that promotes

floral reversion (Pérez-Ruiz et al., 2015). These observations make *AGL24* a potential candidate to mediate the abnormal phenotypes in reproductive development observed in the *ebs* mutant.

4.3.1 Influence of EBS on the chromatin organization of the *AGL24* locus

To assess the possible involvement of AGL24 in the regulatory mechanism of the inflorescence development mediated by EBS, we analyzed the binding of this chromatin factor to four regions of *AGL24* containing or localized close to CarG box motifs in the promoter and the first intron (Liu et al., 2008). For that we performed ChIP assays using the *EBS::c-myc-EBS ebs* line. As *AGL24* expression is overexpressed in the vegetative tissue, and the collection of seedlings is much easier and faster than the collection of inflorescence samples, we first performed the ChIP analysis in seedlings. Binding of EBS to the promoter region 2 was observed (Fig.45.B), confirming that EBS regulates *AGL24* expression directly, at least during vegetative growth, although no binding was observed in the analyzed regions of DNA immunoprecipitated from inflorescence tissue (Fig.45.C).

We then analyzed the impact of the *ebs* mutation on histone H3 acetylation in the genomic region of *AGL24* in inflorescence tissue. H3K9/14 acetylation was slightly, but reproducibly, increased close to the TSS region of *AGL24* in the *ebs* mutant (Fig.45.D). We also observed higher chromatin openness at this region (Fig.45.E) which is likely to facilitate transcription. These observations show that EBS is required to maintain a repressed state in the chromatin region of *AGL24* and that EBS might be involved in the modulation of histone acetylation dynamics in these genomic regions as previously proposed for *FT* (López-González et al., 2014).

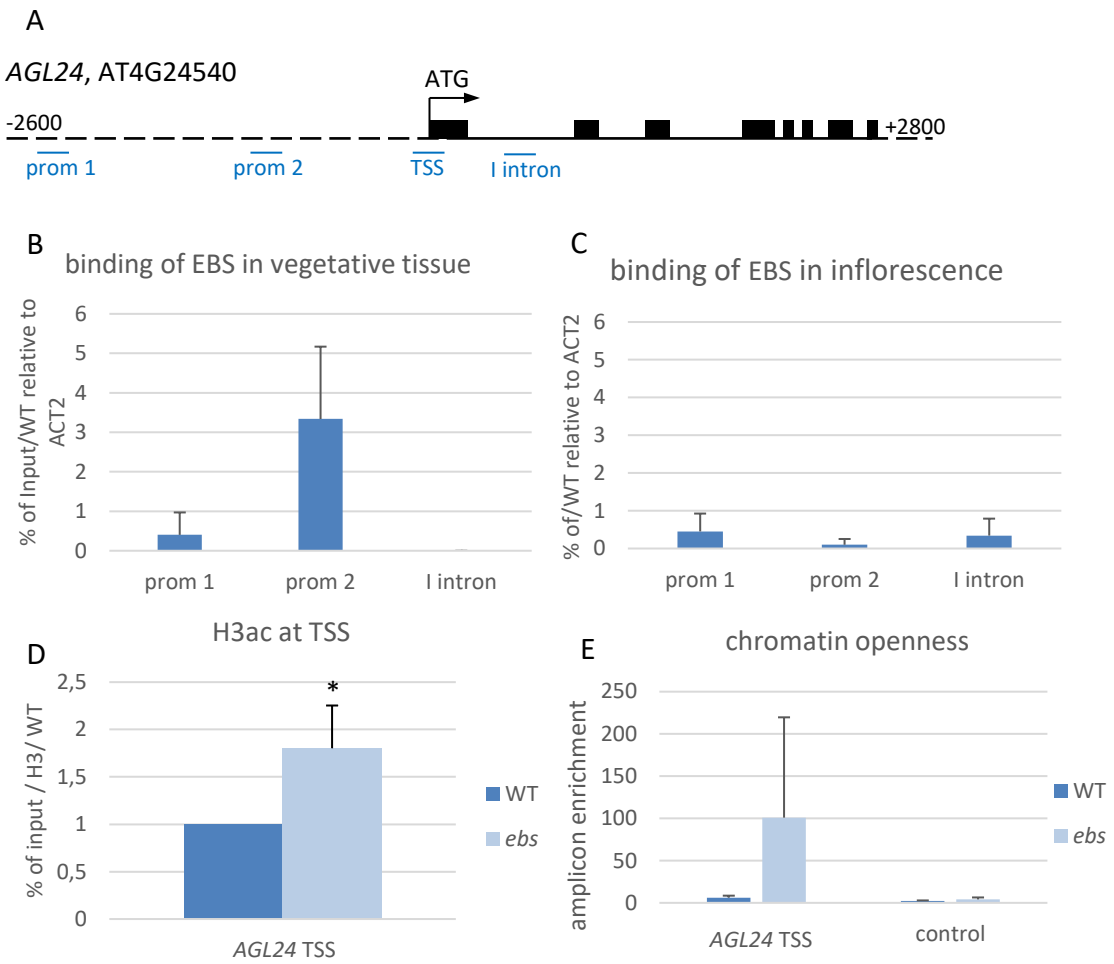

Fig.45 Binding of EBS to the *AGL24* locus and effect of the *ebs* mutation on chromatin features in this gene. A) Schematic representation of the genomic region of *AGL24*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR. B) Binding of EBS to *AGL24* in seedlings; C) Binding of EBS to *AGL24* in inflorescence; D) Comparison of H3 acetylation state at the TSS of *AGL24* in the inflorescence of WT and *ebs* mutant; E) Levels of chromatin openness at the TSS of *AGL24* in the inflorescence of WT and *ebs* mutant. Asterisk represents statistical significance by Student's t-test.

4.3.2 Analysis of genetic interactions between *EBS* and *AGL24*

Based on these observations and previous data discussed above, we hypothesized that *AGL24* overexpression in *ebs* mutant plants could affect flower development and mediate the floral reversion events observed in the *ebs* mutant under SD conditions. To evaluate the involvement of *AGL24* in this pathway mediated by *EBS*, we generated the *agl24 ebs* double mutant and analyzed its phenotype. The results obtained show that *agl24 ebs* flowers slightly later than the *ebs* single mutant under SD and displays no significant changes in inflorescence architecture (Fig.46.A). Interestingly, the phenotypic alterations connected with abnormal flower development in the *ebs* mutant (Fig.46.D) are partially rescued in the *agl24 ebs* double mutant (Fig.46.E). The frequency of floral reversion episodes is also reduced in the *agl24 ebs* double mutant (Fig.47). Altogether these observations suggest that EBS is required for the regulation of *AGL24*, and the function of this gene is necessary for full expression of the floral

alterations observed in the *ebs* mutant under SD, suggesting a possible genetic interaction between both loci in the regulation of late stages of reproductive development.

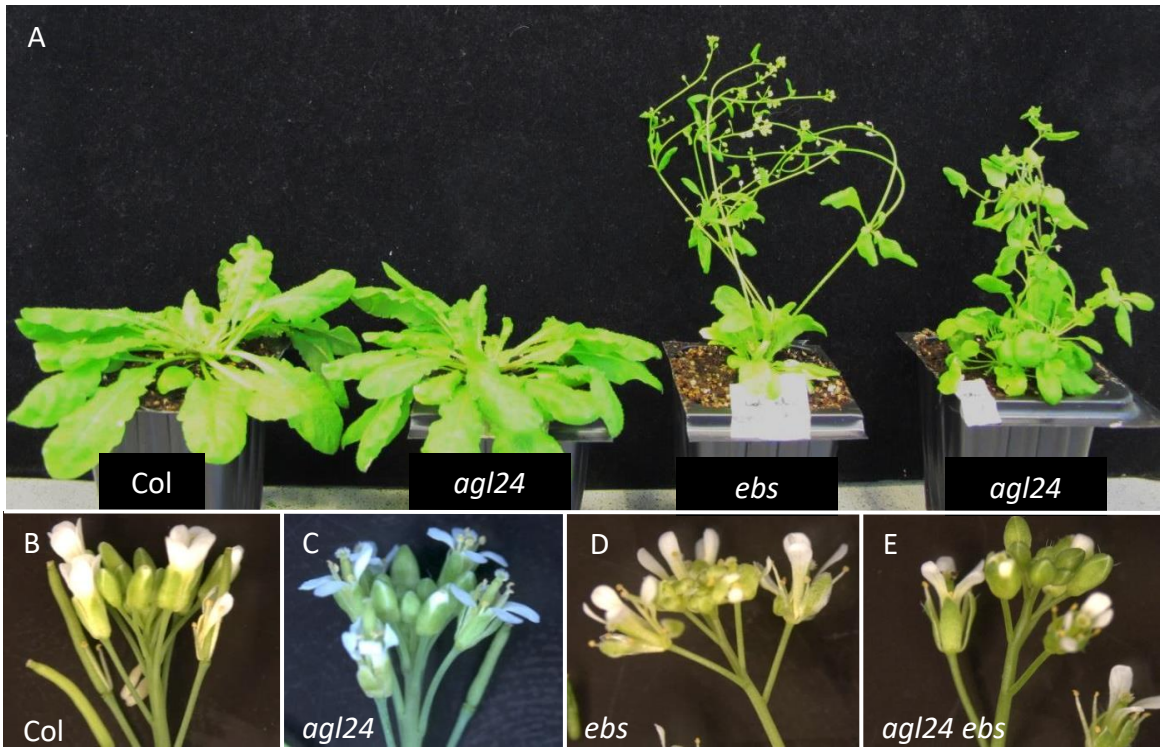

Fig.46 Comparison of the phenotype of WT, *agl24*, *ebs* and *agl24 ebs* in Col background: A) Comparison of the plant architecture. Plant were grown for 7 weeks in SD conditions, B-E) Comparison of the phenotype of reproductive shoot apices of the genotypes analyzed.

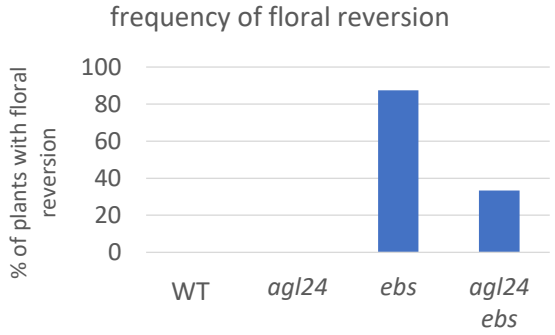

Fig.47 The frequency of floral reversion episodes in WT, *agl24*, *ebs* and *agl24 ebs* plants in Col background.

4.4 Analysis of the possible interaction between EBS and the inflorescence meristem gene *TFL1* in the control of reproductive development

In our transcriptomic analysis, *TFL1* was found to be upregulated in vegetative meristems of the *ebs* mutant. This gene is also upregulated in the later stages of *ebs* mutant growth, during floral reversion. TFL1 opposes the establishment of floral meristem identity but does not antagonize the maintenance of the floral meristem. Upregulation of *TFL1* was linked previously to floral reversion phenotypes of some transgenic lines (Pérez-Ruiz et al., 2015).

4.4.1 Influence of EBS on the chromatin organization of the *TFL1* locus

Given the upregulation of *TFL1* observed in the *ebs* mutant, we analyzed the binding of EBS to four genomic regions previously shown to be important for the regulation of this inflorescence gene, including those located on the 3´end of the locus (Serrano-Mislata et al., 2016). As *TFL1* is upregulated in the vegetative tissue, and collection of seedlings is much easier than the collection of inflorescence samples, we first performed the analysis in seedlings. Our data showed binding of EBS to the *TFL1* promoter region 2, previously shown to bear a binding site for BAF60 (Sacharowski et al., 2015) and XAL2 (Pérez-Ruiz et al., 2015), and to two distant 3'UTR regions named E and F (Figure 48.B; Serrano-Mislata et al., 2016). We then analyzed the binding of EBS to these regions in inflorescence and observed again the binding to region E. This region is responsible for the expression of *TFL1* in lateral meristems and contains a CarG box motif that is bound by AP1, SOC1, AGL24, SVP, SEP4 and XAL2 proteins (Serrano-Mislata et al., 2016). On the other hand, region F is needed for the proper expression of *TFL1* in inflorescence meristem and possesses a binding site for LFY. Together, regions E and F are important for inflorescence architecture and expression of *TFL1* without these regulatory regions drives the formation of secondary and tertiary branches (Serrano-Mislata et al., 2016), a phenotype also observed in the *ebs* mutant.

Next, we analyzed the levels of histone acetylation at the TSS of *TFL1* gene. No significant changes in the levels of H3K9/14 acetylation were observed in the fragment analyzed (Fig.48.D). This observation suggests that EBS could mediate transcriptional regulation of inflorescence genes through a molecular mechanism independent of histone acetylation, although a thorough analysis of H3ac in additional regions of the *TFL1* locus is necessary to rule out the implication of this histone modification in the regulation of *TFL1* by EBS. Furthermore, analyses of chromatin compaction in different regions of this locus showed higher chromatin openness in the vicinity of the TSS region, consistent with the increased expression of *TFL1* observed in the *ebs* mutant (Fig.48.E).

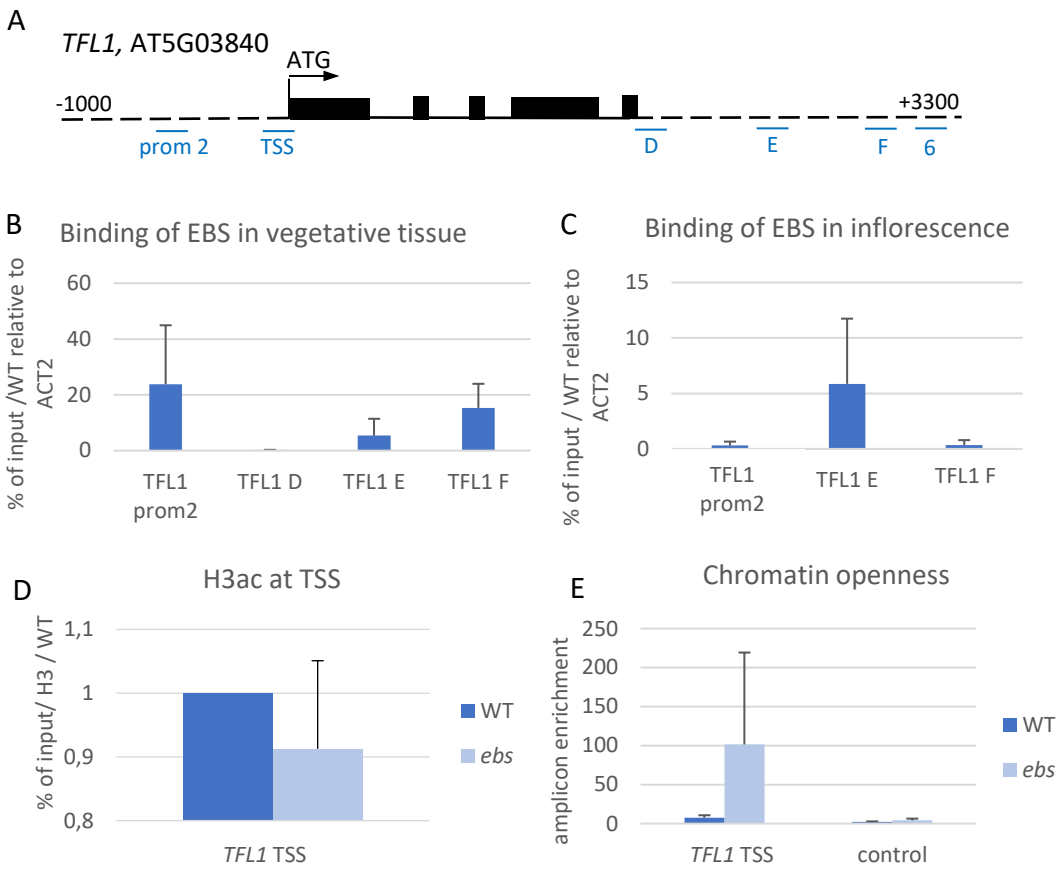

Fig.48 EBS binds regulatory regions of *TFL1*. A) Schematic representation of the genomic region of *TFL1*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR. B) Binding of EBS to the *TFL1* locus in seedlings; C) Binding of EBS to the *TFL1* locus in inflorescence; D) Comparison of H3 acetylation state at the TSS of *TFL1* in the inflorescence of WT and *ebs* mutant; E) Levels of chromatin openness at the TSS of *TFL1* in the inflorescence of WT and *ebs* mutant.

Interestingly, while chromatin at TSS region is less compacted in the inflorescence of the *ebs* mutant (Fig.48.E) chromatin at the 3'UTR of *TFL1* seems to be more compacted in the seedlings of *ebs* mutant than in WT. At the 3'UTR of *TFL1* the nucleosome-depleted regions were not detectable in the assay measuring chromatin openness in seedlings (Fig.49.C). Given the changes in chromatin compaction observed in the genomic regions of the *ebs* mutant, we decided to evaluate the occupancy of unmodified histone H3 over *TFL1* in seedlings. ChIP analyses with α-H3 antibodies revealed an enrichment of H3 at the 3'UTR of this gene in the *ebs* mutant (Fig.49.B), showing that EBS is required for a proper chromatin organization in the regulatory 3´end region of *TFL1*.

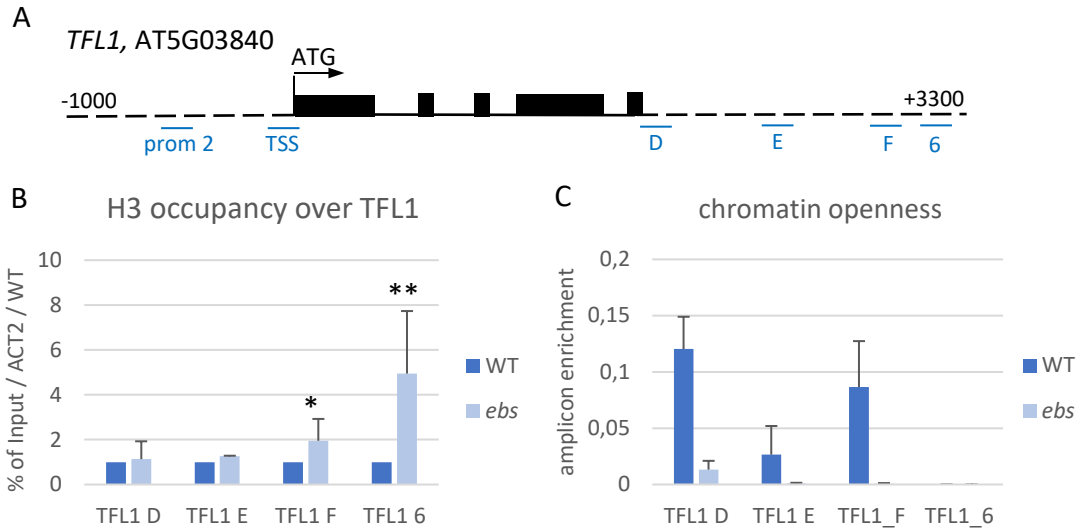

Fig.49 Chromatin conformation in 3' region of *TFL1* in seedlings of WT and *ebs* mutant: A) Schematic representation of the genomic region of *TFL1*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR. B) H3 occupancy; C) Levels of chromatin openness. Note that the number of nucleosome depleted regions is very low in this case, and due to the dilution of the signal we do not expect a perfect correlation with histone H3 occupancy data. Still, in all investigated fragments the chromatin is more compact in case of the *ebs* mutant. Asterisks represent statistical significance by Student's t-test.

4.4.2 Analysis of genetic interactions between *EBS* and *TFL1*

In order to investigate any possible genetic interaction between *EBS* and *TFL1* in the control of inflorescence development, we built the *tfl1 ebs* double mutant and analyzed its phenotype. As in the case of *35S::FT ebs*, these double mutant plants are extremely early flowering under SD (Fig.50.A) and inflorescence development is terminated soon after bolting due to the terminal flower phenotype of the *tfl1* mutation (Fig.50.C,E).

Fig.50 Comparison of the phenotype of WT, *tfl1*, *ebs* and *tfl1 ebs* in L*er* background: A) Comparison of the inflorescence architecture. Plants were grown for 8 weeks in SD conditions, B-E) Comparison of the phenotype of shoot apices in the analyzed genotypes.

tfl1 ebs plants are able to produce only a few more flowers than the *tfl1* single mutant before the formation of terminal flowers in the main shoot and lateral branches (Fig.51). However, this partial rescue of *tfl1* phenotype does not change significantly the overall phenotype of the inflorescence that is very similar in the double mutant and in *tfl1* single mutants. As discussed above for 35S::*FT ebs* plants, the premature termination of inflorescence growth could preclude the observation of inflorescence defects present at later stages in the *ebs* mutant.

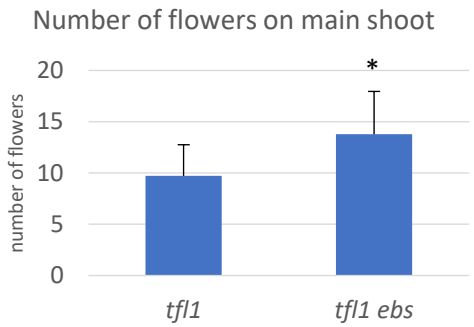

Fig.51 Quantification of the number of flowers formed in *tfl1* and *tfl1 ebs* mutants until the formation of terminal flower on the main shoot. Asterisk represents statistical significance by Student's t-test.

4.5 Analysis of the possible interaction between *EBS* and the floral meristem gene *LFY* in the control of reproductive development

LFY was found in our transcriptomic analysis to be upregulated in vegetative meristems of *ebs*. The expression of this gene is also upregulated in later stages of inflorescence development: high expression was observed in floral buds, flowers and during floral reversion (Fig.27; Fig.18).

4.5.1 Influence of EBS on chromatin organization of the *LFY* locus

We investigated the potential binding of EBS into five different genomic regions of *LFY* that are important for the regulation of this floral meristem identity gene (Pastore et al., 2011). As *LFY* is upregulated in the vegetative tissue in the *ebs* mutant, we first performed the analysis in seedlings. The ChIP analyses with the *EBS::c-myc-EBS* line did not reveal any enrichment for EBS in the regions analyzed (Fig.52.B), although we cannot rule out binding of EBS to other promoter regions not tested in this experiment. We used the *FT* region bound by EBS (FT+) as a positive control and the *FT* region where EBS does not bind (FT-) as a negative control.

We then performed the analysis of the histone H3 acetylation levels around TSS of *LFY* in the WT and *ebs* mutant. We did not observe an increased acetylation of H3K9/14 (Fig.52.C). These observations suggest that *LFY* regulation by the EBS protein could take place indirectly, and thus, additional factors might mediate the changes in *LFY* expression observed in the *ebs* mutant. Still, the results derived from an assay measuring chromatin openness suggest that the *LFY* chromatin structure is less packed in the *ebs* mutant than in WT plants, consistent with the increased level of *LFY* expression observed in the mutant (Fig.52.D).

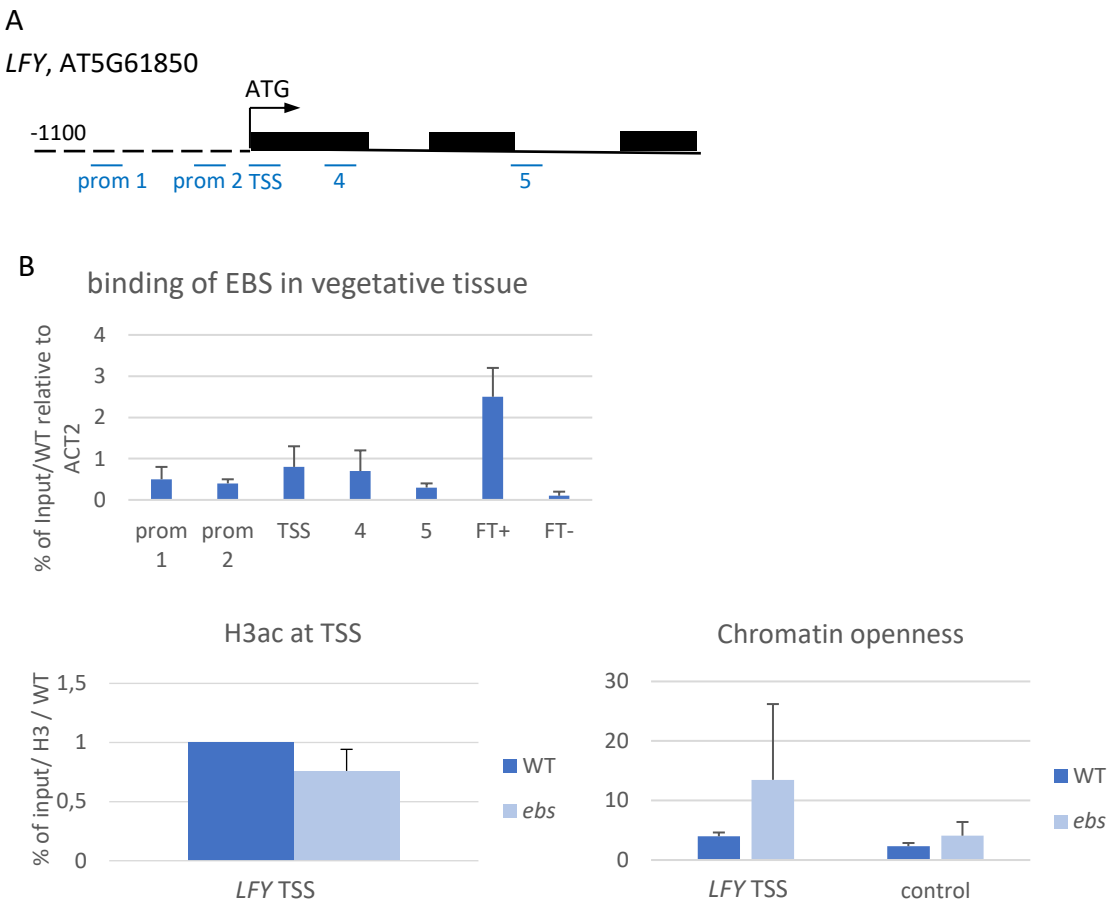

Fig.52 Analysis of EBS binding of to the *LFY* locus and chromatin organization in this gene: A) Schematic representation of the genomic region of *LFY*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR. B) Binding of EBS to *LFY* chromatin in seedlings; C) Comparison of H3 acetylation state at the TSS of *LFY* in the inflorescence of WT and *ebs* mutant; D) Levels of chromatin openness at the TSS of *LFY* in the inflorescence of WT and the *ebs* mutant.

4.5.2 Analysis of genetic interactions between *EBS* and *LFY*

Previous results from our laboratory showed a partial rescue of the floral phenotype of the strong *lfy-6* mutant allele, in the *lfy-6 ebs* double mutant (Gómez-Mena et al., 2001). Petals and stamens are absent in the flower-like structures of the *lfy-6* mutant. In the double mutant *lfy-6 ebs*, however, a variable number of petaloid and staminoid organs and some mosaic organs intermediate between petals and stamens are formed (Gómez-Mena et al., 2001). Due to the severe fertility defects present in *lfy-6* allele and in the double mutant *ebs lfy-6*, we decided to build a new double mutant with a weaker allele of *LFY*, *lfy-5*, to facilitate our analysis of the inflorescence in the double mutant. No significant differences in the overall architecture of the inflorescence were observed between the *ebs* single and *lfy-5 ebs* double mutant plants (Fig.53.A). When analyzing the phenotypes of shoot apices and flowers, we could confirm the partial rescue of *lfy* phenotype by the *ebs* mutation. Leaves that are replacing the flowers in the *lfy* mutant (Fig.53.C), are converted into fertile flowers in the *lfy-5 ebs* double mutant (Fig.53.E), resulting in earlier development of flowers in the double *lfy-5 ebs* than in the *lfy-5* mutant.

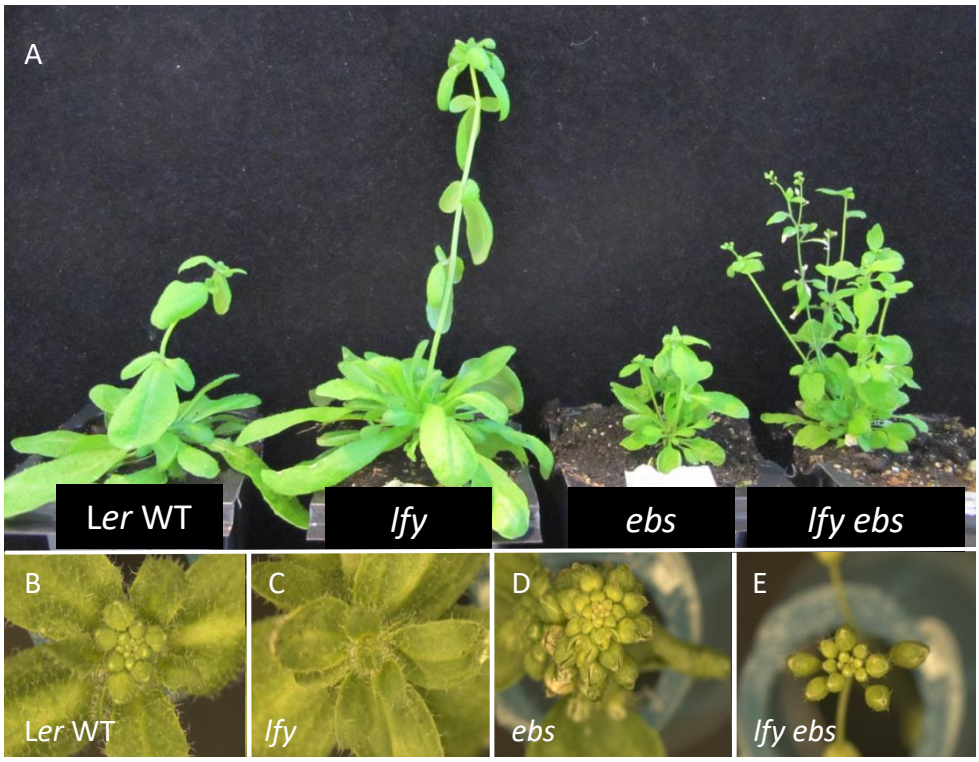

Fig.53 Comparison of the phenotype of WT, *lfy-5*, *ebs* and *lfy-5 ebs* in L*er* background grown for 8 weeks in SD conditions: A) Comparison of the inflorescence architecture, B-E) Comparison of the phenotype of shoot apices in the analyzed genetic backgrounds.

It seems that also *lfy-5* mutation partially rescues flower development abnormalities of the *ebs* mutant. Flowers with prematurely opened sepals of *ebs* mutant, where petals and stamens senescence and die (two first flowers in the *ebs* panel of Fig.54), were not observed in the *lfy-5 ebs* double mutant (Fig.54).

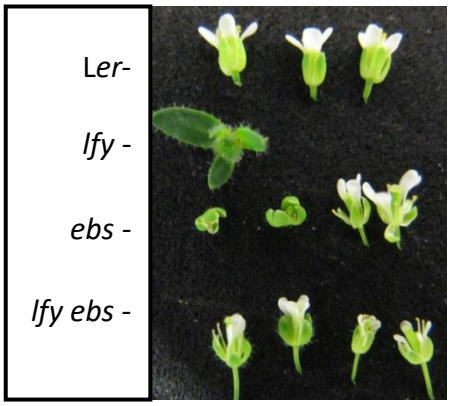

Fig.54 Comparison of the floral phenotype of WT, *lfy-5*, *ebs* and *lfy-5 ebs* in L*er* background grown for 9 weeks in SD conditions. Partial rescue of early flower phenotypes of *lfy-5* and *ebs* mutants in *lfy-5 ebs* double mutant

Thus, analysis of *lfy-5 ebs* double mutant phenotypes revealed that *LFY* and *EBS* are functionally related genes involved in flower development. More detailed analysis with expression-inducible systems will be needed to fully understand the nature of their interactions.

4.6 Analysis of interactions between *EBS* and additional genes involved in floral development: *SEP3*, *AP3* and *HDA19*

4.6.1 Analysis of the possible interaction between *EBS* and the floral identity gene *SEP3* in the control of reproductive development

*SEP*3 is expressed during early flower development. This homeotic gene is involved in the control of floral organogenesis and is needed to ensure proper development of petals, stamens and carpels (Pelaz et al., 2000; Honma and Goto, 2001). In fact, ectopic expression of this floral identity gene in young sepal primordia has been shown to be associated with homeotic transformations in Arabidopsis flowers (Krogan et al., 2012). Our transcriptomic analysis revealed ectopic expression of *SEP3* both in leaves and in vegetative meristems (Fig.27), leading us to hypothesize that partial homeotic sepal-to-petal conversion in *ebs* flowers could be mediated by defects in *SEP3* expression. For that reason, we decided to measure the expression of *SEP3* in young flowers of WT and the *eb*s mutant grown under SD. The results show higher levels of the *SEP3* transcripts in the *ebs* mutant (Fig.55), consistent with the homeotic transformation observed in this mutant and described above (Fig.15.J).

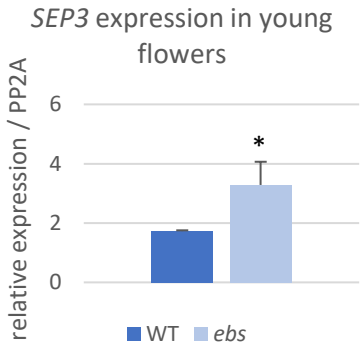

Fig.55 *SEP3* expression is upregulated in young flowers of the *ebs* mutant. Expression levels were quantified by qPCR in young flowers from plants grown under SD conditions. Asterisk represents statistical significance by Student's t-test.

4.6.2 Analysis of EBS influence on the chromatin organization of the *SEP3* locus

In order to determine if EBS-mediated regulation of *SEP3* expression takes place directly, we investigated the binding of EBS to the chromatin of this locus. We performed ChIP experiments using the *EBS::c-myc-EBS* line described above, and analyzed three regions that are important for the transcriptomic regulation of *SEP3*: two regions located in the promoter and one from the first intron. Since the biological material can be obtained faster and more easily from seedlings than from inflorescences, we first optimized the conditions for immunoprecipitation in vegetative tissue. We observed the binding of EBS to a distal upstream region of the promoter in the seedlings that is known to be bound by a number of transcriptional regulators, such as AP1 (Kaufmann et al., 2010), AP2 (Krogan et al., 2012), HISTONE DEACETYLASE 19 (HDA19) (Krogan et al., 2012), LFY (Moyroud et al., 2011), PI (Wuest et al., 2012) and SOC1 (Tao et al., 2012) among others (Fig.56.B). We then repeated the analysis on the apices collected at the inflorescence state and confirmed the binding to the same region (Fig.56.C), corroborating that EBS binds regulatory regions of *SEP3*. This observation together with *SEP3* expression data in the *ebs* mutant indicate that this PHD-containing protein

is required for proper transcriptional regulation of *SEP3* during both vegetative and reproductive phases of development in Arabidopsis, and that this regulation seems to be direct.

The comparison of H3ac levels between WT and *ebs* mutant shown in Figure 56.D reveals that *ebs* plants display increased levels of H3K9/14 acetylation in the *SEP3* locus as compared to WT, consistent with the upregulation of this gene observed in the mutant. These data were further supported by the analysis of chromatin openness at TSS of *SEP3* gene, that revealed a chromatin structure more opened in the *ebs* mutant compared to the WT (Fig.56.E). Altogether, these observations indicate that EBS is required to control *SEP3* expression by maintaining low levels of histone acetylation in the regulatory regions of this gene, establishing an inactive chromatin conformation during different phases of development in Arabidopsis plants.

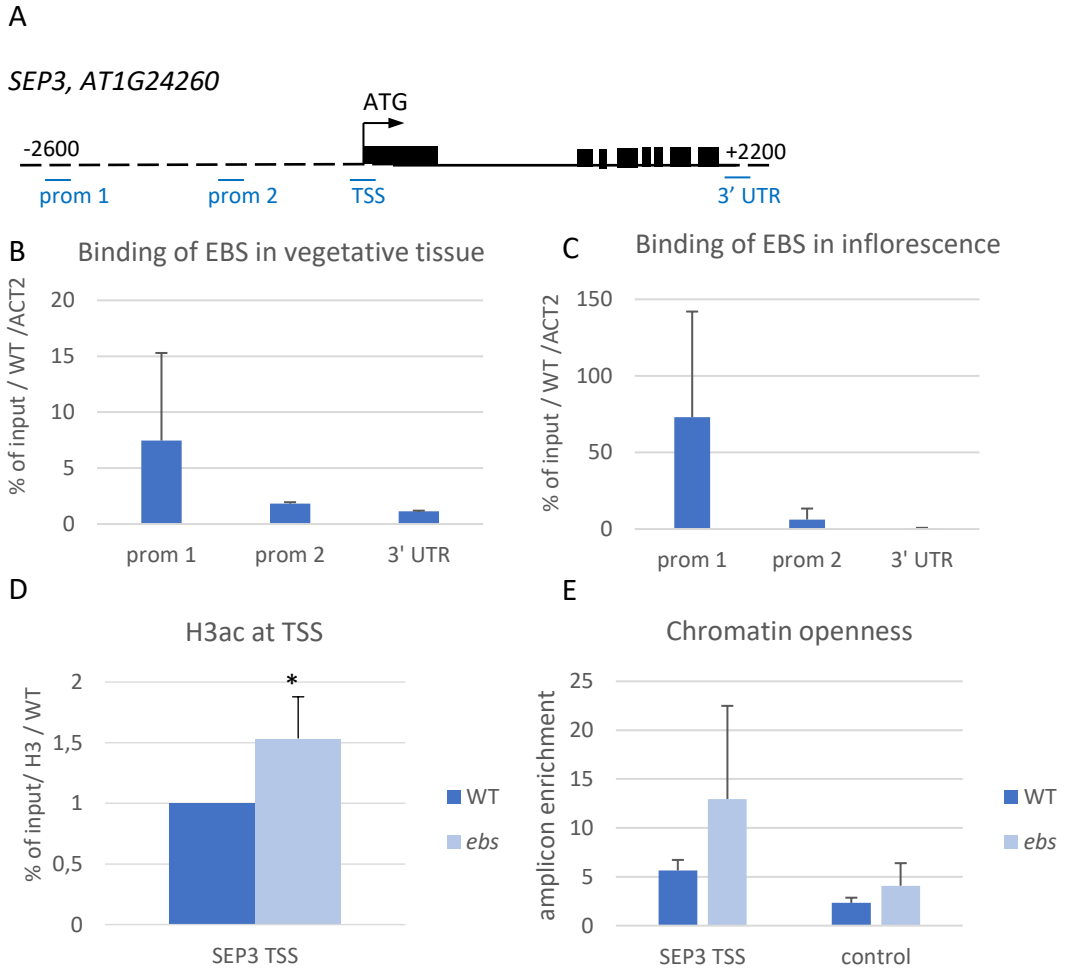

Fig.56 EBS binds regulatory regions of *SEP3*. A) Schematic representation of the genomic region of *SEP3*. Dashed line - promoter, black blocks – exons, continuous line – introns. Blue lines mark regions analyzed by qPCR. B) Binding of EBS to the *SEP3* gene in seedlings; C) Binding of EBS to the *SEP3* gene in inflorescence; D) Comparison of histone H3 acetylation state at the TSS of *SEP3* in inflorescences of WT and *ebs* mutant; E) Levels of chromatin openness at the TSS of *SEP3* in the inflorescence of WT and *ebs* mutant. Asterisk represents statistical significance by Student's t-test.

4.6.3 Analysis of the interactions between *EBS* and *HDA19* in regulation of floral organ development

The partial homeotic conversion of sepals to petals observed in the *ebs* mutant and described above in detail (Fig.15.J) has also been reported for mutants of the Class I RPD3-Like HDA19 histone deacetylase (Krogan et al., 2012). This chromatin remodeling protein was previously identified as an EBS interactor (López-González et al., 2014). HDA19 is also part of a protein complex containing TPL and AP2 proteins, among others. This complex transcriptionally regulates *SEP3*, *AP3* and *AG* to control floral patterning as well as the determination of flower whorl borders (Krogan et al., 2012). In the *hda19* mutant, as well as in the mutants of the other genes encoding members of this protein complex, partial sepal-to-petal transformation is caused by the ectopic expression of *SEP3* in the early sepals' primordia. In view of these observations and the physical interaction between EBS and HDA19, we decided to explore any possible genetic interaction between the genes encoding these proteins in the control of inflorescence or flower development. As expected, we were able to observe this partial homeotic conversion in the *hda19* mutant grown in our conditions, manifested as fair green sepals with white sectors (corresponding to the partial conversion to petals) at the edges (Fig.57.B). Several attempts carried out in our laboratory to generate a double homozygous line bearing concurrently *hda19* and *ebs* mutations failed, suggesting that this double mutant plant is not viable (López-González et al., 2014). Since heterozygous plants for *hda19* and homozygous for *ebs* displayed more dramatic alterations in flower development than the *ebs* single mutants, we decided to analyze the flowers of these *hda19+/- ebs* -/- plants. Our results show that these sesquimutant lines show many strong developmental defects in the flowers with predominant sepal-to-petal conversion (Fig.57.D). This observation suggests a genetic interaction between *EBS* and *HDA19* in the regulation of *SEP3*. Further experiments will be required to confirm this working hypothesis and that a repressive complex containing EBS and HDA19 is required for proper control of *SEP3* expression for correct flower development and floral organ identity determination.

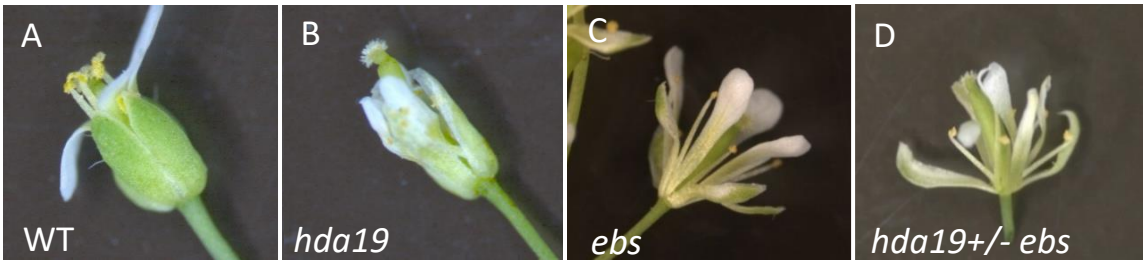

Fig.57 Partial homeotic conversion of sepal to petal in *hda19* and *ebs hda19* mutant plants: A) Wild type flower with green sepals and no traces of homeotic transformation; B-D) Mutant flowers with sepals partially converted to petals that are manifested as fair green sepals white parts (corresponding to petals) at the edges.

4.6.4 Analysis of the influence of EBS on chromatin organization of the *AP3* locus

Besides a role in the regulation of *SEP3* expression, the HDA19 repressive complex also binds the *AP3* genomic region and maintains a proper expression pattern of this B-class gene that specifies petal and stamen fate (Goto and Meyerowitz, 1994). In the *hda19* mutants, *AP3* is expressed precociously in newly initiated sepals primordia and also affects the sepal-to-

petal conversion phenotype. We could confirm previous results from our laboratory showing that *AP3* is upregulated in the inflorescence of *ebs* mutant (Gómez-Mena et al., 2001) (Fig.58.A). ChIP-qPCR experiments with the *EBS::c-myc-EBS* plants were then performed to investigate if EBS binds the *AP3* promoter region as shown for the HDA19 repressive complex. The results show low binding levels for both the *AP3* promoter and the 3' UTR regions, suggesting that the regulation of *AP3* expression by EBS is not direct. However, at this stage it is not possible to rule out that EBS binds additional genomic regions of *AP3*, and that this binding might occur together with the HDA19 repressive complex.

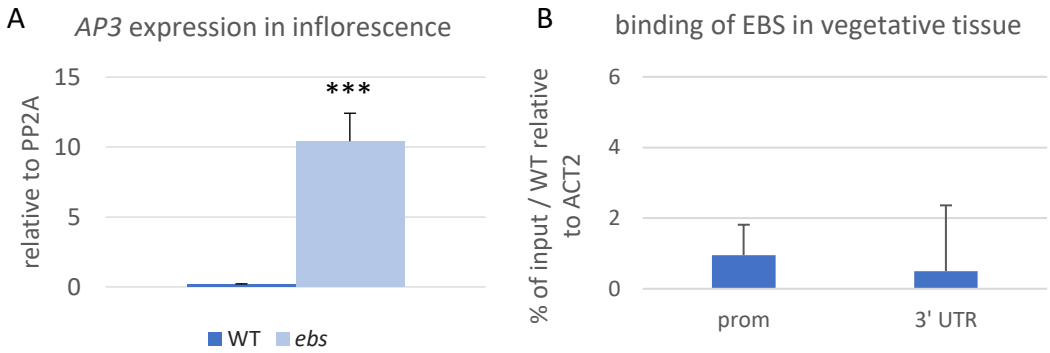

Fig.58 Analysis of *AP3* expression in the *ebs* mutant and binding of EBS to the *AP3* gene: A) *AP3* expression in inflorescence; B) No binding of EBS to two regulatory regions of *AP3* was observed. Asterisks represent statistical significance by Student's t-test.

In summary, the results described above suggest that EBS is involved in the regulation of floral organ identity genes as well as in the establishment of boundaries between sepals and petals. EBS is necessary for proper expression of *SEP3* and *AP3* genes, and at least in the case of *SEP3*, this regulation is direct. Moreover, our results advocate for a shared molecular mechanism for the transcriptional regulation of *FT* and *SEP3* mediated by EBS. Future experiments will address if EBS might be a part of a repressive complex that contains HDA19, or at least interacts with this complex to ensure proper expression patterns of *SEP3* in Arabidopsis inflorescences.

5. Identification of putative EBS-partner proteins

5.1 Co-immunoprecipitation of proteins that may interact with EBS following proteomics approaches

The EBS protein possesses two chromatin binding domains: PHD finger domain (Bienz, 2006) and bromo adjacent homology (BAH) domain (Yang and Xu, 2012) and no DNA binding domain. The presence of these functional motifs suggests that EBS binds chromatin rather than DNA, via histone recognition. Furthermore, no catalytic domain is present in EBS, suggesting that additional partner proteins could be required to mediate the repressive role of EBS. Previous reports from our lab revealed that EBS interacts with HDACs in the context of the regulation of flowering time, and particularly in relation to the repression of *FT* by EBS. (López-González et al., 2014). To look for additional EBS partner proteins, we used a Co-Immunoprecipitation (Co-IP) of protein complexes followed by mass spectrometry (MS) analysis approach (Hubner and Mann, 2011) in cooperation with Gerco Angenent's group

(Wageningen University and Research Center, WUR, Wageningen, Netherlands). A standard protocol for Co-IP of proteins was used for the isolation of EBS-partner proteins. Proteins were then digested in-solution with trypsin. Resulting peptides were dried with acetone and formic acid and analyzed in Orbitrap mass analyzer and identified short peptides were associated with proteins they are components of. For that we used a c-myc-tagged version of EBS protein expressed under the control of the *EBS* promoter purified from *EBS::c-myc-EBS* transgenic plants as a bait. We identified a number of potential EBS interactors that included the Q9FJE8 protein, a histone H2A variant 7, H2A.W.7, the only protein showing a fold change higher than 2, and 8 additional proteins that were slightly overrepresented in the immunoprecipitated material from *EBS::c-myc-EBS* plants: 60S ribosomal proteins P49692 and Q43291; lectin O04311; chromatin remodeler BAF60, Q9FMT4; germin-like protein P94040; Q9FMK9 and Q93XX2 with unknown function, and PIN-domain like family protein Q8L8C2.

Table 13: Label-free quantification (LFQ) results of mass spectrometry analysis that determine the relative amount of proteins with differential abundance between WT and *EBS::c-myc-EBS* samples

PROTEIN IDS	LFQ INTENSITY WT1	LFQ INTENSITY WT2	LFQ INTENSITY WT3	LFQ INTENSITY EBS1	LFQ INTENSITY EBS2	LFQ INTENSITY EBS3
Q9FJE8	5	5	5	7,302634	7,622608	7,458245
P49692	5	5	5	6,932976	5	6,929184
O04311	5	5	5	5	6,669178	7,050573
Q43291	6,845641	5	5	7,807758	6,849806	6,901088
Q9FMT4	5	5	5	6,076822	6,170496	6,184294
P94040	5	5	6,579475	6,471951	6,889643	6,644842
Q9FMK9	5	5	6,701447	6,763563	6,73294	6,555663
Q93XX2	5	7,688304	5	8,342463	7,423819	7,452262
Q8L8C2	5	5	5	5	6,456579	6,55341

In this analysis we could not find peptides belonging to EBS in the list of proteins enriched in *EBS::c-myc-EBS* samples compared with WT. It is possible that the tagged protein was not expressed to high levels, making it difficult to immunoprecipitate protein complexes that contain the EBS protein. Alternatively, since EBS sequence is only 234 amino-acids long and contains 31 cutting sites for trypsin (the enzyme used for the cleavage of proteins in our experiment), the resulted peptides might be too short to associate them significantly with the EBS protein sequence.

5.1.1 Validation of Co-IP MS results by Co-IP followed by Western Blot detection

To confirm the most significant of the protein interactions revealed by the proteomic analysis we pursued Co-IP approaches. In the case of the putative interactor H2A.W.7, we immunoprecipitated EBS protein complexes *in vivo* (with an α-myc antibody) making use of the same EBS-tagged line employed for the proteomic assays, followed by Western Blot (WB) detection of H2A.W.7. This experiment was possible thanks to the antibody prepared against this histone variant kindly provided by Prof. Frederic Berger from Gregor Mendel Institute in Vienna, Austria (Yelagandula et al., 2014). The results confirmed the interaction observed by proteomics between EBS and H2A.W.7 (Fig.59) and provides a new perspective into the possible interaction of the EBS protein with heterochromatic regions.

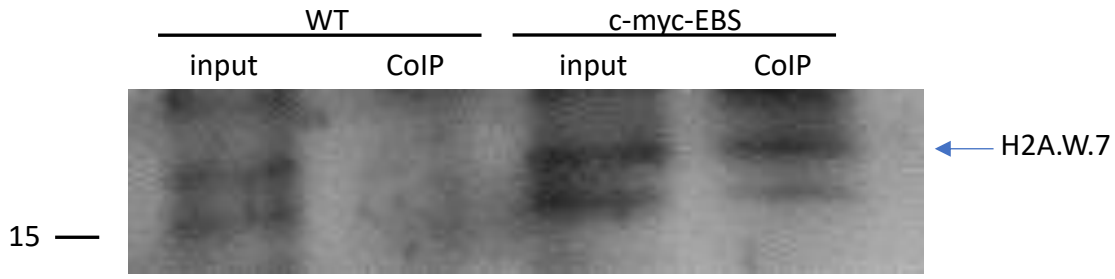

Fig.59 Western Blot showing the result of Co-IP experiment in c-myc-EBS lines with an α-myc antibody and revealing with the antibody against the H2A.W.7 histone variant, what confirms the interaction between EBS and H2A.W.7

Further experiments will be required to confirm the binding of EBS with additional protein candidates identified in the proteomic analysis. Particularly interesting will be the case of BAF60, another chromatin remodeling protein that, as for EBS, is involved in the regulation of genes playing key roles in the control of plant reproductive development (Sacharowski et al., 2015).

5.1.2 Analysis of heterochromatic regions in *ebs* mutant

As mentioned above, H2A.W.7 is involved in the formation of heterochromatin in Arabidopsis nuclei (Yelagandula et al., 2014). There are 3 variants of histone H2A.W: H2A.W.6, H2A.W.7 and H2A.W.12. Triple mutant plants for all H2A.W histone variants display a phenotype of decondensed chromocenters. Two classes of decondensed chromocenters can be identified after DAPI staining: decondensed chromocenters that are enlarged but less intensely stained with DAPI and dispersed chromocenters that are visualized as a big number of small grains after DAPI staining (Yelagandula et al., 2014). To validate the possible biological significance of the interaction revealed between EBS and H2A.W.7, we performed the analysis of the nuclei phenotypes in the *ebs* mutant, measuring the percentage of nuclei with the canonical chromocenter phenotype, decondensed chromocenters and dispersed chromocenters. Interestingly, the *ebs* mutant showed a lower proportion of intact nuclei and increased percentage of decondensed and dispersed nuclei compared to WT (Fig.60).

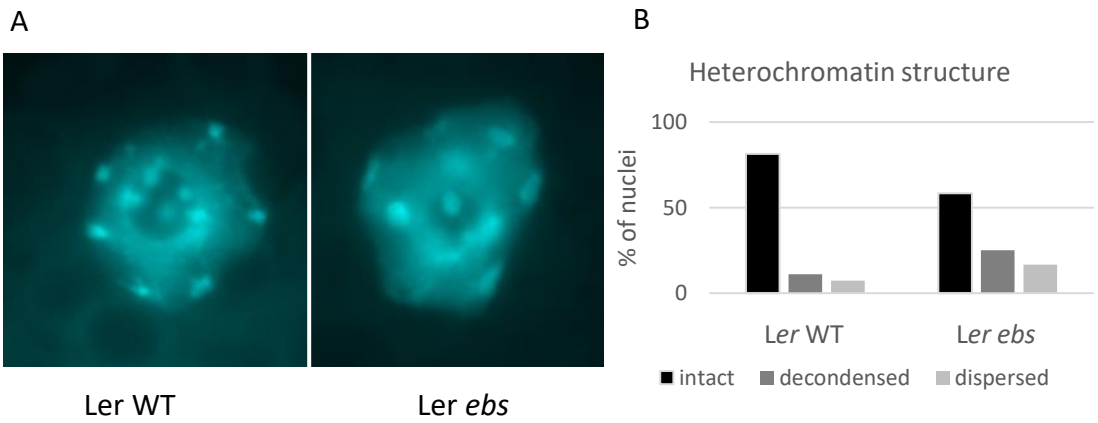

Fig.60 Nuclei phenotype of the *ebs* mutant. A) DAPI staining of representative nuclei of WT and *ebs* mutant, B) Quantification of the nuclei with 3 types of chromocenters: intact (canonical), decondensed and dispersed, represented as percentage of total analyzed nuclei.

Decondensation of chromocenters, causing changed nuclear topography, is required for global transcriptomic changes, as compacted chromatin in previously silenced genes needs to be decondensed to enable further transcription (Bourbousse et al., 2015). This decondensation is expected to occur during the transition to flowering (Rodriguez-Granados et al., 2016). Thus, higher levels of decondensed chromatin in the *ebs* mutant can be a manifestation of the accelerated transition to flowering rather than constitutive rearrangement of heterochromatin. Deeper cytological studies will be necessary to conclude the possible involvement of EBS in heterochromatin formation via interaction with the H2A.W.7 histone variant. Still, this observation can represent a new research avenue in relation to EBS function in the modulation of nuclear organization. This study lies beyond the scope of this work but will be further pursued in our laboratory during coming years.

DISCUSSION

Chromatin remodeling represents a key element in the establishment and maintenance of spatiotemporal gene expression patterns that govern development in eukaryotic organisms, and particularly in plants. In contrast to animal species, organ formation in plants continuously takes place during their whole life cycle. Furthermore, plants can modulate developmental transitions in response to both environmental and endogenous signals, and this represents an important adaptive trait for sessile organisms. However, and despite recent progress, detailed knowledge on how plants establish and sustain gene expression patterns that characterize specific developmental stages is still largely missing. In this PhD thesis, we have aimed at better understanding of the role of chromatin remodeling processes in the control of development in plants and shedding light on how environmental cues shape modifications in plant developmental programs through the modulation of chromatin activity and/or function. For that, we have mainly focused on the characterization of EBS and specifically on the role of this chromatin factor in the regulation of different aspects of reproductive development in Arabidopsis.

1. Alterations in reproductive development observed in the *ebs* mutant

The architecture of the inflorescence depends on its branching pattern and on the relative position where flowers are formed. In *ebs* mutants, the standard layout of inflorescence with main stem holding separated regions of branches and flower formation, is disturbed. The shoot apical meristem of the initial bolting stem is frequently arrested in the mutant, causing growth inhibition or cessation of the main inflorescence. Increased number of secondary and higher order branches is formed that, together with rosette stems (also formed in excessive number), outgrow the main inflorescence. In the early stage of inflorescence growth, leaves are formed instead of flowers. In later stages, regions of branches and flower formation are not separated, as branches are formed in the apical part of the inflorescence. Flowers themselves also develop abnormally: the phyllotaxy is disturbed, floral organs are not properly determined, and floral reversion events occur frequently (Fig.11, 12).

Environmental stimuli such as light, temperature and nutrients availability can influence the shape and identity of inflorescence structures. In fact, environmental factors have an effect on the severity of the defects observed in the *ebs* mutant. Non-inductive conditions for flowering such as SD photoperiod and low ambient temperature (18ºC) increase the frequency and severity of the defects observed in the inflorescences of the *ebs* mutant. SD conditions seem to have a stronger impact than low ambient temperature in the developmental alterations present in the reproductive structures of *ebs* plants, but the concurrence of both environmental cues results in the strongest defects (Table 9, Fig.9, Table 10, Fig.10).

Analysis of the developmental defects observed in the *ebs* mutant suggests that despite early bolting, later processes of reproductive growth, such as the acquisition of floral identity, are delayed. This observation is consistent with distinct roles for EBS at different developmental stages. While EBS acts as a repressor of flowering during the vegetative phase, it is required to promote floral organ identity and flower development at later reproductive stages. The existence of mechanisms that perform contrasting roles at different stages of reproductive development is not unique. For instance, the effect of the plant hormones GAs is

one of the best examples. These signaling molecules promote the termination of vegetative state and initiation of flowering, but their action needs to be repressed for proper flower development (Yamaguchi et al., 2014). Also, inflorescence identity genes must be switched off to ensure floral development, allowing the transition from an indeterminate meristem in the inflorescence to a determinate meristem in the flower (Yu et al., 2004; Hanano and Goto, 2011; Iwata et al., 2012).

In the *ebs* mutant the acquisition of floral organ identity and the determination of the floral meristem are disturbed, possibly due to the accumulation of signals from previous developmental stages, by deficiency of flowering signals, or both. The results of transcriptomic analyses performed in this work revealed a number of misregulated genes in the inflorescence of the *ebs* mutant that are consistent with defects in the identity determination of reproductive structures that partially revert to vegetative organs. Detailed observation of the inflorescence architecture is also consistent with this hypothesis since in the *ebs* mutant branches are formed in positions corresponding to the apical part of inflorescence, where flowers should be generated. This altered configuration changes the structure of typical Arabidopsis raceme, into an inflorescence structure similar to a panicle. Recent theoretical model assumes that raceme and panicle inflorescence structures are genetically closely related. In this model, inflorescences develop as a series of metamers, each consisting of a stem section bearing an apical and a new lateral meristem. Apical meristems have a vegetative (i.e., non-floral) identity, which enables them to generate a new metamer that acquire floral identity and terminate with the formation of the flower. In Arabidopsis, floral signals rise more rapidly in lateral part and a raceme is formed (single flowers in the flank of inflorescence meristem). If floral signals rise with similar kinetics in apical and lateral meristems (so if the flowering signals are reduced in the lateral part as compared to Arabidopsis threshold), a panicle is formed (Prusinkiewicz et al., 2007) with inflorescence branches replacing single flowers.

2. Floral reversion phenomena occur in the *ebs* mutant

A very interesting type of flower development abnormality is floral reversion. Developmental reversion is a return to an earlier phase of development. In the case of floral reversion, a return to leaf or inflorescence production after initiation of flower formation is observed. In the *ebs* mutant plants, during early stages of inflorescence development floral reversion is manifested by the formation of flowers, with floral organs transformed into leaves. In later stages, a new inflorescence is frequently created in the fourth whorl of floral buds, flowers or inside the siliques (Fig.11, Fig.12). Similarly to other developmental abnormalities observed in the *ebs* mutant, the frequency of floral reversion is increased by non-inductive conditions for flowering (Table 9, Fig.9, Table 10, Fig.10). Floral reversion was previously associated with flowering in suboptimal conditions in many plant species (Day et al., 1994; King, 1998; Washburn and Thomas, 2000; Wang, 2001; Wang and Cronk, 2003; Asbe et al., 2015). In Arabidopsis, floral reversion is also enhanced by non-inductive flowering conditions as observed in the *lfy-6*, *ag-1* and *ft-10* mutants that exhibit floral reversion phenotype only in SD conditions (Pontes et al., 1996, Liu et al., 2014). The number of floral reversion episodes in *Arabidopsis suecica* correlates well with the time that is needed to flower (the later the flowering, the more floral reversion, Asbe at al., 2015). The floral reversion phenotype of *lfy-6* and *ag-1* plants was partially suppressed by blocking phytochrome activity, night break

treatments or supplementation with GAs (Okamuro et al., 1996), suggesting that floral reversion can be suppressed by counteracting flowering suppressing signals. These observations, together with the diversity of genetic pathways that cause floral reversion, suggest that floral reversion is the result of an imbalance between flowering promoting and repressing signals that plants face in suboptimal flowering conditions or in flowering mutants.

Our expression analysis of candidate genes at different developmental stages of WT and *ebs* flowers, including *ebs* flower buds undergoing floral reversion, revealed an upregulation of genes connected with inflorescence meristem function such as *AGL24* and *TFL1*, and also of an early floral meristem identity gene, *LFY*, together with a strong upregulation of *FLC* (Fig.18). The misregulation of the inflorescence identity pathway by insufficient repression of those genes might altered the determination of the flower meristem and/or floral organ patterning what would lead to the formation of mosaic structures containing a mix of different identities that were observed during analysis of *ebs* floral reversion episodes under SEM (Fig.16).

3. EBS represses the floral transition, but is required to promote floral identity in later developmental stages

The functional domains present in EBS and previous results obtained in our laboratory strongly suggest that this protein acts as a transcriptional regulator that mediates changes in the organization of the chromatin to modulate gene expression. In fact, EBS was shown to directly repress the expression of the floral integrator gene *FT*, to prevent premature initiation of flowering. Besides assessing the expression of candidate genes during floral reversion events in the flowers of the *ebs* mutant, in order to have a global view of the genes controlled by EBS during different stages of reproductive development, we performed an analysis of the genome-wide transcription profile of shoot apex and leaves at vegetative stage and shoot apex at inflorescence stage of WT and *ebs* mutant by RNA-seq. Our results fit quite well with previous transcriptome analysis obtained in our laboratory with microarray hybridizations (Fig.23)

Analysis of our data with the Genevestigator signature tool (Table 12) revealed that vegetative samples of *ebs* mutant display premature activation of reproductive genes. In contrast, the gene expression signatures obtained in inflorescence samples show characteristics of vegetative structures that fit well with some of the observed developmental defects of the *ebs* mutant. These results suggest distinct roles for EBS at vegetative and inflorescence stage. While during vegetative growth EBS functions as a repressor of the floral transition, this chromatin protein is needed to promote floral identity in later developmental stages. One of the possible molecular mechanisms to explain this phenomenon would be that there is a gene, or a group of genes regulated by EBS with opposed transcriptomic outcome between vegetative and inflorescence stages. To identify potential candidates, we compared genes upregulated at the vegetative stage and downregulated at inflorescence stage, or downregulated at the vegetative stage but upregulated at the inflorescence stage. However, the overlaps between investigated gene fractions were not statistically significant and did not include any promising candidate gene that could mediate this opposite effect of EBS on reproductive genes (Fig.25), suggesting that *EBS* does not have contrasting functions in the regulation of the same genes during vegetative and reproductive development.

A number of genes were found to be differentially expressed in the different tissues analyzed in the *ebs* mutant as compared with WT plants. Among the genes misregulted in the *ebs* mutant there are various involved in the regulation of reproductive growth. This group includes, among others, *FT*, *FLC*, *AGL24*, *TFL1*, *LFY* and *SEP3*, genes important for the inflorescence stage and early floral meristem phase. These genes are differentially expressed in *ebs* as shown by RNA-seq analysis (Fig.27), and some of them also during flower development, as shown by qPCR studies (Fig.18). Misregulation of those genes was previously reported to affect both vegetative and reproductive growth phases and could mediate the effect of EBS on the control of vegetative and reproductive development in Arabidopsis. However, at this stage we cannot rule out that additional genes identified as misregulated in our analysis could be involved in the regulatory mechanism in which EBS participates to control different aspects of reproductive development in Arabidopsis. Factors that affect chromatin features like components of DNA methylation pathways (*FDM2, AGO9*) or factors modifying nucleosome properties (*H1.3, HDA7* and *18*) that are misregulated in *ebs* mutant (Fig.28) can influence transcriptional profile during reproductive growth. Also, several members of the MADS-box family of transcription factors such as *SEP4*, *AG*, *PI*, *FUL*, *AGL19*, *MAF4* and *MAF5*, together with various *SPL* regulators were misregulated in one or more of the tissues assessed in our transcriptional analysis (Fig.27). All of them are known to play additional key roles in the regulation of inflorescences and flowers development, and therefore are plausible candidates to mediate EBS function in reproductive growth.

4. Chromatin-mediated mechanism of gene expression regulation by EBS during reproductive development

A molecular mechanism for the transcriptional regulation of *FT* by EBS has been already described. EBS binds the promoter region of the *FT* locus, indicating that EBS is a direct repressor of this floral integrator gene before the floral transition. In addition, EBS interacts with histone deacetylases, leading to the hypothesis that EBS is required to maintain low levels of H3 acetylation at the TSS of *FT*, ensuring a transcriptionally inactive chromatin conformation to prevent a premature initiation of flowering (López-González et al., 2014). In order to further understand the mechanism of gene expression control mediated by EBS, interesting candidate genes to mediate the developmental abnormalities observed in the *ebs* mutant during reproductive development were subjected to more detailed analysis. Based on the *FT* regulation model described above, the analysis of EBS binding to regulatory regions of the selected loci and H3 acetylation level at their TSS was included in our study. Acetylation of histone H3 is connected with weakening of histone-DNA interactions and local formation of accessible chromatin structures that facilitates the binding of the transcriptional machinery (Hong et al., 1993). For that reason, analysis of the chromatin openness was included in the study. A summary of the analysis performed including expression changes of the selected genes in the different tissues analyzed as well as chromatin features assessed can be found in Table 14.

Table 14: Summary of the molecular analysis performed to shed light on the transcriptional regulation mechanism mediated by EBS on selected candidate genes. VM – vegetative shoot apex, L – rosette leaves, IM – inflorescence shoot apex. ↑ means upregulation, ↓ means downregulation, - means data not available.

gene	VM	L	IM	Flowers	Reverting bud	EBS binding	H3 acetylation	Chromatin openness
FT	-	↑	↓	no	slight ↑	yes*	yes	yes
FLC	↑	↑	↑	↑	↑	no	yes	no
AGL24	↑	↑	no	↑	↑	yes	yes	yes
TFL1	↑	no	no	no	↑	yes	no	yes
LFY	↑	no	no	↑	↑	no	no	yes
SEP3	↑	↑	no	↑	↑	yes	yes	yes

In most of the selected candidate genes analyzed, (*FLC, AGL24, TFL1*, *LFY* and *SEP3*) the lack of functional EBS protein causes upregulation of transcription, indicating that EBS acts as a repressor for the expression of those genes. The only exception is *FT* expression in inflorescence shoot apex that displays lower levels in the *ebs* mutant in comparison to WT (Fig.33.A). This is in contrast with the increased expression observed for *FT* in seedlings of the *ebs* mutant (Piñeiro et al., 2003; López-Gonzalez et al., 2014). The reasons for this different behavior of *FT* expression in the *ebs* mutant during early stages of inflorescence development remain unclear. *FT* has been shown to perform an essential role in the commitment to flowering during inflorescence development, and expression of *FT* in inflorescences and particularly fruits is required to sustain normal reproductive growth in Arabidopsis plants (Liu et al., 2014; Müller-Xing et al., 2014). Given the early transition to flowering and bolting that takes place in *ebs* mutants, it is possible that other factors required for *FT* expression during inflorescence development are still absent in early stages of reproductive development in the *ebs* mutant and this results in abnormally low levels of *FT* expression. Further analysis will be required to explore this hypothesis.

The activity of PcG proteins was shown to be important for the repression of *FLC* and *SVP* during inflorescence development (Müller-Xing et al., 2014). In fact, mutations in genes encoding PcG chromatin remodeling factors cause upregulation of these floral repressors that in turn inhibit *FT* expression what results in floral reversion episodes. The expression of *FLC* is also upregulated during different stages of development in the *ebs* mutants (Fig.27) and it is therefore possible that high levels of this *FT* repressor are related to the observed decrease of *FT* mRNA levels in the *ebs* mutant. Further experiments will be required to test this possibility, and genetic approaches are currently underway to clarify the relationship between *EBS* and *FLC* in the regulation of *FT* during different phases of reproductive growth.

Binding of EBS to regulatory regions of the investigated loci indicating direct transcriptional control was observed for *FT, AGL24, TFL1* and *SEP3* genes. In contrast, based on the data available at present, EBS-mediated regulation of *FLC* and *LFY* expression appears to be indirect. Since only discrete regulatory regions of these loci have been analyzed, additional experiments will be needed to corroborate this conclusion. Among the four loci that were identified as direct targets of EBS, in most cases (namely *FT, AGL24,* and *SEP3*) binding was accompanied by an increased acetylation of histone H3 around the TSS, suggesting that the repression mechanism proposed for the HDAC-mediated repression of *FT* by EBS could also

be operating for other targets. The only exception is *TFL1* where, despite the multiple binding sites for EBS detected in the genomic region of this gene, no changes in histone H3 acetylation levels were observed. On the other hand, in all investigated genes where binding of EBS was observed, we could document changes in chromatin conformation consistent with the transcriptomic outcome observed: chromatin was more open at the TSS of upregulated genes in the *ebs* mutant, and less accessible at the TSS of the only downregulated gene studied, *FT*. Altogether, the observations described above suggest that the mechanism of *FT* repression mediated by EBS could also be active for the regulation of other loci involved in the control of later stages of reproductive development. In particular, the results obtained with *TFL1* suggest that additional molecular mechanisms could intervene in the transcriptional repressive function of EBS. Recent results published during the writing of this report have proposed additional roles for SHL, an EBS homologue also involved in the regulation of developmental processes in Arabidopsis. According to these observations, beside the role as H3K4me3 reader, SHL could also mediate the recognition of another histone mark, specifically H3K27me3. While recognition of the first mark depends on the PHD domain present in this protein, the binding to the second one relies on the BAH domain also found in SHL, making this protein a dual histone reader that can bind both active and inactive chromatin marks (Qian et al., 2018). Since the functional domains present in EBS are the same as in SHL, additional interactions of this histone reader with other chromatin remodeling complexes could have a role in balancing the action of different chromatin factors to finely modulate the expression of important regulators of plant development.

In view of the data discussed above, together with the results obtained in genetic approaches carried out to understand the role of *EBS* in the regulation of different aspects of reproductive development, we have generated working hypotheses to explain how different genes could mediate the effect of this chromatin protein on the control of inflorescence and flower production in Arabidopsis. As previously described, EBS binds regulatory regions of *FT* prior to the floral transition, although so far binding during later stages has not been observed. However, the alterations in *FT* transcription observed in the *ebs* mutant could still potentially explain at least some of the developmental abnormalities present in this mutant. Plants depleted of *FT* function caused either by *ft* mutations (Liu et al., 2014) or Polycomb-Group mutations in which overexpression of *FLC* leads to *FT* repression (Müller-Xing et al., 2014), displayed events of floral reversion. In order to assess whether low *FT* expression might also be the cause of the reproductive reversion episodes observed in the *ebs* mutant, we analyzed the phenotype of *ebs* plants with constitutive *FT* overexpression, *35S::FT ebs.* These plants display an extremely early flowering phenotype and premature termination of the inflorescence (Fig.39). These features precluded drawing conclusions on the genetic interactions between *FT* and *ebs* in the control of inflorescence development. Double mutant plants *ft ebs* were also analyzed to investigate possible genetic interaction between both loci in the regulation of inflorescence and flower development. This study also encountered difficulties due to the extremely delayed development of the inflorescence observed in these plants under SD, conditions where the phenotypic alterations present in the *ebs* mutant are more conspicuous (Fig.40). Nevertheless, initial experiments revealed a synergistic relation between *FT* and *EBS* for floral identity determination, as under these photoperiodic conditions the double mutant *ft ebs* continues to produce leaves instead of flowers for a longer period of time than any of the single mutants,

making it impossible to assess the combined effect of these two mutations on floral reversion in this experimental setting. Interestingly, the study unveiled interactions between *FT* and *EBS* in the regulation of plant architecture, and particularly in the development of rosette stems. In LD conditions this interaction is synergistic, as *ft ebs* double mutant generate more rosette stems than the WT or the single mutants (Fig.41.A, B). In SD conditions, *FT* is required for the excessive rosette stems formation of the *ebs* mutant, as *ft ebs* double mutant gives rise to a significantly lower number of stems coming from the axils of rosette leaves (Fig.41.C). The analysis of these double mutant plants is still ongoing in order to try to shed light in the complex interaction that links *EBS* and *FT* loci in the regulation of different features of reproductive growth, but in any case, these observations confirm previously reported engagement of *FT* in the determination of inflorescence architecture (Huang et al., 2013). As an example of this complex relationship, *FT* is downregulated in the reproductive apex of *ebs* plants, but this effect is no longer observed during later developmental steps of reproductive growth, as there is no change in *FT* expression between the flowers of WT and *ebs* mutant. In fact, in flowers where floral reversion is observed, an overexpression of *FT* can be detected, as revealed by *pFT::GUS* transgenic lines (Fig.38). Further experiments will be needed to explore the exact impact of *FT* in the reproductive defects present in *ebs* mutants.

The role of FT in the maintenance of the commitment to flowering has been linked to *FLC*. The overexpression of this MADS-box transcription factor was associated with floral reversion (Hepworth et al., 2002; Müller-Xing et al., 2014), through the downregulation of *FT* in reproductive tissues (Müller-Xing et al., 2014). In addition, *FLC* was reported to be involved in floral pattern regulation (Deng et al., 2011). *flc ebs* double mutant plants show no changes in inflorescence architecture and flower development when compared to *ebs* single mutants (Fig.44). This may exclude a role for *FLC* in mediating the effect of *EBS* on reproductive development. However, the *flc* single mutant does not display any alterations in flower development (Deng et al., 2011), and it might be that the overexpression of *FLC* (as in PcG mutants) impacts floral reversion through *FT* expression. For that reason, the analysis of the genetic interaction between the *ebs* mutant and plants overexpressing *FLC* is underway to explore the possible interplay of these genes to modulate reproductive growth.

In addition to *FT*, *AGL24* is also a putative candidate to mediate the floral developmental abnormalities observed in the *ebs* mutant. In *AGL24* overexpressing lines (*35S::AGL24*) floral reversion episodes can be observed. At early developmental stages, secondary flowers are created in the axils of the sepals (Yu et al., 2004; Liu et al., 2007), and a new inflorescence can emerge from the ovary in later stages. Also, floral organs undergo homeotic transformations toward structures normally found in inflorescence shoots (Yu et al., 2004). Moreover, gene regulatory network analysis revealed that overexpression of *AGL24* can change the identity of floral meristem into inflorescence/floral mixed meristem identity that promotes floral reversion (Pérez-Ruiz et al., 2015). Given that *AGL24* is upregulated in different tissues of *ebs* mutant plants, including flowers, we analyzed the phenotype of the *agl24 ebs* double mutant. Indeed, phenotypes of flower development abnormalities and floral reversion were partially rescued by the *agl24* mutation, confirming that overexpression of *AGL24* contributes to the developmental defects observed in *ebs* mutant (Fig.46). Since EBS appears to regulate directly *AGL24* by binding the genomic region close to the TSS and seems to be required to maintain low levels of H3ac in this locus, we conclude that EBS is necessary to

repress *AGL24* at least in some stages of reproductive development. The expression of this gene is restricted to initial stages of floral formation, and AGL24 protein acts together with SVP and AP1 to maintain a meristematic identity in the floral meristem. This function is necessary to prevent premature expression of floral homeotic genes while activating *LFY* expression. However, later in flower development, the genes *AG*, *SEP3* and *AP1* act to repress *AGL24* expression and allow floral organ identity determination by homeotic genes (Wils and Kaufmann, 2017). It is possible that high levels of *AGL24* in the *ebs* mutant result in misregulation of the different regulatory loops involved in floral patterning, leading to the defects in the development of flowers that we observe in *ebs* mutant plants grown under SD. It will be interesting to analyze the spatial pattern of expression of *AGL24* in inflorescence and floral meristems of the *ebs* mutant in order to establish whether the floral defects present in these plants are related to ectopic expression of *AGL24* in floral primordia or to a longer persistence of the *AGL24* mRNA in the flowers of the mutant. These experiments are currently under way.

As for other candidate genes already discussed, *TFL1*, a key regulator of inflorescence architecture, is also upregulated in the *ebs* mutant, at least in the vegetative meristem, and the EBS protein binds genomic regions of the *TFL1* locus. Upregulation of *TFL1* was linked previously to floral reversion phenotypes of transgenic lines overexpressing the XAL2/AGL14 transcription factor, an activator of *TFL1* expression (Pérez-Ruiz et al., 2015). Also, defects in inflorescence architecture similar to those observed in the *ebs* mutant were reported for the plants with deregulated spatial expression of *TFL1* (Serrano-Mislata et al., 2016). In *TFL1*, beside the TSS region, additional regions located at 3'UTR were shown to be very important for transcriptomic regulation of this gene as they bear binding sites for a number of *TFL1* regulators such as SOC1, AGL24, SVP and SEP3 (Serrano-Mislata et al., 2016). Our results show that although there were no detectable changes in the level of H3ac at the *TFL1* locus in the *ebs* mutant, EBS impacts chromatin structure at TSS and in 3'UTR of this gene (Fig.48, Fig.49). In fact, nucleosomal density and chromatin openness were disturbed in the *ebs* mutant compared to the WT. Deletion analysis and phylogenomic shadowing studies of the 5′and 3′ genomic regions around the *TFL1* locus have defined several elements that play essential roles in the spatiotemporal regulation of this gene. While regulatory sequences in the 5′region are involved in modulating expression levels, the 3′intergenic regions contains most of the evolutionarily conserved elements required to define the temporal and spatial patterns of *TFL1* expression. Among them, the so-called region IV bears the regulatory elements that mediate the inflorescence meristem expression of the gene (Serrano-Mislata et al., 2016). Interestingly, in the *ebs* mutant this region shows a more condensed chromatin structure measured as a stretch of reduced chromatin openness. Moreover, partially complementing transgenic lines in which this region is missing, display phenotypic alterations similar to those observed in the *ebs* mutant, namely, inhibition of main stem growth and formation of tertiary and higher order branches. It is noteworthy that this regulatory block contains the binding sites for LFY and although LFY is known to repress *TFL1* expression in flowers, a positive role for LFY in the regulation of *TFL1* has also been suggested. This function of LFY would be necessary for the maintenance of *TFL1* expression in inflorescence meristems (Serrano-Mislata et al., 2016). Therefore, it is tempting to speculate that EBS could be involved in mediating accessibility of transcriptional regulators such as LFY to cis-elements that are crucial for proper spatiotemporal expression of

TFL1. The putative molecular mechanism mediating this role of EBS on chromatin organization remains to be elucidated.

Genetic approaches in which *ebs* and *tfl1* were combined in double mutant plants did not shed light on the possible interaction between both loci for the control of inflorescence architecture. Similarly to *35S::FT ebs* plants, *ebs tfl1* mutants display extremely early flowering and premature termination of inflorescence growth, making it difficult to assess possible genetic interactions of *EBS* and *TFL1* (Fig.50). Further studies are currently ongoing, including the analysis of *pTFL1::GUS* reporter (kindly provided by Dr. F. Madueño, IBMCP, Valencia, Spain) in *ebs* background, to try and unveil the possible relationship of *EBS* and *TFL1* in the control of reproductive development, as well as the possible links with LFY-mediated control of *TFL1* expression. In fact, previous results suggested an interaction between *EBS* and *LFY* in the control of floral identity. While petals and stamens are absent in the flower-like structures of the strong *lfy-6* mutant allele, in the double mutant *lfy-6 ebs* a variable number of petaloid and staminoid floral organs and some mosaic organs intermediate between petals and stamens are formed (Gómez-Mena et al., 2001), indicating that *ebs* mutations partially suppress the floral defects present in *lfy* mutants. Our current analysis readily confirmed these observations, showing that double mutants combining the *ebs* mutation with weaker alleles of *lfy* (*lfy-5*) also rescues the floral *lfy-5* phenotype, as *lfy-5 ebs* double mutant already develops fertile flowers when the *lfy-5* mutant still forms leaves (Fig.53, Fig.54). These results are consistent with an impact of *LFY* function in mediating developmental defects observed in *ebs* mutant. Indeed, while constitutive overexpression of *LFY* in *35S::LFY* plants led to premature termination of inflorescence development (Weigel and Nilsson, 1995), gain of function *LFY* lines carrying a fusion of the virus activation domain VP16 to *LFY* coding sequences (*LFY:VP16*) caused ectopic expression of A and C class floral identity genes and misregulation of B class genes, causing flower development defects (Parcy et al., 2002). LFY and EBS share some of the genes they act upon, but at least in some cases they regulate them in opposite direction. For example, LFY can act as an activator of *TFL1* (Goslin et al., 2017) and also *SEP3* (Winter et al., 2011), while EBS (as shown in this study) has a repressive function over their expression. Also other genes, like *AP3* or *SEP4* that were shown to be upregulated in *ebs* mutant (this study, Gómez-Mena et al., 2001), were downregulated in *lfy* mutants (Winter et al., 2011). This suggests that EBS could have an antagonistic role to LFY in the regulation of some target genes with important roles in the control of reproductive development. In this scenario, both EBS and LFY are required to promote proper development of the flowers, LFY by activation of the genes and EBS by ensuring their proper and/or timely repression. Since EBS binds the same regulatory regions of *TFL1* and *SEP3* loci as LFY, it will be informative to investigate whether mutations in either of these two transcriptional regulators influence the binding properties of the other. Further studies will be necessary to address this possibility.

Finally, *SEP3* is also a direct target of EBS and this floral homeotic gene is upregulated in *ebs* mutants. We hypothesize that increased expression of *SEP3* in young sepals primordia could be responsible for the partial homeotic transformation of sepal-to-petal observed in *ebs* mutant flowers and perhaps other floral developmental defects present in this mutant. The levels of histone H3ac are abnormally high around the TSS region of *SEP3* in the *ebs* mutant (Fig.56). Given that EBS was previously shown to interact *in vivo* with HDACs it is conceivable to postulate that EBS is required for the recruitment of these transcriptional repressors to

regulatory regions of floral homeotic genes, similarly to what has been proposed for the EBS-mediated repression of *FT* prior to the floral transition (López-González et al., 2014). As a matter of fact, HDA19, one of the HDACs that have been shown to interact with EBS, is involved in the repression of floral organ identity genes such as *AP3* and *SEP3* together with AP2 and the transcriptional corepressor TPL (Krogan et al., 2012). In this scenario, EBS and the HDAC repressor complex would be necessary to maintain an inactive chromatin conformation in certain floral organ identity genes to ensure their expression in a timely and spatially controlled manner. Additional experiments will be needed to confirm this hypothesis and to assess whether *ebs* mutations cause ectopic expression of *SEP3* outside its normal expression domain within the floral meristem, affecting the determination of identity of sepals and petals, as observed in the *ebs* mutants where mosaic organs with mixed identity have been observed. Additionally, further studies concerning the possible interaction of the EBS protein with the members of the abovementioned protein complex repressing *SEP3* expression are being pursued.

5. Additional mechanisms could mediate the impact of EBS on chromatin remodeling processes that control the expression of developmental genes

Previous reports from our lab as well as the results obtained in this work indicate that EBS acts as a transcriptional regulator that binds genomic regions of target genes to modulate their expression, likely through the recruitment of additional chromatin remodeling complexes such as HDACs that modify the transcriptional status of underlying genes. But the existence of additional mechanisms to affect indirectly gene expression is suggested by the observation that the lack of functional EBS protein causes a misregulation of chromatin remodelers. This is the case of *BAF60* (*AT5G14170*), a gene that was found downregulated in the vegetative samples of the *ebs* mutant. Moreover, ChIP experiments confirmed that *BAF60* is a direct target of EBS (Fig.32). The BAF60 protein (also called SWI/SNF ASSOCIATED PROTEIN 73B (SWP73B/CHC1), is a component of the SWI/SNF chromatin-remodeling complex that mediates seedling growth control by modulating DNA accessibility (Jégu et al., 2017). This protein is also involved in the control of flower development via regulation of a number of flowering genes. In fact, some genes such as *AGL24*, *FLC*, *SEP3*, *TFL1*, and *SPEECHLESS* (*SPCH*) among others, transcriptionally regulated by this complex, were found to be misregulated in the *ebs* mutant. BAF60 binds the genomic regions of those genes and the lack of the functional protein in the *baf60* mutant affects nucleosomal density in the chromatin of target genes (Sacharowski et al., 2015). When we compared the 377 genes that are bound by BAF60 and display altered nucleosomal density in the seedlings of *baf60* mutant with the 1163 misregulated genes in the seedlings of *ebs* mutant, we found an overlap of approximately 16% (Fig.61). This overlap is statistically significant as revealed by hypergeometric test (representation factor = 4.57, p-val < 6.05e-23), supporting that EBS could mediate in the regulation of a number of genes through *BAF60*.

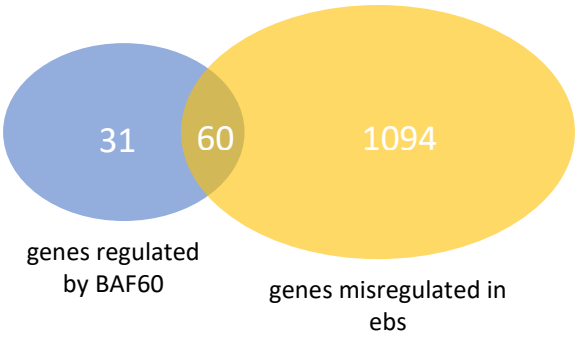

Fig.61 Venn diagram of the overlap between genes misregulated in *ebs* and genes misregulated in *baf60* mutant, with altered nucleosomal density and directly bound by the BAF60 protein (data from Sacharowski et al., 2015).

In addition, the physical interaction revealed between EBS and the histone variant H2A.W.7 (Fig.59) suggests additional chromatin-mediated regulatory mechanisms for the EBS protein. H2A.W.7 histone variant has been involved in heterochromatin formation (Yelagandula et al., 2014). In the Arabidopsis genome there are 3 variants of histone H2A.W: H2A.W.6, H2A.W.7 and H2A.W.12, and plants depleted for all H2A.W histone variants display conspicuous decondensed chromocenters (Yelagandula et al., 2014). Interestingly, a similar nuclear decondensation phenotype was observed in the chromocenters of the *ebs* mutant (Fig.60). Altogether, these data indicate that EBS has also an impact on chromatin organization. However, the exact molecular mechanism mediating the effect of EBS on chromatin compaction is unknown and will be investigated in our lab in the coming years.

6. EBS participates in the regulation of different aspects of reproductive growth to ensure proper progression of development

The various phases of reproductive development are strictly controlled to optimize reproductive success of plant species. This control includes tight regulation of spatial and temporal patterns of gene expression that results in selective activation or silencing of genes involved in different pathways of reproductive growth. The chromatin factor EBS is involved in the regulation of every step throughout reproductive development, from the time of floral initiation, through regulation of inflorescence architecture till the development of the flowers and floral organs. During vegetative growth the activity of EBS is required to repress *FT* expression in order to ensure the right timing for the floral transition. After bolting EBS is necessary to finely tune the expression of a number of genes that play a central role in the establishment of inflorescence and floral meristems such as *AGL24* and *LFY*, both repressed by EBS (directly the first one and indirectly the second). Later in development EBS function is also essential for the control of floral homeotic genes that are repressed by this PHD-containing protein, as shown by the upregulation of these loci observed in the inflorescences of the *ebs* mutant. Many of these key genes involved in the control of reproductive development are part of regulatory networks in which these loci influence each other´s expression. Consistent with this intricate network of genetic circuits in which EBS acts at different levels, *ebs* mutants display complex alterations in multiple aspects of inflorescence and flower development. Altogether, the data presented in this work indicate that EBS represents a central hub in the control of reproductive growth, orchestrating the expression of different genes that need to be

coordinately expressed to guarantee successful completion of the Arabidopsis developmental plan and adequate production of fruits and seeds to safeguard efficient transmission of genetic information to the following generation. Further analyses will be necessary to fully disentangle the multifaceted implication of EBS in the modulation of different phases of reproductive growth to ensure a proper developmental progression.

CONCLUSIONS

1) The EBS chromatin factor is involved in the regulation of multiple steps throughout reproductive development, ranging from the regulation of the floral initiation through the acquisition of proper inflorescence architecture to the control of flower development and floral organ patterning.
2) During vegetative growth, the activity of EBS is required to repress *FT* expression in order to ensure the right timing for the floral transition. After bolting EBS is necessary to finely tune the expression of a number of genes that play a central role in the establishment of inflorescence and floral meristems. Later in development EBS function is also essential for the control of the expression of floral homeotic genes.
3) *ebs* mutants display complex alterations in multiple aspects of inflorescence and flower development. Non-inductive conditions for flowering such as short photoperiod (SD) and low ambient temperature (16ºC) increase the frequency and severity of the phenotypic defects observed in the inflorescences of the *ebs* mutant regarding inflorescence architecture, floral meristem maintenance and function, as well as flower development and floral organ patterning. Reproductive reversion is also a frequent developmental abnormality in *ebs* mutant grown under SD.
4) Many key regulators of reproductive growth in Arabidopsis are misregulated in the *ebs* mutant. This differential expression may contribute to the phenotypic alterations displayed by the *ebs* mutant during reproductive development.
5) Our results demonstrate direct transcriptional control for the *FT*, *AGL24*, *TFL1* and *SEP3* genes by EBS. In contrast, our data suggest that EBS-mediated regulation of *FLC* and *LFY* expression could occur indirectly.
6) The molecular mechanism of EBS-mediated regulation of transcription described for *FT* that is proposed to involve binding to H3K4me2/3 in promoter regions and engaging HDAs to stabilize H3ac level at the TSS appears to also function for some other EBS targets. However, additional mechanisms mediated by EBS and connected with the regulation of chromatin compaction and changes in chromatin accessibility are also conceivable.
7) Genetic interactions between *EBS* and genes encoding key regulators of reproductive growth in Arabidopsis such as *FT*, *AGL24* and *LFY* have been revealed, supporting their participation in common regulatory pathways.
8) Our data support a role for EBS as a central hub in the control of reproductive growth, orchestrating the expression of different genes that need to be coordinately expressed to guarantee successful completion of the Arabidopsis developmental plan and adequate production of fruits and seeds to safeguard efficient transmission of genetic information to the following generation.

CONCLUSIONES

1) El factor cromatínico EBS está implicado en la regulación de múltiples eventos asociados al desarrollo reproductivo tales como el control de iniciación floral, la adquisición de la arquitectura de la inflorescencia o la regulación del desarrollo floral y del patrón de los órganos florales.
2) Durante el desarrollo vegetativo, la actividad de EBS es necesaria para reprimir la expresión de *FT*, asegurando que la transición a la floración ocurra en el momento más apropiado. Después del brote del tallo floral, la proteína EBS es necesaria para el ajuste fino de la expresión de un número de genes que juegan un papel central en el establecimiento de la inflorescencia y de los meristemos florales. Posteriormente en el desarrollo reproductivo, EBS es también esencial para el control de la expresión de genes homeóticos florales.
3) Los mutantes *ebs* desarrollan alteraciones complejas en múltiples aspectos del desarrollo de la inflorescencia y de las flores. En condiciones no inductoras de la floración tales como fotoperiodos cortos o temperaturas de crecimiento bajas (16ºC) se observan un incremento en la frecuencia y en el grado de severidad de los defectos desarrollados en la inflorescencia del mutante, relativos a la arquitectura de la misma, la función y el mantenimiento de los meristemos florales, así como el desarrollo floral y el patrón de los órganos florales. La reversión floral es otra de las alteraciones del desarrollo observadas en mutantes *ebs* cultivados en condiciones de fotoperiodos cortos.
4) La expresión de muchos de los genes reguladores claves en el desarrollo reproductivo se encuentra alterada en los mutantes *ebs*. Estos cambios de expresión podrían contribuir a las alteraciones fenotípicas desarrolladas por los mutantes *ebs* durante el desarrollo reproductivo.
5) Los resultados de este trabajo demuestran un control transcripcional directo de EBS en la expresión de los genes *FT*, *AGL24*, *TFL1* y *SEP3*. Por otra parte, nuestros resultados sugieren que la regulación mediada por EBS de la expresión de *FLC* y *LFY* podría ocurrir de manera indirecta.
6) Los mecanismos moleculares implicados en la regulación transcripcional de *FT* mediada por EBS, y que involucran el reconocimiento de marcas H3K4me2/3 en la región promotora del mismo y el reclutamiento de Histonas Deacetilasas, que modulan los niveles de acetilación en histona H3, parecen también funcionar en la regulación de la expresión de otros genes diana de EBS. Sin embargo, nuestras observaciones apoyan la existencia de mecanismos adicionales de regulación mediados por EBS que están conectados con la regulación de la compactación y la accesibilidad de factores de transcripción a la cromatina.
7) Las interacciones genéticas reveladas entre *EBS* y genes reguladores claves del desarrollo reproductivo como *FT*, *AGL24* y *LFY* corroboran su participación en rutas reguladoras comunes.
8) Nuestros resultados apoyan un papel para EBS como nodo central del desarrollo reproductivo, orquestando la expresión coordinada de diferentes genes para garantizar el plan de desarrollo de Arabidopsis en su totalidad, así como una adecuada producción de flores y frutos que asegure la transmisión eficiente de la información genética a la siguiente generación.

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ANNEXES

Annex 1 RNA-seq.xlsx

RNA-seq results

Annex 2 ChIP-seq.xlsx

ChIP-seq results