UNIVERSITÀ DEGLI STUDI DI MILANO

Dipartimento di BioScienze
Corso di Dottorato di ricerca in Scienze Biologiche e Biomolecolari
Ciclo XXIV

UNRAVELING THE ROLE OF BPC PROTEINS IN INFLORESCENCE AND FLOWER DEVELOPMENT IN *A. THALIANA*

Tutor: Martin Kater

Simonini Sara

3 *SUMMARY*

4 *CHAPTER 1: INTRODUCTION*

4 1. *Arabidopsis thaliana*
4 2. Plant hormones
5 2.A Auxin
6 2.B Cytokinin
8 3.Regulation of gene expression in *Arabidopsis thaliana*: the transcription factors
8 3.A MADS box transcription factors
8 3.B Homeobox factors
9 3.C B3 superfamily members
9 Auxin responsive factors (ARF)
10 3.D GAGA binding proteins
11 4. Snapshot on the reproductive phase: Inflorescence and Floral Meristems
12 4.A Hormonal fluxes in the SAM
12 Auxin
12 Cytokinin
12 Crosstalk between auxin and cytokinin in the SAM
13 4.B MADS-box Transcription Factor involved in IM and FM development: the floral meristem identity genes
14 5. Flower development in *Arabidopsis thaliana*
14 5.A The ABCDE model
14 5.B Flow and auxin biosynthesis during development of floral organs
15 6. The ovule of *Arabidopsis thaliana*
16 6.A Genetic regulation of ovule development

24 *CHAPTER 2*
BPC Proteins Mediate MADS-domain complex Binding To The DNA For Tissue Specific Expression Of Target Genes In *Arabidopsis thaliana*

44 *CHAPTER 3*
BPC Proteins Regulate HOMOEBOX Transcription Factors Involved In Meristem Size Maintenance

56 *CHAPTER 4*
The Transcription Factors BEL1 and SPL Are Required for Cytokinin and Auxin Signaling During Ovule Development in *Arabidopsis*

77 *CHAPTER 5*
Identification of Pathways Directly Regulated by SHORT VEGETATIVE PHASE During Vegetative and Reproductive Development in *Arabidopsis*

79 *CHAPTER 6*

MONOPTEROS (MP) Is Required As Central Integrator Of Auxin And Cytokinin Pathways For Ovule Primordia Formation

81 *CHAPTER 7*

TAF13 Interacts with PRC2 Members and Is Essential for *Arabidopsis* Seed Development

83 *DISCUSSION*

Summary

During my PhD training period I studied different aspects of *Arabidopsis* development, with a focus on the reproductive phase. In Chapter 1, the **Introduction,** I provide an overview of floral meristem, inflorescence meristem, flower and ovule development, and how these processes are regulated. I also discuss the roles of hormones in these processes. The subsequent **Chapters** describe the research I have been involved in.

My main research project focused on the role of BPC proteins in the regulation of the expression of *STK,* a homeotic gene controlling ovule identity. This research is described in **Chapter 2**: I demonstrated that BPC binding sites located in the *STK* promoter are necessary for its correct spatial and temporal expression. BPCs showed to play a crucial role in the recruitment of a multimeric repressor complex containing the MADS-box transcritpion factor SVP, which prevents the expression of *STK* in the floral meristem.

Detailed analysis of the *bpc1 bpc2 bpc3* triple mutant described in **Chapter 3** showed that BPCs play a role in determining the shape of the inflorescence and control inflorescence meristem activity. In particular I focused on the regulation of HOMEOBOX genes involved in meristem size maintenance such as *STM* and *BP*. A link between these HOMEOBOX factors and the cytokinin pathway (in which they are involved) has been investigated.

I have also contributed to other research projects: in **Chapter 4** ovule development in respect to two hormonal pathways is discussed. In this chapter the role of Cytokinin and Auxin in ovule development through the activity of the two transcription factor BEL1 and SPL has been elucidated. The **Chapter 5** focused on the genome wide identification of targets of SVP, a MADS-domain transcription factor involved in the floral transition and floral meristem identity determination. The list of putative targets includes genes involved in meristem development and I have especially contributed to the analysis of those genes. In **Chapter 6** For the manuscript which talk about the role of MP in the regulation of ovule number I performed the in situ hybridization to show *CUC1*, *CUC2* and *ANT* expression in different background. For the manuscript regarding the role of the general transcription factor TAF13 factor in seed development reported in **Chapter 7**, I performed the co-immuno precipitation experiments to show that TAF13 interacts with PCR2 components.

In the **Discussion** I briefly summarize all the data obtained and future research prospectives.

CHAPTER 1: INTRODUCTION

1. *Arabidopsis thaliana*

Arabidopsis thaliana (Figure 1) is a small herbaceous eudicot, member of the family of the *Brassicaceae*; it can be found in temperate areas and it is the model organism for developmental, biochemical and physiological studies in plants.

Its life cycle is relatively short: starting from the seeds, in 4-6 weeks the adult plant is developed and, in turn, it produces hundred of seeds.

The *Arabidopsis* genome, which was the first plant genome to be entirely sequenced (the *Arabidopsis* Genome Project in 2000), is small (146 Mb) and it contains a large amount of genetic redundancy, about 60% of it is probably derived from single event of duplication. The genes encoded by the genome are more than 30.000, but only to a small portion a clear function has already been assigned.

To study gene functions, a huge number of single-nucleotide and/or insertional mutants are available: to date more than 320.000 mutants are present in the NASC (Nottingham *Arabidopsis* Stock Centre) and ABRC (*Arabidopsis* Biological Resource Centre) stock centres.

For these (and many more) reasons, *Arabidopsis* is one of the most used model organism for plant research.

Figure 1. *Arabidopsis thaliana*

2. *Plant hormones*

Hormones are key factors involved in all aspects of plants growth and development (Figure 2). Two of the best studied and most important classes of plant hormones are auxins (AUXs) and cytokinins (CKs). The predominant form of auxin present in nature is Indole-3-Acetic Acid (IAA), while the most common forms of natural cytokinins are N6-(Δ2-isopentenyl) adenine (iP), trans-zeatin (tZ), cis-zeatin(cZ), and dihydrozeatin (DZ).

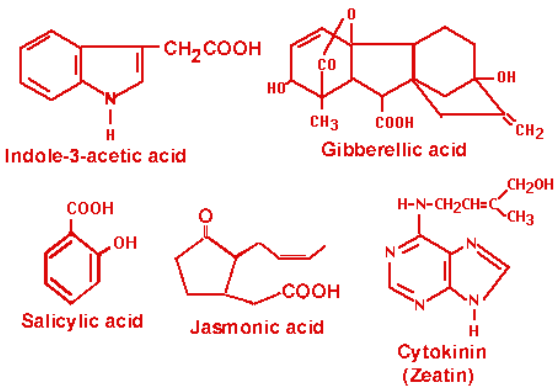


Figure 2. Few plant hormones: auxin and cytokinin but also giberellin are the most studied.

The CK/IAA concentration ratio is the key to modulate different developmental processes such as apical dominance (Nordstrom *et*

al., 2004), SAM initiation (Gordon *et al.,* 2007), axillary buds outgrowth (Tanaka *et al.,* 2006), and lateral root formation (Werner *et al.,* 2003; Laplaze *et al.,* 2007). However, the complicated nature of auxin-cytokinin crosstalk is still far from being completely resolved.

2.A *Auxin*

Auxin is involved in a wide spectrum of functions such as: apical dominance, fruit ripening, root meristem maintenance, hypocotyl and root elongation, shoot and lateral root formation, tropisms, cellular division, elongation and differentiation, embryogenesis and all types of organogenesis (Laskowski *et al.,* 1995; Reinhardt *et al.,* 2000; Benkova *et al*., 2003).

Auxin can be synthesized through two different biosynthetic pathways: the **tryptophan (Trp) dependent** and **Trp-independent** pathways, the latter is still mainly uncharacterized. The 11 ***YUCCA*** (***YUC***) genes encoding flavin mono-oxigenases belong to the first pathway; they are expressed mainly in meristems, young primordia, vascular tissues and reproductive organs suggesting that their expression has precise spatio-temporal profiles (Zhao *et al.,* 2001; Cheng *et al.,* 2006).

Auxin distribution is a balance between production, inactivation and degradation and the **Polar Auxin Transport** (**PAT**) is central for the creation of fine, dynamic regulation of auxin gradients. A major role in PAT is played by ***PIN-FORMED*** (***PIN***) genes, a gene family composed by 8 partially functionally redundant members encoding auxin efflux carriers (Petrasek *et al.,* 2006). The best characterized among *PIN* genes is ***PIN1***, which is expressed in cotyledons, seedlings, roots, leaves, flowers and siliques (Okada *et al.,* 1991). Despite its broad expression profile, the *pin1* mutant shows defects mainly in the inflorescence. Flowers of a mild *pin1* mutant lack stamens, have wide petals and abnormal pistil-like structures with no ovules in the ovary (Bencivenga *et al.,* 2012). Strong *pin1* mutants develop a naked inflorescence stem with a complete lack of lateral floral meristems (Okada *et al.,* 1991). By now several evidences support the involvement of PIN1 in vascular development, phyllotaxis, embryogenesis (like PIN4 and PIN7) and lateral organ formation (like PIN2 and PIN3) (Benkova *et al*., 2003; Galweiler *et al.,* 1998; Reinhardt *et al*., 2003; Scarpella *et al.,* 2006; Weijers *et al.,* 2005).

PIN genes are also involved in root (PIN2 and PIN3) and shoot gravitropism (PIN3), phototropism (PIN3), root patterning and development (PIN4 and PIN7) (Petrasek and Friml, 2009), and even intracellular auxin homeostasis (PIN5; Mravec *et al.,* 2009).

Once auxin cellular concentration increases, due to local biosynthesis or transport, the signaling triggered by the hormone starts rapidly (Figure 3). The auxin signalling pathway is composed by three components: the 29 auxin primary response ***Auxin/Indole-3-Acetic Acid*** (***Aux/IAAs***) genes, the 23 ***Auxin Response Factors*** genes (***ARFs***), and the **SCFTIR1** (**SKP1**, Cullin, F-box protein TRANSPOR INHIBITOR RESPONSE1) complex for specific ubiquitylation of substrates to a 26S proteasome-dependent degradation (Weijers *et al.,* 2004). Auxin binds to its TIR1 receptor and this facilitates the interaction, and then degradation, of Aux/IAA

proteins through ubiquitination. Aux/IAA proteins normally form hetero-dimers with the ARFs transcription factors. When this interaction is lost, then the free ARF transcription factor can regulate transcription by binding to specific **Auxin Response Elements** (**AuxREs**, TGTCTC) in the cis-regulatory regions of their target genes. At low cellular concentrations of auxin, the ARFs are linked to the DNA and interact through the corepressore TPL with the repressors Aux / IAA (stable at low concentrations of auxin): this prevents the ARF to begin transcriptional activity.

After increase of auxin concentration, the AUX/IAA repressors become unstable and ubiquitinated. Once free from inhibition, the ARF bound to DNA, dimerize with another ARF, thereby regulating the transcription of target genes (reviewed in Lyser *et al*., 2002; Rogg and Bartel, 2001).

Multiple AuxREs have been cloned in direct (or reverse) orientation up-stream a minimal -46 cauliflower mosaic virus (CaMV) (Ulmasov *et al.,* 1997b; Sabatini *et al.,* 1999; Friml *et al.,* 2003) to create the **DR5** synthetic auxin sensitive promoter. DR5::GUS and DR5rev::GFP constructs are by now commonly used as markers for auxin presence *in vivo* (Sabatini *et al.,* 1999; Benkova *et al*., 2003).

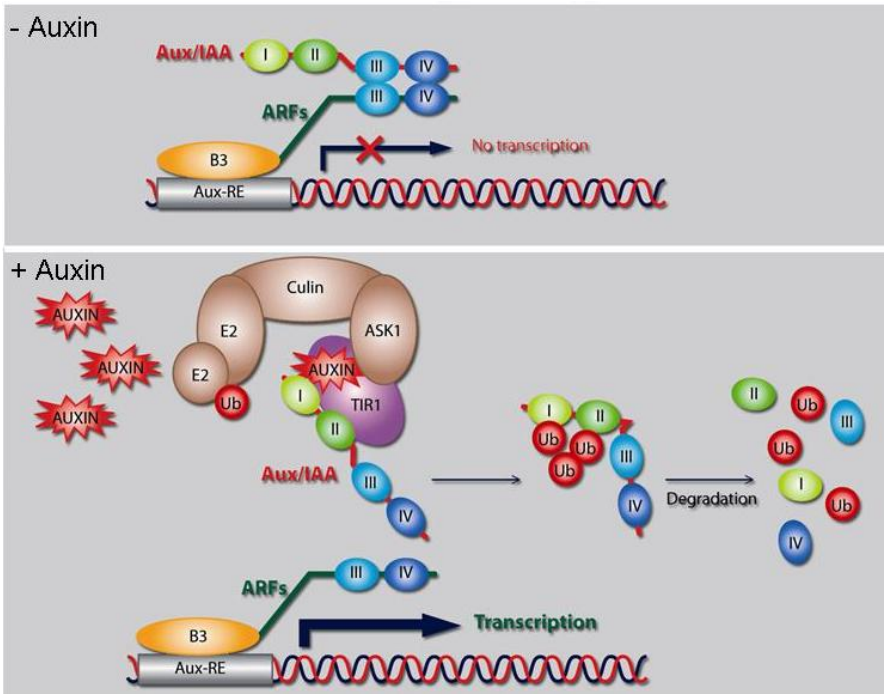


Figure 3. The Auxin signaling.

2.8 *Cytokinins*

Cytokinins act as positive and negative regulation of respectively shoot and root meristem formation and activity (Werner *et al.,* 2003), vascular tissue formation (Aloni *et al.,* 1987; Mahonen *et al*. 2000; Mahonen *et al*. 2006), apical dominance, leaf senescence, chloroplast biogenesis (Mok, 1994), cell differentiation (Mahonen *et al.,* 2006; Dewitte *et al,*. 2003; Dello Ioio *et al*., 2007) and cell division (Dewitte *et al*., 2003; Dewitte *et al,*. 2007).

Apparently roots and shoots have two distinct CKs biosynthetic pathways (the **iPMP-dependent** one, dominant in roots, and the **iPMP-independent** one, dominant in shoot) differing for the nature of the sidechain donor for the adenine, and the presence of chloroplasts seems to be a prerequisite for the second pathway to work (Astot *et al*., 2000; Nordstrom *et al*., 2004). A central role in CK synthesis is played by the products of the seven ***IPT*** genes, which show different specific expression patterns (Kakimoto *et al*., 2001; Takei *et al*. 2001; Miyawaki *et al*. 2004).

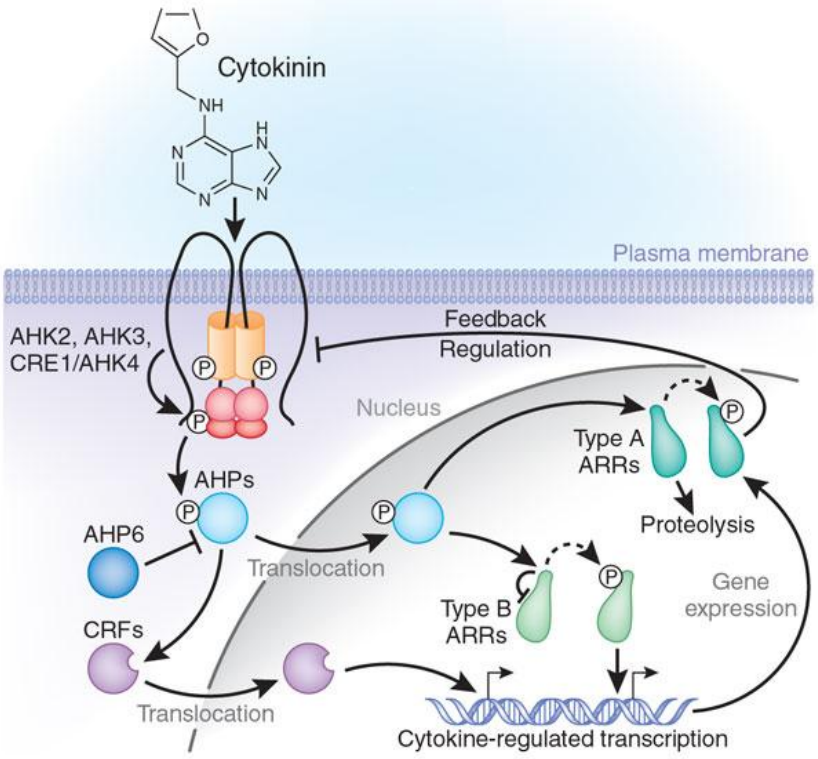


Figure 4. The Cytokinin singaling.

As for the other plant hormones, CK homeostasis requires a balance among biosynthesis, conjugation and degradation rates, and transport. CKs signalling pathway is similar to the two component bacteria signal transduction, where a sensor kinase perceives the stimulus and, via phosphotransfer, a response regulator propagates it (Figure 4). The above mentioned proteins in *Arabidopsis* are:

- hybrid-type sensor kinases (**AHKs**), three in *Arabidopsis* (AHK2; AHK3; CRE1 /AHK4);
- histidine phosphotransfer proteins (**AHPs**), five in total (AHP1-5);
- response regulators (**ARRs**), which are more than 20 and are divided into two main groups: 10 type-A and 11 type-B ARRs.

Type-B ARRs are positive regulators of CK signalling whereas the **type-A ARRs**, once activated by CK, trigger the signaling in a negative feedback loop manner, switching down the type-B ARRs (Heyl *et al.,* 2008). A good example of type-A ARRs gene is ARR5: it is a direct target of type-B ARRs (Heyl *et al.,* 2008), it is a CK primary response gene since it is rapidly up-regulated by CKs, it is expressed both in SAM and RAM (D'Agostino *et al,.* 2000), and acts negatively in CK signalling pathway counteracting type-B ARRs action (To *et al.,* 2008).

3. Regulation of gene expression in Arabidopsis thaliana by transcription factors

3.A MADS-box transcription factors

The *Arabidopsis* genome encodes 107 **MADS-domain transcription factors** (Parenicova *et al*., 2003). The MADS-domain is a highly conserved 58-aminoacids DNA binding domain able to recognize a specific sequence called **CArG-box** (CC(A/T)$_6$GG) (Schwarz-Sommer *et al*., 1992: Huang et al., 1993; Shiraishi et al., 1993; Riechmann and Meyerowitz 1997), and is also involved in nuclear localization and dimerization (de Bodt *et al*., 2003).

The Arabidopsis MADS-box gene family was divided in two classes: **MADS-box type I**, that can be further divided into the **Mα**, **Mβ** and **Mγ** clades, and **type II**, that comprises the **Mδ** and **MIKC** clades (Alvarez-Buylla *et al*., 2000; Parenicova *et al*., 2003).

The type I subfamily comprises 60 members and except for the MADS-box domain doesn't share any sequence similarity with the type II class. Among the type II MADS-box genes the Mδ class hasn't been well characterized so far, while many members of the MIKC clade have been intensively studied. The MIKC transcription factors share a conserved structure constituted by the **MADS domain** (**M**), the **intervening-domain** (**I**) involved in dimer formation, the **keratin-like domain** (**K**) involved in protein-protein interactions and the **C-terminal domain** (**C**) involved in ternary complexes formation and transcriptional activation (Becker and Thiessen, 2003: de Bodt *et al*., 2003; Masiero *et al*., 2002).

The first *Arabidopsis* MADS-box gene that was characterized is ***AGAMOUS***: it belongs to the MICK clade, which includes the most important MADS-domain transcription factors involved in flower development.

The ability of MADS-domain proteins to form heterodimers and ternary complexes strongly increases the complexity of their regulation, since a single transcription factor may change its activity simply by forming complexes with different partners (de Folter *et al*., 2005). Many floral organ identity genes and many floral meristem identity genes are MADS-box transcription factors (Parenicova *et al*., 2003).

3.B The HOMEOBOX gene family

Homeobox genes encode a typical DNA-binding domain of 60 amino acids, known as homeodomain (HD), that characterizes a large family of transcription factors. The first homeobox gene was isolated from *Drosophila melanogaster* and was subsequently found to be involved in many aspects of development (Gehring *et al*. 1994; Burglin 2005). A distinction has been made between ''typical'' homeodomains, characterized by a length of 60 amino acids, versus ''atypical'' ones of different lengths (Burglin 1994). The latter include a group characterized by homeodomains of 63 aa, with three extra residues inserted between helix 1 and 2 (Burglin 1995), that have been named TALE (Three Amino acid Loop Extension) homeobox genes (Bertolino *et al*. 1995). Both TALE and typical homeobox genes were found to be present in all major eukaryotic lineages including plant, fungi, and animals, suggesting that these two types of homeobox were present in the eukaryote ancestor (Burglin 1995; Bharathan *et al*.

1997; Burglin 1997, 1998a; Derelle *et al*. 2007). Plant homeodomain proteins have been classified in the literature into various groups based on sequence similarity of their homeodomains: **KNOX** (KNOX I and KNOX II) and **BEL**, belonging to the TALE superclass, **ZM-HOX**, **HAT1**, **HAT2**, **WOX**, **ATHB8**, and **GL2**. The HAT1, HAT2, ATHB8, and GL2 genes are all characterized by a leucine-zipper motif downstream of the homeodomain (Ruberti *et al*. 1991) and have been successively renamed HD-ZIP I, HD-ZIP II, HD-ZIP III, and HD-ZIP IV, respectively (Bharathan *et al*. 1997; Meijer *et al*. 1997; Aso *et al*. 1999; Sakakibara *et al*. 2001). An alternative classification was also proposed and divides the members into five groups (HD-ZIP, GLABRA, KNOTTED, PHD, and BEL).

The 16 *Arabidopsis* genes encoding WOX proteins are characterized by distinctive conserved motifs both upstream and downstream of the homeodomain, including the WUS Box (Haecker *et al*. 2004).

TALE genes have been extensively studied and classified into the two classes KNOX and BEL. Among the homeobox gene classes, *KNOX* and *BEL* appeared to be the oldest classes, with members present in single-cell green algae and in red algae.

From the analysis of the codomain structure of *KNOX* genes from flowering plants, we could further classify *KNOX I* genes into two subclasses, *KNAT 2/6* and *KNAT1*, while the *KNOX II* class could be separated into the two subclasses *KNAT7* and *KNAT3/4/5*.

3.C The Arabidopsis B3 superfamily

The B3 DNA binding domain was first identified in the maize gene VIVIPAROUS (VP1) (McCarty *et al.,* 1991), it is composed of about 110 aa and it is widespread in plant genomes. In *Arabidopsis thaliana* four gene families are part of the B3 superfamily: the **LAV** (LEAF COTYLEDON2 [LEC2] Abscisic acid-INSENSITIVE3 [ABI3]-VAL) family, the **ARF** (AUXIN RESPONSIVE FACTORS) family, the **RAV** (REALETED AND TO ABI3 VP1) family and the **REM** (REPRODUCTIVE MERISTEM) family. Hormone regulation seems to be a key aspect for understanding the function of the first three groups: the ARF family is strongly involved in responses to auxin, the RAV family seems to be involved in brassinosteroids pathways and the LAV family seems to respond to abscisic acid (Swaminathan *et al*., 2008).

Auxin Responsive Factors (ARF)

There are 23 **ARF** encoding genes present in the genome of *Arabidopsis* and all of them (except *ARF23* which is probably a pseudogene) bind DNA in correspondence of regions called **Auxin Response Elements** (**AuxRE**) characterized by the consensus sequence TGTCTC (Guilfoyle *et al*., 2007; Ulmasov *et al*., 1997) The ARFs are composed of a B3 DNA binding domain (**DBD**) located at the N-terminal. (Ulmasov *et al*., 1997), a middle region (**MR**) responsible of transcriptional activation or repression, a C-terminal domain (**CTD**) divided into two domains (III and IV) and responsible for homo-and heterodimerization between the ARF-ARF and ARF-AUX/IAA (Guilfoyle *et al*., 2007).

For several ARFs a role in *Arabidopsis* development has already been assigned: **ARF1** and **ARF2** are involved in flower development and floral organ abscission (Ellis *et al*., 2005;

Schruff *et al*., 2006); **ARF3** and **ARF4** are involved in the development and polarity establishment of vegetative and reproductive structures (Pekker *et al*., 2005; Session *et al*., 1997); **ARF5** is expressed in the embryo, in the vascular tissues and carpel and it has a crucial role in establishing the cell division plane in embryo cells (Hardtke *et al*., 1998); **ARF6** and **ARF8** have been implicated in the development of flowers and fruits (Nagpal *et al*., 2005); **ARF7** is expressed in shoots, roots and in the embryo during development; (Hardtke *et al*., 2004). **ARF12**, **13**, **15**, **20**, **21**, **22** are involved in embryogenesis and development of seeds (Okushima *et al*., 2005).

3.D GAGA binding proteins

The first member of this class of proteins that was characterized is the **GAGA factor** (**dGAF**) of *Drosophila melanogaster*, but further analyses demonstrated that **GAGA Binding Proteins** (**GBPs**) are present in various organisms including plants. The GBPs are able to bind the DNA at (GA)n or (CT)n sequences playing important roles in many processes such as regulation of genes transcription and chromatin remodeling through their physical interactions with subunits of **NURF** and **FACT** complexes, involved in nucleosome spacing (Orphanides *et al*., 1998). The function of these higher order complexes is to move the nucleosomes, thus making transcription factors' binding sites accessible, and interact directly with the transcriptional machinery or recruit other proteins which mediate the activation or repression of transcription. The binding sites for GAFs are contained in many promoters, in particular in the 200 bases upstream from the start of transcription (Katsani *et al*., 1999), but there are many exceptions that see binding sites contained in introns or in correspondence to elements characteristic of the GA-rich satellite DNA (Platero *et al*., 1998). When multiple GAGA sites are close to each other, a strong cooperative effect is observed: the binding of a GAF recruits other GAFs to the adjacent binding sites (Espinas *et al*., 1999), suggesting that the GAF should not be considered only as a factor involved in regulation of gene expression, but also as a structural protein.

In several plant species genes encoding GAGA Binding Protein have been identified: **GBP** in soybean (Sangwan and O'Brian, 2001), **B Recombinant barley** (**BBR**) in barley (Santi *et al*., 2003) and **Basic Pentacysteine** (**BPC**) in *Arabidopsis thaliana* (Meister *et al*., 2004). These proteins have appart from the fact that they bind to GAGA sites nothing in common with GAFs found in other species. The broad pattern of expression of these factors and the high number of potential target sequences present in the plant genome, suggests that these proteins could be involved in the regulation of the expression of many genes involved in many different processes.

The BPCs are seven (Meister *et al*., 2004) and they can act as transcription factors. The C-terminal domain is highly conserved between all the members and it contains an unusual arrangement of 5 cysteines (Cys, C). The BPCs can be divided, on the basis of similarities of the entire amino acid sequence, into three classes:

- Class I: **BPC1**, **BPC2**, **BPC3** which have a conserved N-terminal domain;

- Class II: **BPC4**, **BPC5**, **BPC6** characterized by a conserved region capable of forming a coiled-coil structure due to the presence of hydrophobic areas;
- Class III: **BPC7**, which doesn't show any conserved region apart from the C-terminus.

At least for BPC1, the RGARAGRRA consensus binding site has been proposed: through this binding site BPC1 binds to DNA inducing conformational changes which may have a consequence on transcription (Kooiker *et al*., 2005; Simonini *et al.,* 2012).

Research outside the plants kingdom produced no results: this finding supports the hypothesis that the Basic PentaCysteine is a specific gene family of plants.

4. Snapshot on the reproductive phase: Inflorescence and Floral Meristems

During embryo development in *Arabidopsis thaliana*, the primary meristems are established and develop along the longitudinal axis of the embryo body. The one that develops between the two cotyledons is named **Shoot Apical Meristem** (**SAM**) and after germination it will give rise to all the areal plant organs and tissues.

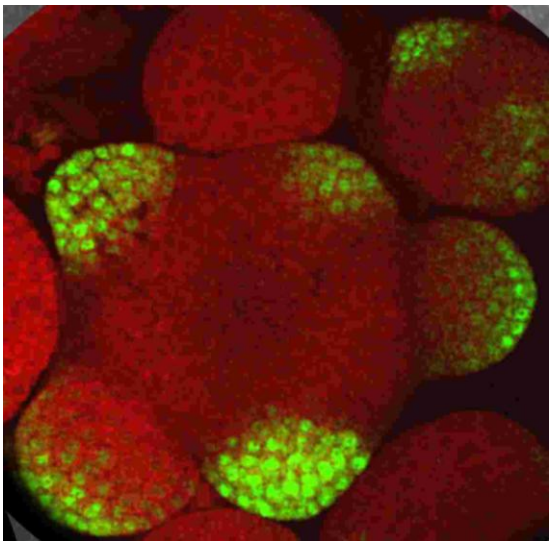


Figure 5. Organization of a shoot apical meristem: IM at the centre and several floral meristems, the green ones which arise from the FM surface.

The SAM is a typical undifferentiated meristem and it is organized in three cell layers: L1 -the upper one- L2 and L3. Moreover three zones -**central**, **peripheral** and **rib**, can be distinguished based on the cell activities that take place in each of them. The central zone (CZ), localized at the apex of the SAM, is a small cluster of enlarged, highly vacuolated cells that usually have a slow rate of division, as they are the reservoir of pluripotent stem cells. The peripheral zone (PZ) surrounds the central zone and it is the site of organ formation; its cells are small and divide more frequently. The rib zone (RZ) is beneath the central zone in the deeper layers of the meristem, it is characterized by large, vacuolated cells organized in columns, and it contributes to the bulk of the meristem.

After germination, the SAM enters in the **vegetative phase** starting to produce leaves; after the floral transition the SAM becomes **Inflorescence Meristem** (**IM**) that, instead of leaves, produces **Floral Meristems** (**FM**) positioned in a spiral pattern tightly regulate by hormones fluxes (Figure 5). The floral meristem is a determinate meristem from which the floral organs differentiate.

4.A Hormonal fluxes in the SAM

Auxin

In the SAM Auxin induces **organogenesis** and regulates **phyllotaxis**. According to the current models auxin is transported through the L1 surface layer of the meristem through a mechanism called reverse-fountain and the efflux facilitator PIN1 plays a central role in this process (Benkova *et al.*, 2003). PIN1 directs the auxin flux toward the area where a new floral primordia has to grow. This results in an accumulation of auxin at the tip of the emerging floral primordia, depleting the surroundings area of the meristem from auxin. The auxin concentration remains high at the tip of the primordia until stage 2-3 of flower development; later, the auxin starts to efflux far from the primordia and it is conducted sub-epidermally towards new areas of the meristem to positioning a new primordia buldge. This mechanism represents a combination of positive feedback -auxin accumulation- and lateral inhibition -withdrawal of auxin from adjacent tissues- (Reinhardt *et al*., 2003).

Cytokinin

Stem cells are a source of cytokinins (Gordon *et al.,* 2009) which up-regulates *WUS* expression. WUS is regulated via a classical negative-loop and two positive-loops. In the first scenario stem cells secrete CLAVATA3 (CLV3) ligand that binds to the complex formed by CLV1/CLV2 proteins leading to WUS downregulation. The positive circuit can be either CLV-dependent and/or -independent, and in both cases it is cytokinin driven. In the CLV-independent positive loop CK directly promotes (via an AHK2/AHK4 circuit) WUS expression, which in turn negatively regulates the expression of several type-A ARRs genes (*ARR5-7*, and *ARR15*). The final result is a positive regulation of CK signalling. In the CLV-dependent positive loop, CK directly downregulates *CLV1* expression, thus indirectly promoting WUS expression; in this way cytokinin positively regulates its own signalling. The effect of both positive loops is to maintain the stem cells in the Central Zone, which, as mentioned, are a cytokinin source (Gordon *et al.,* 2009).

Crosstalk between auxin and cytokinin in the SAM

At the SAM, synthesis and signalling pathways for both auxin and cytokinin are present and highly active. In the SAM a high IAA/CK concentration ratio induces organ formation, while a low IAA/CK concentration ratio is necessary for the meristem identity maintenance of cells.

Both hormones regulate each other at different levels (Figure 6): auxin, indirectly reduces CK synthesis in the SAM by repressing in the CZ the gene *SHOOTMERISTEMELESS*

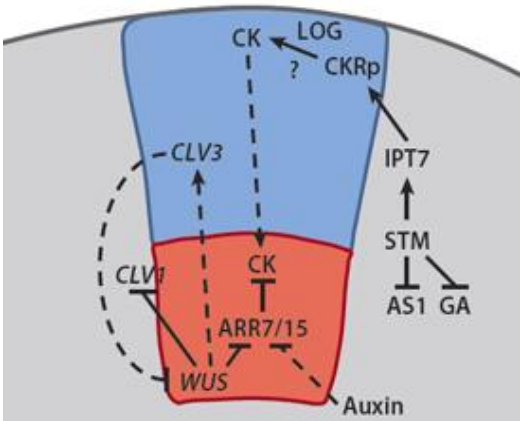


Figure 6. Aux and CK at the SAM regulate each other.

(*STM*) which induces *IPT* genes expression: as a consequence CK signalling is negatively regulated (Shani *et al.,* 2006). Moreover auxin is also able to induce expression of genes encoding for enzymes involved in CK degradation, and by regulating CKs conjugation rate, thus acting on CK concentration at multiple levels. (Nordstrom *et al.,* 2004).
Finally, in recent years evidences have been collected that CKs can regulate auxin homeostasis indirectly, by modulating IAA polar efflux. Studies in *Arabidopsis* have in fact shown that CK has no effect on *AUX1*, which encodes an IAA influx carrier, while it is able to affect the spatial expression of *PIN* genes (Laplaze *et al.,* 2007, Bencivenga *et al*., 2012, Ruzicka *et al.,* 2009; Pernisova *et al.,* 2009) and transcripts levels.

4.8 MADS-domain Transcription Factors involved in IM and FM development: the floral meristem identity genes

Several genes are involved in the control of **Floral Meristem Identity** (**FMI**) and among them ***SHORT VEGETATIVE PHASE (SVP)***, ***AGAMOUS-LIKE24 (AGL24)*** and ***APETALA1* (*AP1*)**, play a fundamental and redundant role in floral meristem specification.

The paralogous genes *SVP* and *AGL24* encode MADS-box transcription factors involved both in floral transition and floral meristem determination. They are expressed in the flower primordia at stages 1 and 2. While the *svp* and *agl24* single mutants don't display any flower phenotype, in the *svp agl24* double mutant the flowers have a very mild phenotype in the first developing flowers. These flowers have mainly a reduced number of floral organs. However, when this double mutant was grown at high temperatures >30°C, all flowers were severely affected and displayed reduced number of organs and homeotic conversions (Gregis *et al*., 2006) suggesting an involvement of these genes in flower development.

APETALA1 was firstly discovered as a MADS-domain transcription factor with a fundamental role in floral organ identity and, although the *ap1* mutant displays phenotype defects only during flower development, *AP1* has a role also in floral meristem development. Indeed the triple mutant *svp agl24 ap1* displays a complete loss of floral meristem identity as indeterminate inflorescence meristems only give rise to new inflorescence meristems, leading to the formation of cauliflower-like structures (Gregis *et al*., 2008). Moreover, when the *svp agl24* double mutant was combined with a weak allele of *ap1*, the floral meristem differentiated precociously due to the ectopic expression of the homeotic genes *APETALA3*, *PISTILLATA*, *AGAMOUS* and *SEPALLATA3* (which are involved in floral organs development and identity) strongly suggesting that in the flower primordium the meristem identity is maintained through the repression of genes involved in the floral organ differentiation (Gregis *et al.,* 2006). This repression is mediated by the repressor complex made by AP1-SVP/AGL24 with the SEUSS and LEUNIG co-repressors complex (Gregis *et al*., 2006) forming a mulitmeric complex able to bind to the promoters of the floral homeotic genes *AP3*, *PI*, *AG* and *SEP3*.

5. Flower development in Arabidopsis thaliana

The *Arabidopsis* flower is composed by four types of floral organs positioned in a whorled manner: four green **sepals**, four white **petals**, six **stamens** (two of them are shorter than the others) and a pistil composed by two fused **carpels**. The flower of *Arabidopsis* contains both the male and female reproductive organs, so it is a "perfect flower" as it can self-pollinate to obtain the fertilization of the ovules and the consequent development of the seeds.

5.A The ABCDE model

At stage 3 of flower development, the floral meristem has reached the correct size to start to differentiate into floral organs. The identification of many floral homeotic mutants in *Arabidopsis thaliana* and *Antirrhinum majus* (Bowman *et al*., 1989; Yanofsky *et al*., 1990; Coen *et al.*, 1990, Schwarz-Sommer *et al*., 1990, 1992) led to the formulation of a genetic model, called ABC model that was further integrated with two additional classes, the D- and E- classes. The resulting **ABCDE model** (Figure 7) explains how the specification of the different floral organ whorls occurs through different combinations of ABCDE genes:

- A-Class genes ***APETALA1*** and ***APETALA2***: specify sepal identity in whorl 1;
- B-Class genes ***APETALA3*** and ***PISTILLATA***: together with the A-genes and C-genes specify petal identity in whorl 2 and stamen identity in whorl 3 respectively;
- C-Class genes ***AGAMOUS, SHATTERPROOF1*** and ***SHP2***: specify carpel and ovule identity in whorl 4 and floral meristem determinacy.
- D-Class gene ***SEEDSTICK***: controls ovule identity
- E-class genes are ***SEPALLATA1***, ***SEP2***, ***SEP3*** and ***SEP4***: effect redundantly the identity of all floral organs; they are necessary for the function of the A, B and C class genes interacting with them for the formation of multimeric complexes (Honma and Goto 2001; Theissen and Saedler, 2001).

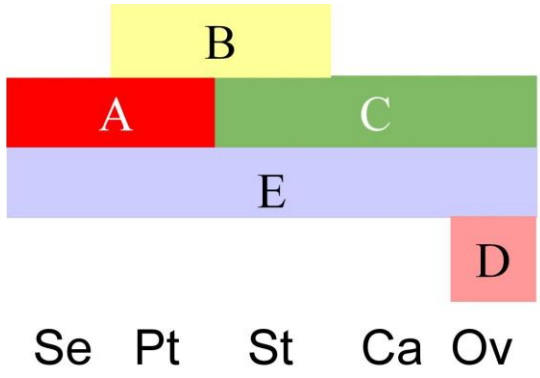


Figure 7. The ABCDE model.

All these genes belong to the MADS-box transcription factor family, except for *AP2* that belongs to the AP2-like transcription factors (Okamuro *et al*., 1997; Aukerman and Sakai, 2003).

5.B Flow and auxin biosynthesis during development of floral

At early stages of flower development, auxin is concentrated in correspondence at the **apical region** of the floral organs: sepals and petals show a weak distribution of the hormone while the anthers and stigma are the sites affected by the more intense auxin biosynthesis. Auxin is also essential for the timing of floral organs development, thus flower can develop organs

that terminate their growth correctly synchronized (Aloni *et al.*, 2005). Several evidences suggest that the synthesis of auxin in a particular area, can both promote and inhibit the growth of organs belonging to the adjacent whorls. In support to this hypothesis, the elimination of some manual floral organs triggers the active auxin synthesis in the remaining organs: the elimination of the stamens causes an abrupt increase of the hormone in the petals, which reach greater dimensions, and an increase of the growth of carpel and stigmatic papillae.

6. The ovule of Arabidopsis thaliana

Arabidopsis thaliana ovules (Figure 8) are lateral organs emerging from the subepidermal meristematic tissue of the pistil, the placenta. Ovule development consists of 3 stages (further divided into sub-stages) (Schneitz *et al*. 1995), first the ovule emerges as a protrusion as result of periclinal divisions in cells of the subepidermal placenta. During progressive elongation, three zones differentiate: the **nucellus**, the **chalaza**, and the **funiculus**. The most apical cell harbored by the nucellus is the **Megaspore Mother Cell** (**MMC**), which has a large nucleus and vacuole. Subsequently, both integuments develop forming a continuous ring around the nucellus. During this stage, megasporogenesis takes place: the MMC undergoes meiosis producing four haploid megaspores (Reiser *et al*., 1993; Schneitz *et al*., 1995; Christensen *et al*., 1997) among which only the one closest to the chalaza area of the nucellus becomes the **Functional Megaspore** (**FM**). After three mitotic divisions the female gametophyte has eight nuclei/seven cell -two **synergid cells**, one **egg cell**, on **binucleated central cell**, and three **antipodal cells**. Both inner and outer integuments have now completely enclosed the nucellus. At this stage, the inner layer of the inner integument gives rise to a third layer of cells, the endothelium (Schneitz *et al*., 1995).

When fertilization occurs ovules turns into seeds: the pollen tube penetrates one of the two synergid cells which bursts, and delivers into the embryo sac two sperm cells. One sperm cell fuses with the egg cell. From this event the zygote is formed. The second sperm cell fuses with the central cell producing a triploid nucleus from which the endosperm is subsequently formed.

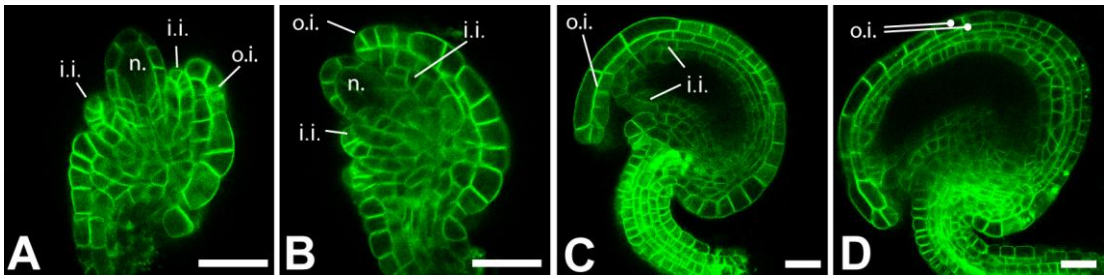


Figure 8. Ovule development. N, nucella; ii, inner integument; oi, outer integument.

6.A *Genetic regulation of ovule development*

Ovule identity is determined by transcription factor complexes, composed of different combinations of MADS-domain proteins (Brambilla *et al.,* 2007; Favaro *et al.,* 2003). SEEDSTICK (*STK*), SHATTERPROOF 1 (SHP1), SHATTERPROOF 2 (SHP2), SEPALLATA3 (SEP3) (Favaro *et al* 2003).

SEEDSTICK is expressed in placental tissue and during ovule development in the funiculus and in the integuments. When the ovule is fully formed *STK* expression is clearly maintained in the funiculus, and it becomes weaker in both integuments (Pinyopich *et al* 2003). The *stk* mutant develops seeds that show defective abscission zone formation; moreover the funiculus of *STK* ovules are formed by more cells and are larger then those of wild type ovules (Pinyopich *et al.,* 2003).

To properly determine ovule identity the *STK* protein complex must interact with another complex formed by SEP3, AGAMOUS (AG) and BEL1 (Reiser *et al*., 1995).

BEL1 is expressed in ovules, in the chalaza and in both integuments throughout their early development, but not in the nucellus and embryo sac (Reiser *et al.,* 1995). In the *bel1* mutant the funiculus are thicker then normal, consisting of more cells then wild type, and the most striking phenotype is the formation at the base of the nucellus of a single integument-like structure (Robinson-Beers *et al.,* 1992).

This integument-like structure enlarges and converts into a carpelloid structure suggesting that BEL1 has a central function in ovule integuments determination (Robinson-Beers *et al.,* 1992; Brambilla *et al.,* 2007). Moreover, like all sporophytic mutants and combinations of them (Colombo *et al.,* 2008), *bell1* ovules fail the megagametogenesis since the development within the nucellus starts to deviate after stage 3-I and never ends up with a functional embryo sac (Battaglia *et al*., 2008; Schneitz *et al.,* 1997).

Another sporophytic mutant with female gametophyte defects is *aintegumenta* (*ant*). *ANT* encodes for an *Arabidopsis* transcription factor (TF) of the AP2-like TF family involved in the control of growth during lateral organ development by positively regulating cellular proliferation (Mizukami *et al.,* 2000). *ANT* is expressed in all organ primordia but not in roots. In ovules it is first expressed in the placenta, then in the funiculus and chalazae, and later in the endothelium, while no *ANT* expression is detected in the nucellus (Elliot *et al.,* 1996).

The *ant* mutant lacks integuments (FIG6). The integument-ridge is formed, however it is unable to further expand (Baker *et al.,* 1996; Elliot *et al.,* 1996). Moreover ant mutants produce 50% less ovules than wild type, and they are completely female sterile (Baker *et al.,* 1996) since megagametogenesis does not occur properly (Elliot *et al.,* 1996) and embryo sac development is blocked at the FG1 stage
(Schneitz *et al.,* 1997).

Aloni, R. (1987). Differentiation of vascular tissues. Annual Review of Plant Physiology and Plant Molecular Biology 38, 179-204.

Aloni R, Langhans M, Aloni E, Dreieicher E, Ullrich CI. (2005). Root-synthesized cytokinin in Arabidopsis is distributed in the shoot by the transpiration stream. J Exp Bot. 56:1535-1544.

Alvarez-Buylla ER, Pelaz S, Liljegren SJ, Gold SE, Burgeff C, Ditta GS, Ribas de Pouplana L, Martínez-Castilla L, Yanofsky MF. (2000). An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. Proc Natl Acad Sci U S A. May 9;97(10):5328-33

Aso K, Kato M, Banks JA, Hasebe M. (1999). Characterization of homeodomain-leucine zipper genes in the fern Ceratopteris richardii and the evolution of the homeodomain-leucine zipper gene family in vascular plants. Mol Biol Evol. 16:544-452.

Astot, C., Dolezal, K., Nordstrom, A., Wang, Q., Kunkel, T., Moritz, T., Chua, N. H., and Sandberg, G. (2000). An alternative cytokinin biosynthesis pathway. Proc Natl Acad Sci U S A 97, 14778-14783.

Aukermann MJ, Sakai H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes;15(11):2730-41. 16:555.

Battaglia R, Brambilla V, Colombo L, Stuitje AR, Kater MM. (2006). Functional analysis of MADS-box genes controlling ovule development in Arabidopsis using the ethanol-inducible alc gene-expression system. Mech Dev. 123:267-276.

Becker A, Theissen G. (2003). The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol. 29: 464-489.

Bencivenga S., Simonini S., Benkova E. and Colombo L. (2012). The Transcription Factors BEL1 and SPL are Required for Cytokinin and Auxin Signaling During Ovule Development in Arabidopsis. The Plant Cell 24:2886-2897.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115, 591-602.

Bertolino E, Reimund B, Wildt-Perinic D, Clerc RG. (1995). A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. J Biol Chem. 270:31178-31188.

Bharathan G, Janssen BJ, Kellogg EA, Sinha N. (1997). Did homeodomain proteins duplicate before the origin of angiosperms, fungi, and metazoa? Proc Natl Acad Sci U S A. 94: 13749-13753.

Bowman JL, Smyth DR, Meyerowitz EM. (1989). Genes directing flower development in Arabidopsis. Plant Cell 1:37-52.

Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S., Kater, M. M., and Colombo, L. (2007). Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in Arabidopsis. Plant Cell 19, 2544-2556.

Bürglin TR, Cassata G. (2002) Loss and gain of domains during evolution of cut superclass homeobox genes. Int J Dev Biol.46:115-123.

Bürglin TR. (1994). A Caenorhabditis elegans prospero homologue defines a novel domain. Trends Biochem Sci. 19:70-71.

Bürglin TR. (1997). Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. Nucleic Acids Res. 25: 4173-4180.

Bürglin TR. (1998). The PBC domain contains a MEINOX domain: coevolution of Hox and TALE homeobox genes? Dev Genes Evol. 208:113-116.

Cheng, Y., Dai, X., and Zhao, Y. (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes Dev 20, 1790-1799.

Christensen, C. A., King, E. J., Jordan, J. R., and Drews, G. N. (1997). Megagametogenesis in Arabidopsis wild type and the Gf mutant. Sexual Plant Reproduction 10, 49-64.

Coen ES, Romero JM, Doyle S, Elliott R, Murphy G, Carpenter R. (1990). Floricaula: homeotic gene required for flower development in Antirrhinum majus. Cell 63, 1311-1322.

Colombo, L., Battaglia, R., and Kater, M. M. (2008). Arabidopsis ovule development and its evolutionary conservation. Trends Plant Sci 13, 444-450.

D'Agostino, I. B., Deruere, J., and Kieber, J. J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol 124, 1706-1717.

de Bodt S, Raes J, Van de Peer Y, Theissen G. (2003). And then there were many: MADS goes genomic.Trends Plant Sci. 8:475-83. Review.

de Folter S, Immink RG, Kieffer M, Parenicová L, Henz SR, Weigel D, Busscher M, Kooiker M, Colombo L, Kater MM, Davies B, Angenent GC. (2005). Comprehensive interaction map of the Arabidopsis MADS Box transcription factors. Plant Cell. 17:1424-1433.

Dello Ioio, R., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and Sabatini, S. (2007). Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. Curr Biol 17, 678-682.

Derelle R, Lopez P, Le Guyader H, Manuel M. (2007). Homeodomain proteins belong to the ancestral molecular toolkit of eukaryotes. Evol Dev. 9:212-219.

Dewitte, W., and Murray, J. A. (2003). The plant cell cycle. Annu Rev Plant Biol 54, 235-264.

Dewitte, W., Riou-Khamlichi, C., Scofield, S., Healy, J. M., Jacqmard, A., Kilby, N. J., and Murray, J. A. (2003). Altered cell cycle distribution, hyperplasia, and inhibited differentiation in Arabidopsis caused by the D-type cyclin CYCD3. Plant Cell 15, 79-92.

Dewitte, W., Scofield, S., Alcasabas, A. A., Maughan, S. C., Menges, M., Braun, N., Collins, C., Nieuwland, J., Prinsen, E., Sundaresan, V., and Murray, J. A. (2007). Arabidopsis CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc Natl Acad Sci U S A 104, 14537-14542.

Elliott, R. C., Betzner, A. S., Huttner, E., Oakes, M. P., Tucker, W. Q., Gerentes, D., Perez, P., and Smyth, D. R. (1996). AINTEGUMENTA, an APETALA2-like gene of Arabidopsis with pleiotropic roles in ovule development and floral organ growth. Plant Cell 8, 155-168.

Ellis CM, Nagpal P, Young JC, Hagen G, Guilfoyle TJ, Reed JW. (2005). AUXIN RESPONSE FACTOR1 and AUXIN RESPONSE FACTOR2 regulate senescence and floral organ abscission in Arabidopsis thaliana. Development. 132:4563-4574.

Espinás ML, Jiménez-García E, Vaquero A, Canudas S, Bernués J, Azorín F. (1999). The N-terminal POZ domain of GAGA mediates the formation of oligomers that bind DNA with high affinity and specificity. J Biol Chem. 274: 16461-16469.

Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M. F., Kater, M. M., and Colombo, L. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell 15, 2603-2611.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature 426, 147-153.

Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science 282, 2226-2230.

Gehring WJ, Affolter M, Bürglin T. (1994). Homeodomain proteins. Annu Rev Biochem. ;63:487-526.

Gordon SP, Chickarmane VS, Ohno C, Meyerowitz EM. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsisshoot meristem. Proc Natl Acad Sci U S A. 106:16529-16534.

Gordon, S. P., Heisler, M. G., Reddy, G. V., Ohno, C., Das, P., and Meyerowitz, E. M. (2007). Pattern formation during de novo assembly of the Arabidopsis shoot meristem. Development 134, 3539-3548.

Gregis V, Sessa A, Colombo L, Kater MM. (2006). AGL24, SHORT VEGETATIVE PHASE, and APETALA1 redundantly control AGAMOUS during early stages of flower development in Arabidopsis. Plant Cell, 18, 1373-1382.

Gregis V, Sessa A, Colombo L, Kater MM. (2008). AGAMOUS-LIKE24 and SHORT VEGETATIVE PHASE determine floral meristem identity in Arabidopsis. Plant J. Dec;56(6):891-902.

Guilfoyle TJ, Hagen G. Auxin response factors. Curr Opin Plant Biol. 10:453-460.

Haecker A, Gross-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T. (2004). Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in Arabidopsis thaliana. Development. 131:657-668.

Hardtke CS, Berleth T. (1998). The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. EMBO J. 17:1405-1411.

Hardtke CS, Ckurshumova W, Vidaurre DP, Singh SA, Stamatiou G, Tiwari SB, Hagen G, Guilfoyle TJ, Berleth T. (2004). Overlapping and non-redundant functions of the Arabidopsis auxin response factors MONOPTEROS and NONPHOTOTROPIC HYPOCOTYL 4. Development. 131:1089-1100.

Heyl, A., Ramireddy, E., Brenner, W. G., Riefler, M., Allemeersch, J., and Schmulling, T. (2008). The transcriptional repressor ARR1-SRDX suppresses pleiotropic cytokinin activities in Arabidopsis. Plant Physiol 147, 1380-1395.

Honma T, Goto K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature 409, 525-529.

Huang H, Mizukami Y, Hu Y, Ma H. (1993). Isolation and characterization of the binding sequences for the product of the Arabidopsis floral homeotic gene AGAMOUS. Nucleic Acids Res., 21, 4769-4776.

Kakimoto, T. (2001). Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. Plant Cell Physiol 42, 677-685.

Katsani KR, Hajibagheri MA, Verrijzer CP. (1999). Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology. EMBO J. 18:698-708.

Kooiker M., Airoldi C.A., losa A., Finzi L., Kater M.M. and Colombo L. (2005). BASIC PENTACYSTEINE1 a GA-binding protein that induces conformational changes in the regulatory region of the homeotic Arabidopsis gene SEEDSTICK. The Plant Cell 17:722-729.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M. B., *et al.* (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell 19, 3889-3900.

Laskowski, M. J., Williams, M. E., Nusbaum, H. C., and Sussex, I. M. (1995). Formation of lateral root meristems is a two-stage process. Development 121, 3303-3310.

Mahonen, A. P., Bishopp, A., Higuchi, M., Nieminen, K. M., Kinoshita, K., Tormakangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y. (2006). Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science 311, 94-98.

Mahonen, A. P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P. N., and Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. Genes Dev 14, 2938-2943.

Masiero S, Imbriano C, Ravasio F, Favaro R, Pelucchi N, Gorla MS, Mantovani R, Colombo L, Kater MM. (2002). Ternary complex formation between MADS-box transcription factors and the histone fold protein NF-YB. J Biol Chem. 27:26429-26435.

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK. (1991). The Viviparous-1 developmental gene of maize encodes a novel transcriptional activator. Cell. 66:895-905.

Meijer AH, Scarpella E, van Dijk EL, Qin L, Taal AJ, Rueb S, Harrington SE, McCouch SR, Schilperoort RA, Hoge JH. (1997). Transcriptional repression by Oshox1, a novel homeodomain leucine zipper protein from rice. Plant J. 11:263-276.

Meister R.J., Williamsa L.A., Monfared M.M., Gallagher T.L., Kraft E.A., Nelson C.G. and Gasser C.S. (2004). Definition and interaction of a positive regulatory element of the Arabidopsis INNER NO OUTER promoter. The Plant Journal 37: 426-438.

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J 37, 128-138.

Mizukami, Y., and Fischer, R. L. (2000). Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. Proc Natl Acad Sci U S A 97, 942-947.

Mok M C (1994) Cytokinins: Chemistry, Activity and Function, eds Mok D W S, Mok M C (CRC, London), pp 155–166.

Mravec, J., Skupa, P., Bailly, A., Hoyerova, K., Krecek, P., Bielach, A., Petrasek, J., Zhang, J., Gaykova, V., Stierhof, Y. D., *et al.* (2009). Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. Nature.

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, Ecker JR, Reed JW. (2005). Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development. 132:4107-4118.

Nordstrom, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Astot, C., Dolezal, K., and Sandberg, G. (2004). Auxin regulation of cytokinin biosynthesis in Arabidopsis thaliana: a factor of potential importance for auxin-cytokinin-regulated development. Proc Natl Acad Sci U S A 101, 8039-8044.

Okada, K., Ueda, J., Komaki, M. K., Bell, C. J., and Shimura, Y. (1991). Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. Plant Cell 3, 677-684.

Okamuro JK, den Boer BG, Lotys-Prass C, Szeto W, Jofuku KD. (1996). Flowers into shoots: photo and hormonal control of a meristem identity switch in Arabidopsis. Proc Natl Acad Sci U S A. Nov 26;93(24):13831-6.

Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologis A. (2005). Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. Plant Cell. 17:444-463.

Orphanides G., LeRoy G., Chang C.H., Luse D.S. and Reinberg D. (1998). FACT, a factor that facilitates transcript elongation through nucleosomes. Cell 92: 105-116.

Parenicová L, de Folter S, Kieffer M, Horner DS, Favalli C, Busscher J, Cook HE, Ingram RM, Kater MM, Davies B, Angenent GC, Colombo L. (2003). Molecular and Phylogenetic Analyses of the Complete MADS-Box Transcription Factor Family in Arabidopsis New Openings to the MADS World.Plant Cell. 15(7):1538-51.

Pekker I, Alvarez JP, Eshed Y. (2005). Auxin response factors mediate Arabidopsis organ asymmetry via modulation of KANADI activity. Plant Cell. 17:2899-2910.

Pernisova, M., Klima, P., Horak, J., Valkova, M., Malbeck, J., Soucek, P., Reichman, P., Hoyerova, K., Dubova, J., Friml, J., *et al.* (2009). Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc Natl Acad Sci U S A.

Petrasek, J., and Friml, J. (2009). Auxin transport routes in plant development. Development 136, 2675-2688.

Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., *et al.* (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312, 914-918.

Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E., and Yanofsky, M. F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424, 85-88.

Platero JS, Csink AK, Quintanilla A, Henikoff S. (1998). Changes in chromosomal localization of heterochromatin-binding proteins during the cell cycle in Drosophila. J Cell Biol. 140:1297-1306.

Reinhardt, D., Mandel, T., and Kuhlemeier, C. (2000) Auxin regulates the initiation and radial position of plant lateral organs.

Reinhardt, D., Pesce, E. R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. Nature 426, 255-260.

Reiser, L., and Fischer, R. L. (1993). The Ovule and the Embryo Sac. Plant Cell 5, 1291-1301.

Riechmann JL, Meyerowitz EM. (1997). MADS domain proteins in plant development. Biol Chem.;378(10):1079-101. Review.

Robinson-Beers, K., Pruitt, R. E., and Gasser, C. S. (1992). Ovule Development in Wild-Type Arabidopsis and Two Female-Sterile Mutants. Plant Cell 4, 1237-1249.

Ruberti I, Sessa G, Lucchetti S, Morelli G. (1991). A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. EMBO J. 10:1787-1791.

Ruzicka, K., Simaskova, M., Duclercq, J., Petrasek, J., Zazimalova, E., Simon, S., Friml, J., Van Montagu, M. C., and Benkova, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc Natl Acad Sci U S A 106, 4284-4289.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., and Scheres, B. (1999). An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell 99, 463-472.

Sakakibara K, Nishiyama T, Kato M, Hasebe M. (2001). Isolation of homeodomain-leucine zipper genes from the moss Physcomitrella patens and the evolution of homeodomain-leucine zipper genes in land plants. Mol Biol Evol. 18:491-502.

Sangwan I. and O'Brian M.R. (2002). Identification of a soybean protein that interacts with GAGA element dinucleotide repeat DNA. Plant Physiol. 129: 1788-1794.

Santi L., Wang Y., Stile M.R., Berendzen K., Wanke D., Roig C., Pozzi C., Müller K., Müller J., Rohde W. and Salamini F. (2003). The GA octodinucleotide repeat binding factor BBR participates in the transcriptional regulation of the homeobox gene Bkn3. Plant J. 34: 813-826.

Scarpella, E., Marcos, D., Friml, J., and Berleth, T. (2006). Control of leaf vascular patterning by polar auxin transport. Genes Dev 20, 1015-1027.

Schneitz, K., Hulskamp, M., and Pruitt, R. E. (1995). Wild-Type Ovule Development in Arabidopsis-Thaliana - a Light-Microscope Study of Cleared Whole-Mount Tissue. Plant Journal 7, 731-749.

Schneitz, K., Hulskamp, M., Kopczak, S. D., and Pruitt, R. E. (1997). Dissection of sexual organ ontogenesis: a genetic analysis of ovule development in Arabidopsis thaliana. Development 124, 1367-1376.

Schruff, M. C., Spielman, M., Tiwari, S., Adams, S., Fenby, N., and Scott, R. J. (2006). The AUXIN RESPONSE FACTOR 2 gene of Arabidopsis links auxin signalling, cell division, and the size of seeds and other organs. Development 133, 251-261.

Schwarz-Sommer Z, Hue I, Huijser P, Flor P J, Hansen R, Tetens F, Lönning W-E, Saedler H, Sommer H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens : Evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J. 11, 251-263.

Schwarz-Sommer Z, Huijser P, Nacken W., Saedler H., Sommer H. (1990). Genetic control of flower development by homeotic genes in Antirrhinum majus. Science 250, 931-936.

Sessions A, Nemhauser JL, McColl A, Roe JL, Feldmann KA, Zambryski PC. (1997). ETTIN patterns the Arabidopsis floral meristem and reproductive organs. Development. 124:4481-4491.

Shani, E., Yanai, O., and Ori, N. (2006). The role of hormones in shoot apical meristem function. Curr Opin Plant Biol 9, 484-489.

Shiraishi H, Okada K, Shimura Y. (1993). Nucleotide sequences recognized by the AGAMOUS MADS domain of Arabidopsis thaliana in vitro. Plant J. Aug;4(2):385-98.

Simonini S., Roig-Villanova I., Gregis V., Colombo B., Colombo L. and Kater M.M. (2012) BPC proteins mediate MADS-domain complex binding to the DNA for tissue specific expression of target genes in Arabidopsis thaliana. The Plant Cell. IN PRESS.

Swaminathan K, Peterson K, Jack T. (2008). The plant B3 superfamily. Trends Plant Sci. 13:647-55.

Takei, K., Sakakibara, H., and Sugiyama, T. (2001). Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. J Biol Chem 276, 26405-26410.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., and Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. Plant J 45, 1028-1036.

Theissen G, Saedler H. (2001). Plant biology. Floral quartets. Nature. 409:469-471.

To, J. P., and Kieber, J. J. (2008). Cytokinin signaling: two-components and more. Trends Plant Sci 13, 85-92.

Ulmasov, T., Hagen, G., and Guilfoyle, T. J. (1997a). ARF1, a transcription factor that binds to auxin response elements. Science 276, 1865-1868.

Ulmasov, T., Hagen, G., and Guilfoyle, T. J. (1997a). ARF1, a transcription factor that binds to auxin response elements. Science 276, 1865-1868.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T. J. (1997b). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9, 1963-1971.

Weijers, D., and Jurgens, G. (2004). Funneling auxin action: specificity in signal transduction. Curr Opin Plant Biol 7, 687-693.

Weijers, D., Sauer, M., Meurette, O., Friml, J., Ljung, K., Sandberg, G., Hooykaas, P., and Offringa, R. (2005). Maintenance of embryonic auxin distribution for apical-basal patterning by PIN-FORMED-dependent auxin transport in Arabidopsis. Plant Cell 17, 2517-2526.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 2532-2550.

Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. Nature 346, 35-39.

Zhao, Y., Christensen, S. K., Fankhauser, C., Cashman, J. R., Cohen, J. D., Weigel, D., and Chory, J. (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science 291, 306-309.

CHAPTER 2

BPC proteins mediate MADS-domain complex binding to the DNA for tissue specific expression of target genes in *Arabidopsis thaliana*

Sara Simonini[1], Irma Roig-Villanova[1], Veronica Gregis[1], Bilitis Colombo[1], Lucia Colombo[1,2] and Martin M. Kater[1]

[1] Dipartimento di BioScienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy
[2] CNR, Istituto di Biofisica, Università di Milano, Via Celoria 26, 20133 Milano, Italy

Plant Cell 2012, 9 October

ABSTRACT

BASIC PENTACYSTEINE (BPC) transcription factors have been identified in a large variety of plant species. In *Arabidopsis thaliana* there are seven BPC genes, which, except for BPC5, are expressed ubiquitously. BPC genes are functionally redundant in a wide range of developmental processes. Recently, we reported that BPC1 binds to guanine and adenine (GA)– rich consensus sequences in the *SEEDSTICK* (*STK*) promoter in vitro and induces conformational changes. Here we show by chromatin immunoprecipitation experiments that *in vivo* BPCs also bind to the consensus boxes, and when these were mutated, expression from the *STK* promoter was derepressed, resulting in ectopic expression in the inflorescence. We also reveal that SHORT VEGETATIVE PHASE (SVP) is a direct regulator of *STK*. SVP is a floral meristem identity gene belonging to the MADS box gene family. The SVP-APETALA1 (AP1) dimer recruits the SEUSS (SEU)-LEUNIG (LUG) transcriptional cosuppressor to repress floral homeotic gene expression in the floral meristem. Interestingly, we found that GA consensus sequences in the *STK* promoter to which BPCs bind are essential for recruitment of the corepressor complex to this promoter. Our data suggest that we have identified a new regulatory mechanism controlling plant gene expression that is probably generally used, when considering BPCs' wide expression profile and the frequent presence of consensus binding sites in plant promoters.

INTRODUCTION

Transcriptional regulation is still poorly understood in plants. Recently, a new class of transcription factors, named BASIC PENTACYSTEINE (BPC), was identified (Meister *et al.*, 2004). In *Arabidopsis thaliana*, BPCs belong to a small gene family of seven members that encode activators and repressors of transcription (Meister *et al.*, 2004; Monfared *et al.*, 2011; Berger and Dubreucq, 2012). Based on their sequence similarity, they were divided into three classes: class I (containing BPC1 to BPC3), class II (containing BPC4 to BPC6), and class III (containing only BPC7) (Meister *et al.*, 2004). All genes, except for BPC5, which is probably a pseudogene, are expressed ubiquitously. BPC genes belonging to these different classes were shown to be functionally redundant, and combining multiple bpc mutant alleles together results in a wide range of developmental defects (Monfared *et al.*, 2011). Recently, we identified BPC1 as a regulator of the ovule identity gene *SEEDSTICK* (*STK*), which is specifically expressed in ovules (Rounsley *et al.*, 1995; Pinyopich *et al.*, 2003; Brambilla *et al.*, 2007). BPC1 binds to the *STK* promoter at multiple guanine and adenine (GA)–rich boxes, which have the RGARAGRRA consensus sequence (Kooiker *et al.*, 2005). Its cooperative binding was shown to induce conformational changes in the *STK* regulatory region, suggesting that multiple consensus sites are required for the regulation of *STK*. Furthermore, it was shown that *STK* expression was upregulated in the *bpc1-1* single mutant, although the spatial expression profile of *STK* was not changed (Kooiker *et al.*, 2005). Here we describe the identification of the MADS domain factor SHORT VEGETATIVE PHASE (SVP) as another regulator of *STK*. This transcription factor was first identified as a repressor of the floral transition (Hartmann *et al.*, 2000). By combining the strong *ap1-10* allele with the *agamous*-

like24 (*agl24*) *svp* double mutant, it was shown that, after the floral transition, SVP also acts as a floral meristem identity gene, because in this triple mutant, floral meristem identity was not specified, and inflorescence meristems developed in place of floral meristems, resulting in a phenotype that resembles a cauliflower curd-like structure (Gregis *et al.*, 2008). During reproductive growth, SVP is expressed only in the floral meristem (stage 1 and 2 of flower development), which during this phase increases in size. In the floral meristem, SVP interacts with AP1 to recruit the LEUNIG (LUG)-SEUSS (SEU) corepressor (Gregis *et al.*, 2006, 2009). This complex is important to repress homeotic gene expression during the first stages of flower development to prevent precocious differentiation of the floral meristem into floral organs. For instance, it was shown that SVP and AP1 directly bind and repress MADS box floral organ identity genes, such as *APETALA3* (*AP3*), *PISTILLATA* (*PI*), *SEPALLATA3* (*SEP3*), and *AGAMOUS* (*AG*) (Gregis *et al.*, 2006; Gregis *et al.*, 2009). Here we show that the regulation of *STK* is dependent on the binding of a MADS domain protein–containing repressor complex to its promoter and that BPC binding sites are essential for the recruitment of this complex. We provide evidence that this is probably a general mechanism by which BPCs regulate gene expression in plants.

RESULTS

Class I BPC proteins bind the *STK* promoter *in vivo*

Electrophoretic mobility shift assay (EMSA) experiments using primers based on the *STK* promoter sequence predicted that BPC1 binds to seven out of 12 GAGA boxes (Kooiker *et al.*, 2005). Based on these experiments, the RGARAGRRA consensus BPC1 binding sequence (consensus [C]-box) was proposed. The seven BPC1 binding sites (C-boxes) were numbered as 1, 4, 7, 8, 9, 10, and 12, based on their order in the *STK* promoter sequence (Figure 1A). In particular, C-boxes 4 and 12 showed the strongest binding. Previous analysis of the *STK* promoter sequence showed that apart from the seven C-boxes, there are five nonconsensus (NC) boxes (NC-boxes 2, 3, 5, 6, and 11), which have one mismatch with respect to the consensus sequence. These NC-boxes did not bind

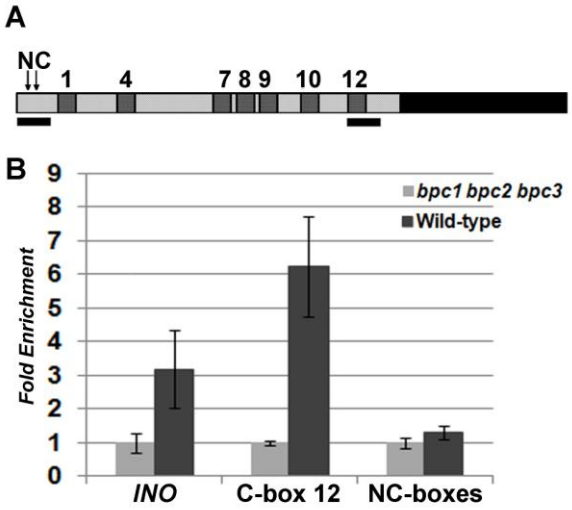


FIGURE 1. BPC proteins of class I bind the *STK* promoter at C-boxes.

(A) Schematic representation of *STK* promoter indicating the regions tested by ChIP (labeled bars). The two arrows indicate the position of the two NC boxes tested.

(B) ChIP assays conducted on wild-type (dark gray bars) chromatin compared with the *bpc1 bpc2 bpc3* triple mutant (light gray bars) and analyzed by Real-time PCR testing the regions indicated in A, and a region of the *INO* promoter.

BPC1 in EMSA studies (Kooiker *et al.*, 2005). We carefully reanalyzed the *STK* promoter region and discovered the presence of five additional NC-boxes (see Supplemental Figure 1 online). To verify the ability of class I BPC proteins (BPC1, BPC2, and BPC3) to bind C- and NC-boxes in the *STK* promoter *in vivo*, three independent biological replicates of chromatin immunoprecipitation (ChIP) assays were performed using a polyclonal antibody that specifically recognizes class I BPC proteins (see Supplemental Figure 2 online). These experiments were conducted using chromatin extracted from wild-type inflorescences. Two different regions of the *STK* promoter of 250 bp were analyzed (Figure 1A), allowing us to distinguish between C-box and NC-box binding. One region was at the beginning of the promoter, containing two NC-boxes, and the other region was at the end of the promoter and contained only C-box 12, which showed strong binding in the EMSA experiments.

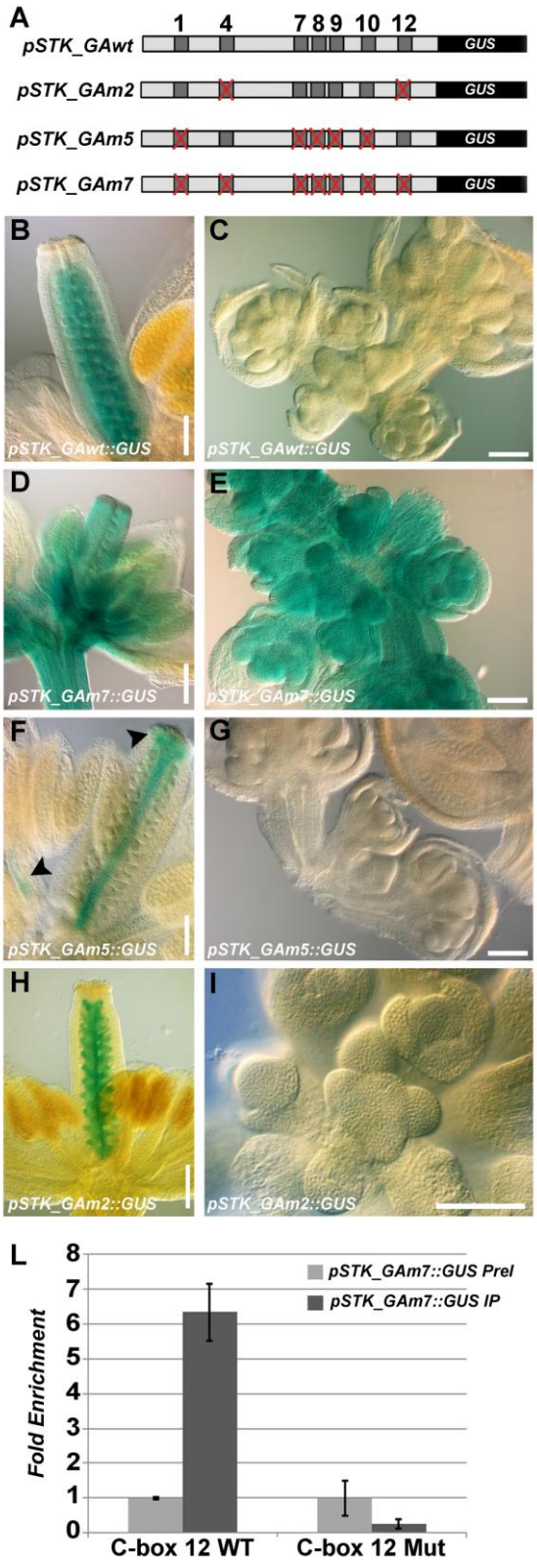
As a positive control, binding to the promoter of *INNER NO OUTER* (*INO*) was tested (Meister *et al.*, 2004), and as a negative control, the *bpc1-3 bpc2 bpc3* triple mutant was used (Figure 1B).

ChIP analysis on the region containing C-box 12 showed high enrichment, confirming strong binding to this consensus site. When we tested the NC-boxes for BPC binding, we did not observe any enrichment, confirming the EMSA experiment and showing that the NC-boxes do not bind BPC1, BPC2, and BPC3 *in vivo*.

The C-boxes are necessary for correct spatial and temporal regulation of *STK* expression

Recently, functional analysis of different classes of BPC genes in *Arabidopsis* showed that they have redundant functions in plant developmental processes (Monfared *et al.*, 2011). Analysis of *STK* expression in the bpc1-1 mutant confirmed this, because *STK* was only slightly upregulated, but its expression profile was not affected (Kooiker *et al.*, 2005). Therefore, considering redundancy between BPC factors and to avoid pleiotropic effects of higher-order *bpc* mutant combinations, we decided to investigate the role of BPC proteins in the regulation of *STK* by mutating the seven C-boxes in the *STK* promoter by changing in each box two purines essential for BPC1 binding into pyrimidine residues. This mutated promoter fragment of 2900 bp upstream of the *STK* translation start site was cloned in frame with the uidA reporter gene that encodes *b-glucuronidase* (*GUS*), and the resultant *STKpro_GAm7:GUS* construct (Figure 2A) was used for *Arabidopsis* transformation. As a positive control, we used the wild-type *STK* promoter (*STKpro_GAwt:GUS*; Figure 2A), which drives specific expression in the placenta and all stages of ovule development (Figure 2B; Pinyopich *et al.*, 2003). Of the 28 independent transgenic lines that were transformed with the wild-type promoter construct, 89% showed the expected wild-type *STK* expression profile (Figures 2B and 2C), whereas in 11% of the lines, an aberrant profile was observed (see Supplemental Table 1 online). In these lines, expression was random in all parts of the plant. Those lines in which no GUS signal was detected (n = 3) were not considered for further analysis. Of the 125 independent lines transformed with the *STKpro_GAm7:GUS* construct, 94 plants (75%) showed strong deregulation of the GUS reporter gene. Strong blue staining was observed not only in all the floral organs but also in inflorescence and floral

meristems (Figures 2D and 2E; see Supplemental Table 1 online), suggesting a pivotal role for the C-boxes in the regulation of spatial and temporal *STK* expression. Kooiker *et al.* (2005) showed by tethered particle motion (TPM; Finzi and Dunlap, 2003) analysis that BPC1 is able to induce loops in the *STK* promoter and that prominent loop formation occurs between C-boxes 4 and 12, which were the strongest BPC1 binding sites in the EMSA assays. However, TPM experiments using a *STK* promoter fragment in which only C-boxes 4 and 12 were maintained and the other five were mutated showed that no conformational changes were introduced, suggesting that Cboxes 4 and 12 alone are not enough to induce looping. To investigate in more detail the importance of the C-boxes for *STK* regulation taking into consideration the previously performed TPM analysis, a *STK* promoter-GUS construct was prepared in which only C-boxes 4 and 12 were maintained and all five other C-boxes were mutated (*STKpro_GAm5:GUS*; Figure 2A). This *STKpro_GAm5:GUS* construct was introduced in *Arabidopsis*, and of the 71 independent transgenic lines that were analyzed, 27 plants (38%) showed deregulation of the GUS reporter (Figures 2F and 2G; see Supplemental Table 1 online), suggesting that C-boxes 4 and 12 are important for proper *STK* expression and that the mutation of five C-boxes led to more instability in the regulation of the expression of the reporter gene. It is important to notice that deregulation in these lines was observed only in the anthers and the style and stigma of the carpel (Figure 2F). The reporter line did not show expression in the inflorescence or floral meristem (Figure 2G). To answer the question of whether C-boxes 4 and 12 are essential for proper *STK* expression, a promoter construct was generated in which only these two C-boxes were mutated (*STKpro_GAm2:GUS*; Figure 2A). Of the 57 independent transformants that were analyzed, only 14% showed deregulation of the GUS reporter gene (Figures 2H and 2I; see Supplemental Table 1 online), indicating that C-boxes 4 and 12 seem not to be essential for proper *STK* expression. These results showed that multiple C-boxes are important for the proper regulation of *STK* by BCP proteins. To ensure that the mutations that were introduced in the C-boxes prevented BPC binding *in vivo*, three independent ChIP assays using BPC class I–specific antibodies were performed. The experiments were conducted using inflorescences of homozygous *STKpro_GAm7:GUS* lines. The wild-type endogenous C-box 12 was tested as a positive target sequence, and as a negative control, the preimmune serum was used. To discriminate between the endogenous wild-type C-box 12 and the mutated one located on the *STKpro_GAm7:GUS* construct, specific primers that differed in two 39 nucleotides were used. In all three ChIP experiments, no enrichment was detected when binding to the mutated C-box 12 was tested (Figure 2J), whereas the endogenous C-box 12 was highly enriched. This result confirmed the efficacy of the mutations to abolish BPC binding *in vivo* and is consistent with the previous in vitro analysis (Kooiker *et al.*, 2005). Furthermore, it supports the observation that deregulation of the *STK* promoter activity is caused by the absence of BPC binding to the mutated C-boxes.



Class I BPC proteins interact with each other

BPC proteins, at least the ones of class I, were shown to bind in vitro and *in vivo* to multiple C-boxes in the *STK* regulatory region. Furthermore, Kooiker *et al.* (2005) showed by TPM analysis that they induce conformational changes in the DNA by inducing loops between the different C-boxes. To investigate whether loops can be induced into the DNA through both the binding to the C-boxes and by direct interactions between these class I BPC proteins, we performed yeast two-hybrid assays. The open reading frames encoding BPC1, BPC2, and BPC3 were fused to the activation domain (AD) and binding domain (BD) and tested for interaction (Table 1). These assays showed that BPC1 interacted with BPC2 and BPC3; BPC2 interacted with BPC3 and also formed homodimers (Figure 3A). These data show that BPC proteins potentially can loop the DNA by binding to C-boxes and interacting with each other (Figure 3B).

FIGURE 2. **Mutation in C-boxes avoids BPC of class I binding to *STK* promoter**.
(A) Schematic representation of the *STK* promoter versions generated: dark grey squares represent C-boxes wild-type and mutated (crossed).
(B-I) GUS staining performed on inflorescence (left panels mature flowers, right panels meristems and young flowers) of *pSTK_GAwt::GUS* **(B-C)**, *pSTK_GAm7::GUS* **(D-E)**, *pSTK_GAm5::GUS* **(F-G)**, *pSTK_GAm2::GUS* **(H-I)**.
(L) ChIP assay conducted on wild-type chromatin and analyzed revealing that BPC of classi I don't bind the mutated C-box 12 in *pSTK:GAm7::GUS* background.
PI: Pre-immune serum; IP: immunoprecipitate.

Table 1. Class I BPC Interactions in Yeast

Plasmid	pGBKT7-BPC1	pGBKT7-BPC2	pGBKT7-BPC3	pGBKT7 empty
pGADT7-BPC1	–	++	++	–
pGADT7-BPC2	++	++	++	–
pGADT7-BPC3[a]	++	++	++	++
pGADT7 empty	–	–	–	–

– indicates no interaction; ++ indicates positive strong interaction.
[a]BPC3 autoactivates as AD fusion.

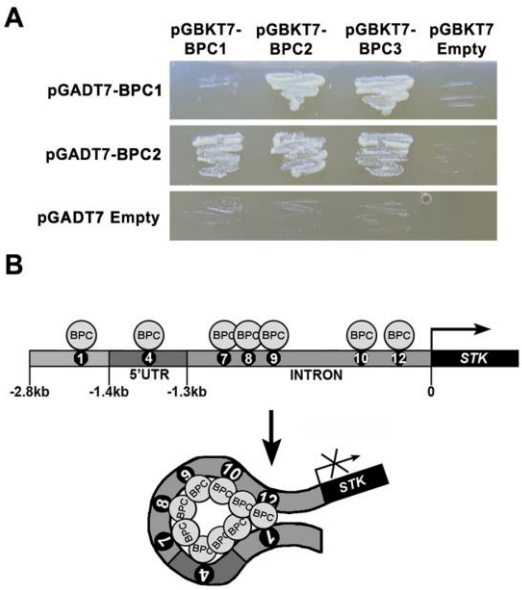


Figure 3. BPC Proteins of Class I Interact with Each Other.
(A) Yeast two-hybrid assays testing interactions between BPC proteins of class I using –W-L-H +5 mM of 3-AT selective media. The empty vectors were used as negative controls.
(B) Putative model of BPC interactions with the STK promoter: BPCs interact with each other and bind the STK promoter at multiple C-boxes; as a consequence, this might induce conformational changes in this region. UTR, untranslated region.

STK is a target of the MADS-domain transcription factor SVP

We recently performed a ChIP-sequencing assay to identify genome-wide targets for the floral meristem identity factor SVP, which resulted in the identification of *STK* (V. Gregis *et al.*, unpublished results). This experiment showed that the MADS domain transcription factor SVP binds to various positions in the *STK* genomic region (Figure 4A). The direct interaction between SVP and the *STK* promoter was confirmed by three ChIP assays (independent biological replicates) using antibodies against green fluorescent protein (GFP) and chromatin extracted from inflorescences of svp homozygous mutant plants complemented with a *SVPpro:SVP-GFP* construct (Gregis *et al.*, 2009). As a positive control, the enrichment of a region of the *AP1* promoter known to interact with SVP was used (Grandi *et al.*, 2011), whereas, as a negative control, inflorescences of wild-type plants were used. The *STK* promoter was divided into three parts (regions A, B, and C; Figure 4A) spanning all putative SVP promoter binding sites. This ChIP analysis revealed that the highest enrichment in all three replicates was in region B (Figures 4A and 4B), which contains three CArG boxes (MADS domain binding sites) surrounded by several C-boxes (Figure 4A; see Supplemental Figure 1 online). *STK* is closely related to *AG*, a gene regulating ovule, stamen, and carpel identity and floral meristem determinacy (Bowman *et al.*, 1991). Recently, we showed that in the *agl24 svp ap1-12* triple mutant, *AG* is ectopically expressed in floral meristems of stage 1 and 2

(Gregis *et al.*, 2008), evidencing the redundant role of these three genes in the repression of *AG* at these very early stages of flower development.

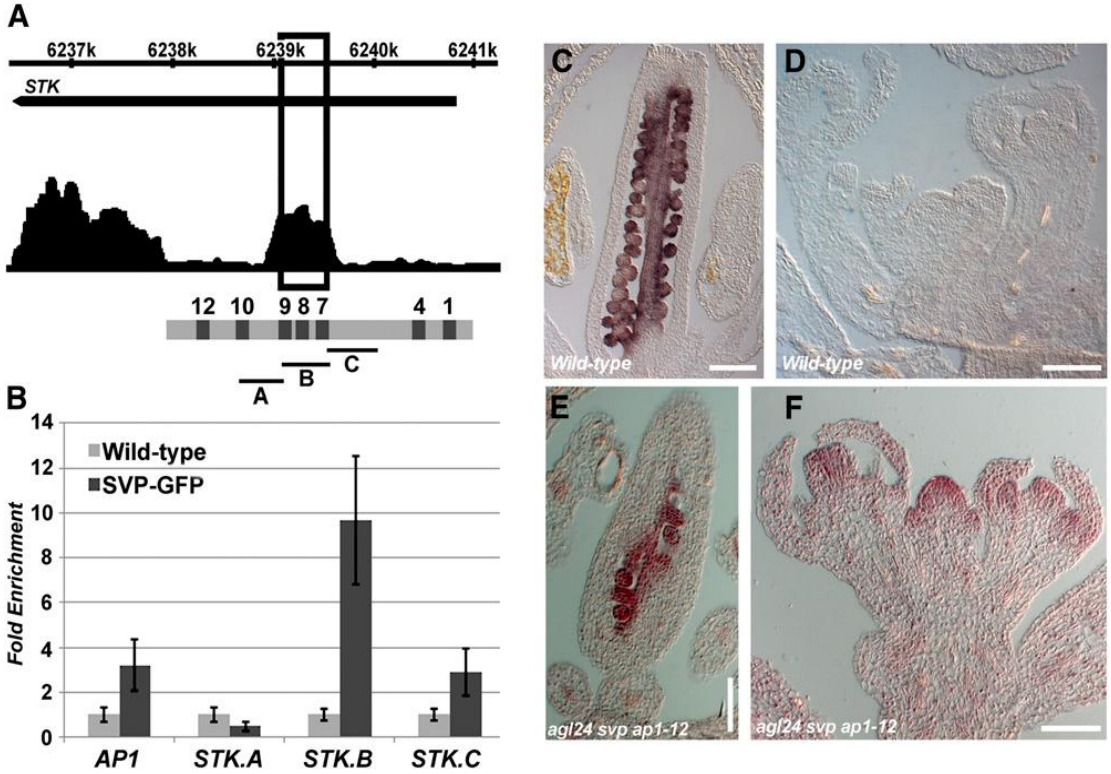


FIGURE 4. *STK* is target of SVP.

(A) Schematic representation of the fragments belonging to the *STK* genomic locus immunoprecipitated through the ChIP sequencing. Each bold line corresponds to a fragment. The *STK* promoter contains few putative SVP binding site localized close to the GAGA boxes 7,8 and 9.

(B) Real-time PCR on *pSVP::SVP-GFP svp/svp* and wild-type chromatin to confirm the SVP binding to the *STK* regions A, B and C and *AP1* promoter used as positive control.

(C-F) *In situ* hybridization on wild-type **(C-D)** and *svp agl24 ap1-12* triple mutant **(E-F)** using *STK* specific antisense probe.

Bars in C and E: 200µm; Bars in D and F: 100µm.

To investigate whether the binding of SVP to *STK* is, as previously observed for *AG*, important for the repression of *STK* in the floral meristem, we analyzed the expression of this gene in the *agl24 svp ap1-12* triple mutant by in situ hybridization. In wild-type plants, *STK* is expressed in ovules and the placenta (Figure 4C; Pinyopich *et al.*, 2003), and no expression was detectable in floral meristems and flowers at early stages of development (Figure 4D). Interestingly, in the *agl24 svp ap1-12* triple mutant, *STK* expression in the carpel was like that in wild-type plants (Figure 4E); however, its expression was also observed in floral meristems and young flowers (Figure 4F). Taken together, these data suggest that SVP prevents STK expression in the floral meristem by direct binding to its promoter regionwas in region B (Figure 4B), which contains three MADS-domain binding sites, CArG boxes, surrounded by several C-boxes (Figure 4A).

STK is closely related to *AG*, a gene controlling ovule, stamen and carpel identity and floral meristem determinacy (Walter *et al.*, 2004).

Recently we showed that in the *agl24 svp ap1-12* triple mutant *AG* is ectopically expressed

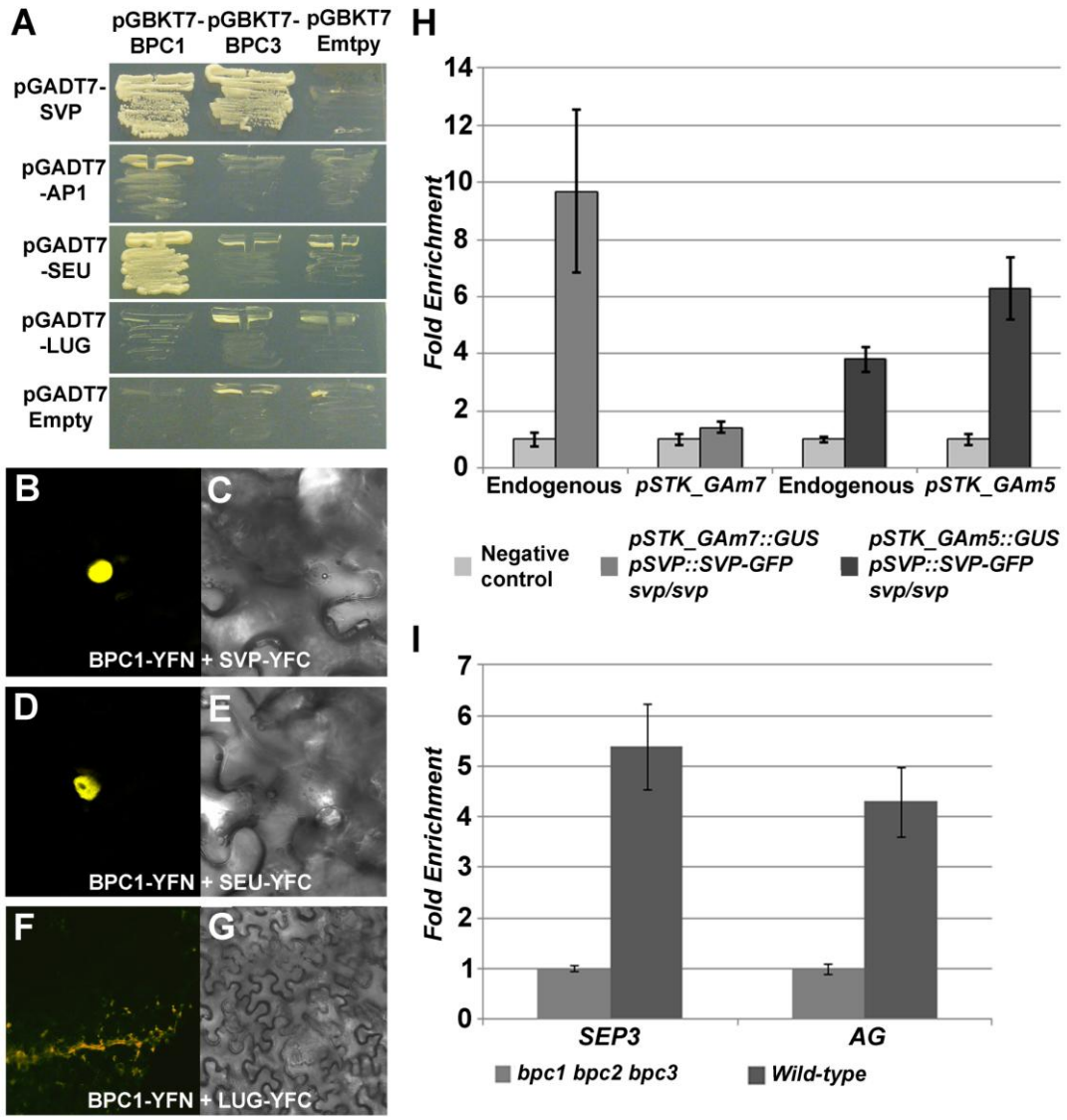


FIGURE 5. BPC1 interacts with SEU and SVP and collaborate with SVP for its DNA recognition.

(A) Yeast two hybrid assay between SEU, SVP and BPC1, BPC3: positive interactions on selective media –W-L-H +5mM 3-AT.

(B-G) Subcellular localization of reconstituted YFP complexes in plant cells. Tobacco leaves were transformed with the indicated YN and YC fusions. Panels above: yellow fluorescence, panels below: bring field images.

(H) Real-time PCR analyses of the ChIP assays to check the SVP binding to endogenous wild-type and mutated versions of fragment B of *pSTK_GAm7::GUS* and *pSTK_GAm5::GUS* promoters.

(I) Real-time PCR analyses of the ChIP assays to check the BPCs of class I binding to *AG* and *SEP3* promoters.

in floral meristems of stage 1 and 2 (Gregis *et al.*, 2008) evidencing the redundant role of these three genes in the repression of *AG* at these very early stages of flower development. To investigate whether the binding of SVP to *STK* is like previously observed for *AG* also important for the repression of *STK* in the floral meristem, we analysed the expression of this gene in the *agl24 svp ap1-12* triple mutant by *in situ* hybridization analysis. In wild-type plants, *STK* is expressed in ovules and the placenta (Figure 4C; Pinyopich *et al.*, 2003) and no expression was detectable in floral meristems and flowers at early stages of development (Figure 4D). Interestingly, in the *agl24 svp ap1-12* triple mutant, *STK* expression in the carpel was like in wild type plants (Figure 4E), however, its expression was also observed in floral meristems and young flowers (Figure 4F). Taken together these data suggest that SVP prevents *STK* expression in the floral meristem by direct binding to its promoter region.

BPC factors facilitate the binding of the AP1-SVP-SEU-LUG repressor complex to the *STK* promoter

The region of the *STK* promoter (region B) to which SVP was shown to bind contains three CArG boxes, which are very close to C-boxes 7 and 8 (Figure 4A). This peculiar arrangement of BPC and MADS domain binding sites suggests that these factors might interact to facilitate binding to this promoter region. To investigate this further, we tested by yeast two-, three-, and four-hybrid experiments whether BPC proteins were able to interact with the AP1-SVP-SEU-LUG corepressor complex (Gregis *et al.*, 2006). First, we tested by yeast two-hybrid assays the interactions between BPC1, BPC2, and BPC3, all cloned as BD fusions, with AP1, LUG, SEU, and SVP, all cloned as AD fusions. This analysis revealed that BPC1 strongly interacted with SEU and weakly with AP1 and SVP, whereas BPC3 interacted only weakly with SVP (Figure 5A). The strength of the interaction was tested by selecting for growth on medium without His and different concentrations of 3-amino-1,2,4triazole (3-AT). BPC2 did not interact with any of the proteins. The strength of the interactions was enhanced when three or four proteins were combined together in three- or four-hybrid assays, using SEU and LUG as bridging proteins and BPC1, BPC3, or SVP as BD or AD fusion proteins (see Supplemental Table 2 online). To validate in planta the results obtained by the yeast interaction experiments, a bimolecular fluorescent complementation assay (BiFC; Walter *et al.*, 2004) in tobacco (*Nicotiana benthamiana*) leaves was performed. The coding sequence of each gene was fused with a part of the yellow fluorescent protein, and then they were introduced into the cells through *Agrobacterium tumefaciens*–mediated transient transformation. As positive control, the SNF1 protein kinase was used (data not shown; Ferrando *et al.*, 2001), whereas, as negative control, BPC1 and BPC3 interactions with LUG were used, because they did not show an interaction in yeast (Figures 5F and 5G). Using this system, both the interactions between BPC1 (or BPC3, data not shown) and SVP (Figures 5B and 5C) or SEU (Figures 5D and 5E) gave a clear nuclear fluorescent signal, supporting the interactions observed in yeast and the hypothesis that a multimeric complex of BPC proteins and AP1, SVP, SEU, and LUG can be formed in plants. The protein interaction data supported the hypothesis that BPC proteins can facilitate and/or stabilize the binding of the AP1-SVP-

SEU-LUG repressor complex to the CArG boxes in the *STK* promoter. To test this hypothesis, ChIP experiments using inflorescence tissue were performed. The *svp SVPpro:SVP-GFP* plants were crossed with plants containing the *STKpro_GAm7:GUS* construct in which all seven C-boxes are mutated. In subsequent generations, plants homozygous for the svp mutation and the two constructs were selected for ChIP experiments, and antibodies against GFP were used for these assays (Figure 5H). As a positive control, the endogenous *STK* promoter (region B) was used, whereas, as negative control, inflorescences of plants containing only the *STKpro_GAm7:GUS* construct were used. Furthermore, specific primers for the mutated C-boxes 7 and 8 were used to assay specifically binding to region B encoded by the *STKpro_GAm7:GUS* construct. In three out of three independent ChIP experiments, no binding of SVP to the mutated region B was observed, suggesting that BPC binding to C-boxes is necessary for binding of SVP to the CArG boxes in region B (Figure 5H). The C-boxes and CArG boxes are very close to each other in region B (see Supplemental Figure 1 online); therefore, we could at this point not exclude the idea that the mutations in the C-boxes directly affected the affinity of SVP for the CArG binding site. To test this hypothesis, ChIP experiments were performed using inflorescences of *svp* mutant plants containing both the *SVPpro:SVP-GFP* and the *STKpro_GAm5:GUS* construct. In the latter construct, the C-boxes surrounding the SVP binding sites (C-boxes 7 and 8) are still mutated, and only the more distant boxes 4 and 12 are wild-type. As described above, these plants showed a low frequency of deregulation of GUS expression in stamens and carpels but no GUS expression in the floral meristem (Figures 2F and 2G). Interestingly, three independent ChIP experiments showed that SVP did bind to region B present in the *STKpro_GAm5:GUS* construct, confirming that the mutations that were introduced in the C-boxes close to the MADS domain binding sites did not influence the binding of SVP to this mutated *STK* promoter region. These experiments show clearly that C-box sequences to which BPC proteins bind are important to facilitate binding of the AP1-SVP-SEU-LUG repressor complex to the CArG boxes in the *STK* promoter.

BPCs regulate other floral homeotic genes

The repressor complex SVP-AP1-SEU-LUG was shown to interact with the promoters of homeotic genes regulating floral organ identity, including the two MADS box transcription factors *AGAMOUS* (*AG*) and *SEPALLATA3* (*SEP3*) (Gregis *et al.*, 2009). The SVP repressor complex prevents expression of these genes at stages 1 and 2 of flower development. Analysis of the *AG* and *SEP3* promoter regions revealed that several C-boxes are positioned within 100 bp from the CArG boxes that previously were shown to bind SVP *in vivo* (Gregis *et al.*, 2009) (see Supplemental Figure 3 online). Three independent preparations of chromatin from wild-type inflorescences and *bpc1-3 bpc2 bpc3* triple mutant inflorescences (used as negative control) were immunoprecipitated using antibodies against class I BPCs, confirming the binding of BPCs to these C-boxes (Figure 5I). These data indicate that the regulatory mechanism involving the BPC proteins is not restricted to *STK*, but rather may be a more general transcriptional regulatory mechanism common to many target genes.

STK is redundantly regulated by BPC proteins of different classes

To investigate whether *STK* regulation is dependent only on the class I BPC proteins (BPC1, BPC2, and BPC3), we introduced the *STKpro_GAwt:GUS* construct into the *bpc1-3 bpc2 bpc3* triple mutant. This analysis revealed that in none of the single,double, or triple mutant combinations GUS expression was deregulated (see Supplemental Figure 4 online). This analysis shows that in addition to the class I BPC proteins, other BPC factors act redundantly in the regulation of *STK*.

DISCUSSION

The molecular mechanisms regulating the expression of homeotic floral genes are poorly understood in plants. The homeotic gene *STK*, which regulates ovule and seed development in *Arabidopsis* (Pinyopich *et al.*, 2003; Brambilla *et al.*, 2007), provides an excellent model for these studies, because, in addition to being a key regulator controlling seed production, its expression is precisely restricted to developing ovules (Pinyopich *et al.*, 2003; Brambilla *et al.*, 2007). Previously, we identified BPC1 as a direct regulator of *STK*, showing that it binds to the RGARAGRRA (C-box) consensus sequence, which is present in multiple copies in the *STK* promoter (Kooiker *et al.*, 2005). Furthermore, in vitro single-molecule studies showed that BPC1 binding to these GA-rich elements causes DNA loop formation. These conformational changes might well be important for the regulation of *STK*. However, analysis of *STK* expression in the bpc1-1 single mutant showed that its expression was only mildly upregulated without changes in its expression profile. This is not surprising, considering that BPC1 shares sequence similarity to BPC2 and BPC3. Redundancy between these genes was further supported by the findings of Monfared *et al.* (2011), who showed that all the ubiquitously expressed BPC genes are likely to have overlapping functions. Furthermore, the analysis of the *bpc1-3 bpc2 bpc3* triple mutant that we show here revealed that the profile of *STK* promoter activity in this triple mutant was the same as in wild-type plants, which further strengthens the idea that redundancy occurs between BPC genes of different classes. To overcome the necessity of making an *Arabidopsis* mutant including all six bpc mutant alleles (BPC5 is considered to be a pseudogene), which based on their broad expression profile will probably also display severe phenotypic defects, we decided to mutate the consensus binding sites, which we called C-boxes, in the *STK* promoter to abolish BPC binding. This allowed us to study the function of BPC in the regulation of *STK* while preventing all kinds of pleiotropic effects caused by mutations in BPC genes. The GUS reporter lines that contain the *STK* promoter with the seven mutated consensus C-boxes showed strong deregulation of the GUS reporter gene throughout the flower during all stages of flower development. Promoter constructs in which only two strong BPC binding sites were mutated showed no deregulation. By contrast, plants that contained the reporter construct in which five C-boxes were mutated displayed ectopic expression in the carpel and stamens, suggesting that the number of BPC binding sites is important for correct gene expression. Our yeast interaction studies showed that BPC proteins of class I can interact with each other, and the fact that those of class II also interact between them and with those of class I (Wanke *et al.*, 2011)

further supports the idea that multiple DNA interactions combined with BPC protein–protein interactions will induce conformational changes into the *STK* promoter region, also corroborated by the previous reported in vitro TPM analysis (Kooiker *et al.*, 2005). An important outcome of our studies is that binding of BPC proteins to the *STK* promoter region is important for the repression of *STK* expression in the floral meristem. We showed that C-boxes to which BPC proteins bind are essential to facilitatebinding of the SVP-AP1-SEU-LUG repressor complex to the *STK* promoter region. However, it is not yet completely clear how this mechanistically works. Do the conformational changes induced by BPC cause the exposure of the CArG boxes so that MADS domain proteins can bind to them, or are protein–protein interactions between BPC proteins and the repressor complex necessary to stabilize the MADS domain repressor complex on the *STK* promoter? Our protein interaction studies, which showed interactions between different members of the repressor complex and BPCs, strongly support the latter scenario, although we cannot exclude that BPC proteins are also important to make the CArG boxes available for MADS domain protein binding. Interesting in this respect is the analogy with the GAGA Associated Factor of *Drosophila melanogaster* (dGAF) (Berger and Dubreucq, 2012), which is involved in the regulation of a wide variety of processes, including the regulation of homeotic *HOX* genes (Botas, 1993; Graba *et al.*, 1997; Lehmann, 2004). Although GAF proteins and BPCs do not show sequence homology similarity, they both bind to (GA)n or (CT)n sequences in promoter regions. Once dGAF binds DNA, it can both activate and repress gene transcription. This is the same for BPC proteins that are, for instance, involved in *STK* repression and INO activation (Meister *et al.*, 2004). In case of activation, dGAF cooperates with the Trithorax-like group (Trx-G) complex (Poux *et al.*, 2001), whereas, in case of repression, it forms a multimeric complex with Polycomb group (Pc-G) members (Horard *et al.*, 2000). Apart from this, several findings suggest that the GAF should not be considered only as a factor involved in regulation of gene expression but also as a structural protein. Indeed, GAF can establish contact with subunits belonging to NURF and FACT complexes (Orphanides *et al.*, 1998; Xiao *et al.*, 2001; Shimojima *et al.*, 2003), which are involved in the nucleosome spacing processes. BPCs share many similarities with GAF (Berger and Dubreucq, 2012); therefore, it is tempting to speculate that BPCs, apart from stabilizing the binding of the AP1-SVP-SEU-LUG repressor complex via protein–protein interactions on the *STK* promoter, also might play a role in the exposure of the CArG boxes by moving nucleosomes. The regulation of *STK* expression is likely not always dependent on BPC binding to its promoter. The promoter constructs containing the mutated C-boxes showed deregulation only in floral tissues but not in vegetative tissues. This indicates that other regulatory mechanisms, independent of BPCs, are important to silence *STK* expression during vegetative development. Furthermore, our analysis of the *AG* and *SEP3* promoters showed that the function of BPCs in facilitating transcription complex binding to promoters, which we discovered using the *STK* promoter, probably accounts for many other genes. This is further strengthened by the following observations: (1) many genes contain C-boxes in their putative promoter regions, (2) plants having multiple *bpc* genes mutated show a broad range of phenotypic effects (Monfared *et al.*, 2011), and (3)

BPC genes are ubiquitously expressed in plants. It is also important to notice that the regulatory functions of BPC factors as we describe here are likely not restricted to *Arabidopsis*, because BPC factors have also been identified in other plant species. In fact, this GAGA binding protein was first described in soybean (*Glycine max*) (GAGA Binding Protein [GBP]; Sangwan and O'Brian, 2002) and subsequently also in barley (*Hordeum vulgare*) (B Recombinant barley [BBR]; Santi *et al.*, 2003) and rice (*Oryza sativa*) (Meister *et al.*, 2004). Conservation of their function in different plant species is further suggested by the observation that in the promoter of the *STK* ortholog in rice, *MADS13* (Dreni *et al.*, 2007), C-box sequences are also present. In conclusion, our data reveal important insights into molecular mechanisms controlling gene expression in plants. We show that a MADS domain repressor complex depends, for binding to a target promoter, on the binding of the ubiquitously expressed BPC factors to the same promoter region. These data therefore provide insight into the role of BPC factors in plant development. The fact that BPCs are functionally but not structurally related to the intensively studied dGAF factor of Drosophila is intriguing and makes BPC proteins particularly interesting for further studies.

METHODS

Plant material and growth conditions

The *Arabidopsis thaliana* ecotype used in this work is Columbia; the plants were directly sown on soil and kept under short day conditions for two weeks (22°C, 8 h light and 16 h dark) and then moved to long-day conditions (22°C, 16 h light and 8 h dark). The svp alg24 ap1-12 triple mutant and the pSVP::SVP-GFP line were previously described in Gregis *et al.* (2008) and Gregis *et al.* (2009), respectively. The bpc1 bpc2 bpc3 triple mutant was kindly provided by C. Gasser.

STK promoter constructs and plant transformation

Each mutated version of the *STK* promoter (*pSTK_GAm2*, *pSTK_GAm5* and *pSTK_GAm7*) was obtained by assembling together three, six and eight fragments respectively, which start and terminate with the appropriate mutated GAGA box replacing two purines (highlighted by the small font) by two pyrimidine residues: Box1 AGAAagAAA; Box4 AGAAAgaAAGAgaAGAGA; Box7 AGAAAgaAAGAgaAAGAAA; Box8 TCTCTTtcCTTTCT; Box9 TTCctCTCT; Box10 TCTctCTCT; Box12 TTTCTCTctCC. All the versions were recombined into the pDONOR207 vector (Life Technologies) and then recombined in the pBGWFS7 vector (Life Technologies), which already contains the GUS sequence. *Arabidopsis thaliana* was transformed with these constructs using the Agrobacterium tumefaciens–mediated floral dip method (Clough and Bent 1998).

GUS staining and *In situ* hybridization

GUS assays were performed as described previously by Liljegren *et al.* (2000). The samples were mounted in 5% glycerol and subsequently observed using a Zeiss Axiophot D1 microscope equipped with DIC optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

In situ hybridization experiments were performed as described previously by Dreni *et al.* (2011) using as probe *STK* antisense RNA, which corresponds to nucleotides 455 to 818 (Brambilla *et al.*, 2007).

Chromatin Immunoprecipitation (ChIP) assay

Chromatin Immunoprecipitation and Real-time PCR were carried out as described by Gregis *et al.* (2009) using for SVP-GFP, the commercial antibodies GFP:Living Colors_ full-length (Clontech, http://www.clontech.com/), whereas for BPC, a polyclonal antibody raised against the full-length purified GST tagged BPC1.

Yeast Two-, three- and four hybrid assay

The two-, three-hybrid assays were performed at 28°C in the yeast strain AH109 (Clontech), whereas the four-hybrid assay was performed at 28°C in strain PJ64-4A (Clontech) using the co-transformation technique (Egea-Cortines *et al.*, 1999). The coding sequences of BPC1, BPC2, BPC3, SVP, SEU, LUG and AP1 were cloned in the Gateway vector GAL4 system (pGADT7 and pGBKT7, Clontech) or pTFT1 Gateway and pRED Gateway (kindly provided by R. Immink) passing through pDONOR207 (Life Technologies). Yeast two-and three-hybrid assays were tested on selective YSD medium lacking leucine, tryptophan, adenine and histidine supplemented with different concentrations of 3-aminotriazole (1, 2.5, 5 mM 3-AT). Four-hybrid interactions were assayed on selective YSD medium lacking leucine, tryptophan, adenine, uracile and histidine supplemented with different concentrations of 3-AT (1, 2.5, 5 mM).

Bimolecular fluorescence complementation (BiFC) assay

The BPC1, BPC2, BPC3, SVP, SEU, LUG and AP1 coding sequences were cloned, passing through the pDONR207 (Life Technologies), into the pYFPN43 and pYFPC43 vectors, which together with the SNF1 protein kinase constructs used as positive controls (Ferrando *et al.*, 2001), were kindly provided by A. Ferrando (IBMCP, Valencia, Spain). BiFC assays were performed in triplicate injecting Agrobacterium tumefaciens expressing viral suppressor p19/experimental constructs. The abaxial surfaces of infiltrated tobacco leaves were imaged 3 days after inoculation.

SUPPLEMENTARY FIGURE

The following materials are available in the online version of this article.

Supplemental Figure 1. Position of C-, NC-, and CArG Boxes in the *STK* Promoter Region.

Supplemental Figure 2. BPC Proteins of Class I Are Specifically Recognized by a Polyclonal Antibody Serum Raised against BPC1.

Supplemental Figure 3. Position of C-Boxes and CArG Boxes in the *AG* and *SEP3* Promoters.

Supplemental Figure 4. *STK* Expression Profile in Single, Double, and Triple Class I *bpc* Mutants.

Supplemental Table 1. GUS Activity in the *STKpro:GAwt, STKpro: GAm2, STKpro:GAm5,* and *STKpro:GAm7* Lines.

Supplemental Table 2. Yeast Two-, Three-, and Four-Hybrid Assays to Test Interactions between BPC1, BPC2, and BPC3 and SVP, AP1, SEU, and LUG.

Supplemental Table 3. Primers Used in This Study.

AUTHORS CONTRIBUTION

SS, IRV, VG and BC performed the experiments; LC and MK designed the research strategy; SS and MK did most of the writing.

ACKNOWLEDGMENTS

We thank Prof. C. Gasser for providing the *bpc1-3 bpc2 bpc3* triple mutant; Dr. R. Immink for the pRED and pTFT gateway vectors; Prof. F. Valverde for supervision with BiFC assay and Dr. A. Ferrando for BiFC positive control constructs.

FUNDING STATEMENT

The research was funded by the project FLOWERPOWER (AGRO-11 e Rif.n° 16976) from the Lombardy region, EMBO and FIRB projects (IRV). SS and BC were funded by a PhD fellowship and VG by a Post Doc fellowship from the Università degli Studi di Milano.

REFERENCES

Berger N, Dubreucq B. (2012) Evolution goes GAGA: GAGA binding proteins across kingdoms. Biochim Biophys Acta 1819: 863-868.

Botas J (1993) Control of morphogenesis and differentiation by *HOM/Hox* genes. Curr Opin Cell Biol 5: 1015-1022.

Bowman JL, Drews GN, Meyerowitz EM (1991) Expression of the *Arabidopsis* floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. Plant Cell 3: 749-758.

Brambilla V, Battaglia R, Colombo M, Masiero S, Bencivenga S, *et al.* (2007) Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in *Arabidopsis*. Plant Cell 19: 2544-2556.

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

Dreni L, Jacchia S, Fornara F, Fornari M, Ouwerkerk PB, *et al.* (2007) The D-lineage MADS-box gene *OsMADS13* controls ovule identity in rice. Plant J. 52: 690-699.

Dreni L, Pilatone A, Yun D, Erreni S, Pajoro A, *et al.* (2011) Functional analysis of all AGAMOUS subfamily members in rice reveals their roles in reproductive organ identity determination and meristem determinacy. Plant Cell 23: 2850-2863.

Egea-Cortines M, Saedler H, Sommer H. (1999) Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*. EMBO J. 18: 5370-5379.

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

Ferrando A, Koncz-Kálmán Z, Farràs R, Tiburcio A, Schell J, *et al.* (2001) Detection of *in vivo* protein interactions between Snf1-related kinase subunits with intron-tagged epitope-labelling in plants cells Nucleic Acids Res 29: 3685-3693.

Finzi L, Dunlap D (2003) Single-molecule studies of DNA architectural changes induced by regulatory proteins. Methods Enzymol 370: 369-378.

Graba Y, Aragnol D, Pradel J (1997) Drosophila Hox complex downstream targets and the function of homeotic genes. Bioessays 19: 379-388.

Grandi V, Gregis V, Kater MM (2011) Uncovering genetic and molecular interactions among floral meristem identity genes in *Arabidopsis thaliana*. Plant J. 69: 881-893.

Gregis V, Andrés F, Sessa A, Guerra RF, Simonini S, *et al.* (2012) Identification of pathway directly regulated by SHORT VEGETATIVE PHASE during vegetative and reproductive development in *Arabidopsis*.

Gregis V, Sessa A, Colombo L, Kater MM (2006) AGL24, SHORT VEGETATIVE PHASE, and APETALA1 Redundantly Control *AGAMOUS* during Early Stages of Flower Development in *Arabidopsis*. Plant Cell 18: 1373-1382.

Gregis V, Sessa A, Colombo L, Kater MM (2008) AGAMOUS-LIKE24 and SHORT VEGETATIVE PHASE determine floral meristem identity in *Arabidopsis*. Plant J. 56: 891-902.

Gregis V, Sessa A, Dorca-Fornell C, Kater MM (2009) The *Arabidopsis* floral meristem identity genes *AP1*, *AGL24* and *SVP* directly repress class B and C floral homeotic genes. Plant J. 60: 626-637.

Hartmann U, Höhmann S, Nettesheim K, Wisman E, Saedler H, *et al.* (2000) Molecular cloning of *SVP*: a negative regulator of the floral transition in *Arabidopsis*. Plant J. 21: 351-360.

Horard B, Tatout C, Poux S, Pirrotta V. (2000) Structure of a polycomb response element and in vitro binding of polycomb group complexes containing GAGA factor. Mol Cell Biol 20: 3187-3197.

Kooiker M, Airoldi CA, Losa A, Finzi L, Kater MM, *et al.* (2005) BASIC PENTACYSTEINE1 a Ga-binding protein that induces conformational changes in the regulatory region of the homeotic *Arabidopsis* gene *SEEDSTICK*. Plant Cell 17: 722-729.

Lehmann M (2004) Anything else but GAGA: a nonhistone protein complex reshapes chromatin structure. Trends Genet 20: 15-22.

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

Meister RJ, Williamsa LA, Monfared MM, Gallagher TL, Kraft EA, *et al.* (2004) Definition and interaction of a positive regulatory element of the *Arabidopsis* INNER NO OUTER promoter. Plant J. 37: 426-438.

Monfared MM, Simon MK, Meister RJ, Roig-Villanova I, Kooiker M, *et al.* (2011) Overlapping and antagonistic activities of BASIC PENTACYSTEINE genes affect a range of developmental processes in *Arabidopsis*. Plant J. 66: 1020-1031.

Orphanides G, LeRoy G, Chang CH, Luse DS, Reinberg D. (1998) FACT, a factor that facilitates transcript elongation through nucleosomes. Cell 92: 105-116.

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

Poux S, Melfi R, Pirrotta V. (2001) Establishment of Polycomb silencing requires a transient interaction between PC and ESC. Genes Dev 15: 2509-2514.

Rounsley SD, Ditta GS, Yanofsky MF (1995) Diverse roles for MADS box genes in *Arabidopsis* development. Plant Cell 7: 1259-1269.

Sangwan I, O'Brian MR (2002) Identification of a soybean protein that interacts with GAGA element dinucleotide repeat DNA. Plant Physiol 129:1788-1794.

Santi L, Wang Y, Stile MR, Berendzen K, Wanke D, *et al.* (2003) The GA octodinucleotide repeat binding factor BBR participates in the transcriptional regulation of the homeobox gene *Bkn3*. Plant J. 34: 813-826.

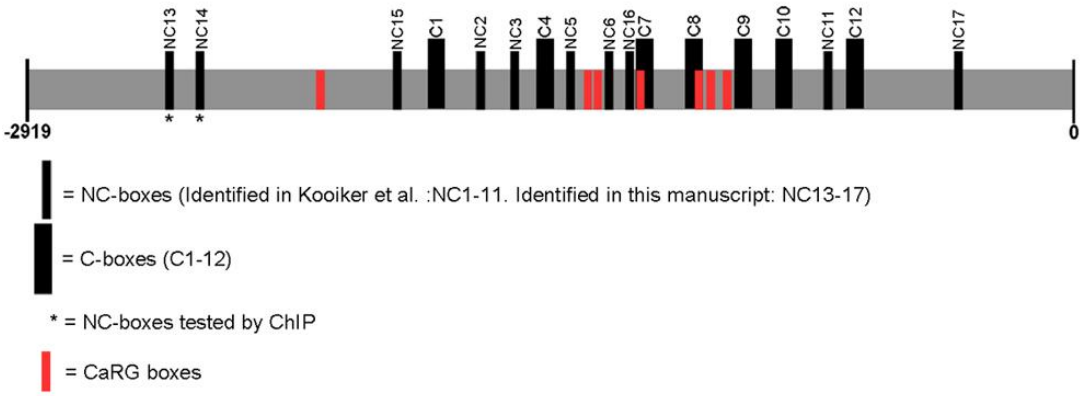
Shimojima T, Okada M, Nakayama T, Ueda H, Okawa K, *et al.* (2003) Drosophila FACT contributes to *Hox* gene expression through physical and functional interactions with GAGA factor. Genes Dev 17: 1605-1616.

Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, *et al.* (2004) Visualization of protein interactions in living plant cells using bimolecularfluorescence complementation. Plant J. 40: 428-438.

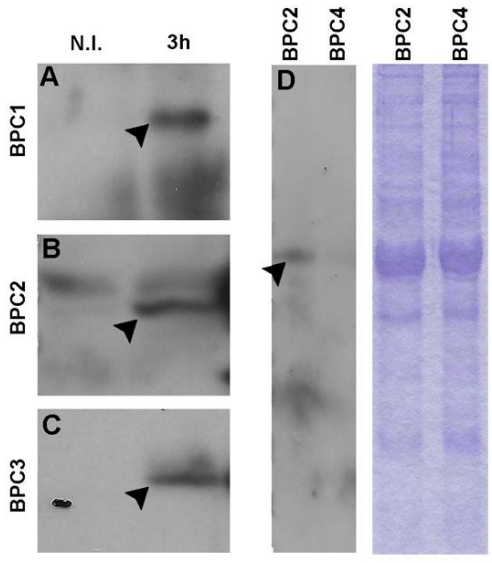
Wanke D, Hohenstatt ML, Dynowski M, Bloss U, Hecker A, *et al.* (2011) Alanine zipper-like coiled-coil domains are necessary for homotypic dimerization of plant GAGA-factors in the nucleus and nucleolus. PLoS One 10:e16070.

Xiao H, Sandaltzopoulos R, Wang HM, Hamiche A, Ranallo R, *et al.* (2001) Dual functions of largest NURF subunit NURF301 in nucleosome sliding and transcription factor interactions. Mol Cell 8: 531-543.

Supplemental Figure 1: Non Consensus (NC) and Consensus (C) boxes in the *STK* promoter

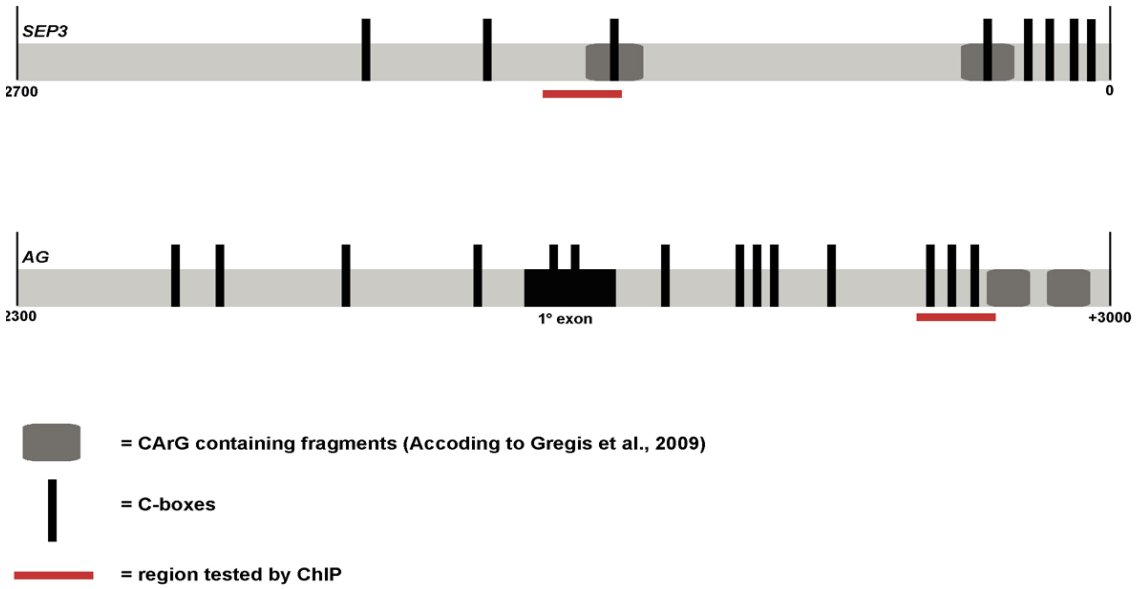


Supplemental Figure 2. BPCs of class I are specifically recognized by polyclonal antibodies which were raised against BPC1

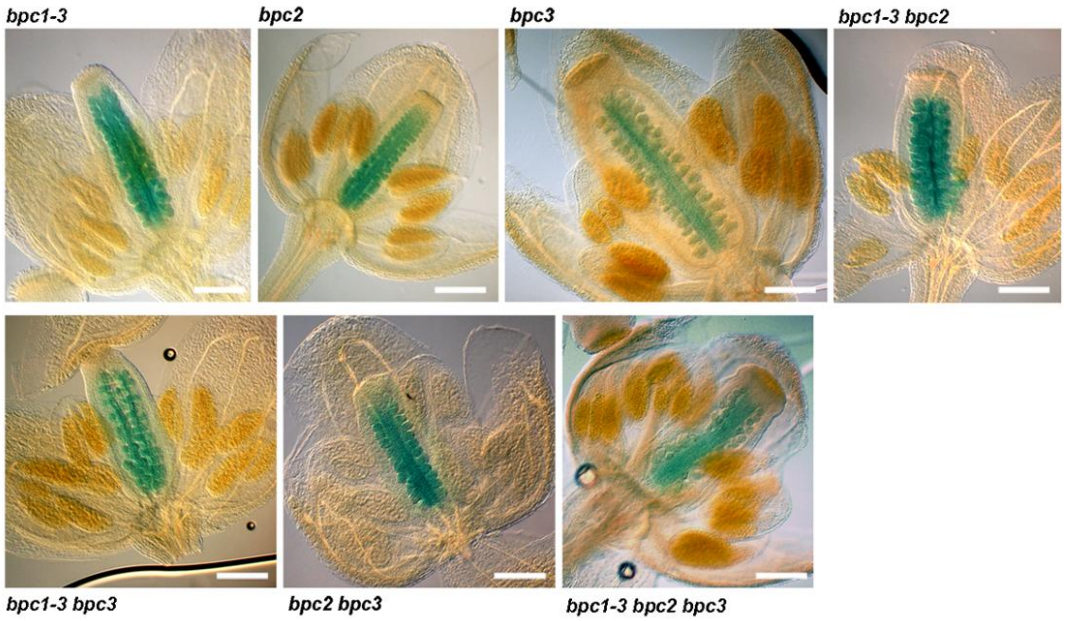


Crude E.coli protein extracts after induction of BPC1-GST (A), BPC2-GST (B), BPC3-GST (C)and pLYS-BPC4 (D).
The comassie stained gel (D) confirmed that BPC4 was strongly induced in the extraxts used for the Western Blot.
The antibidy recognizes all the BPC members of class I (A-C), whereas BPC4 was not recognized (D).
N.I. = non inducted; 3h = 3 hours after induction.

Supplemental Figure 3. C-boxes contained in *AG* and *SEP3* promoters are localized close to CArG containing fragments (According to Gregis et al., 2009).



Supplemental Figure 4.
STK expression profile in single, double and triple *bpc* mutants of class I.



Supplemental Table 1. GUS activity in the *STKpro:GAwt*, *STKpro:GAm2*, *STKpro:GAm5* and *STKpro:GAm7 lines*.

Plant with no staining were discarded from each analyses.

	STKpro::GAwt	*STKpro::GAm2*	*STKpro::GAm5*	*STKpro::GAm7*
Ovules and septum	25	49	44	31
Ectopic expression	3	8	27	94
TOT.	28	57	71	125
% deregulation	11%	14%	38%	75%
No GUS	3	3	6	7

AD	TFT	RED	BD	-H			-H +2,5 triAT			-H +5 triAT		
				Replicates			*Replicates*			*Replicates*		
				1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
AP1	**LUG**	**SEU**	**BPC1**	++	++	++	+	-	-	-	-	-
AP1	LUG	SEU	empty	+	++	++	-	-	-	-	-	-
AP1	LUG	empty	BPC1	++	++	++	-	-	-	-	-	-
AP1	empty	SEU	BPC1	+	++	++	-	-	-	-	-	-
empty	LUG	SEU	BPC1	++	++	++	-	-	-	-	-	-
AP1	**LUG**	**SEU**	**BPC2**	++	++	++	-	-	-	-	-	-
AP1	LUG	SEU	empty	+	+	++	-	-	-	-	-	-
AP1	LUG	empty	BPC2	+	++	++	-	-	-/+	-	-	-
AP1	empty	SEU	BPC2	+	+	+	-	-	-	-	-	-
empty	LUG	SEU	BPC2	+	+	+	-	-	-	-	-	-
AP1	**LUG**	**SEU**	**BPC3**	++	++	++	-/+	-/+	-/+	-	-	-
AP1	LUG	SEU	empty	++	++	++	-	-	-	-	-	-
AP1	LUG	empty	BPC3	+	++	++	-	-	-	-	-	-
AP1	empty	SEU	BPC3	+	+	+	-	-/+	-	-	-	-
empty	LUG	SEU	BPC3	+/-	+	+	-	-/+	-	-	-	-
SVP	**LUG**	**SEU**	**BPC1**	++	++	++	++	++	++	-	+	+
SVP	LUG	SEU	empty	+	++	++	-	-	-	-	-	-
SVP	LUG	empty	BPC1	+	++	++	-	-	-	-	-	-
SVP	empty	SEU	BPC1	++	++	++	-	-/+	-/+	-	-/+	-/+
empty	LUG	SEU	BPC1	++	++	++	-	-	-	-	-	-
SVP	**LUG**	**SEU**	**BPC2**	++	++	++	-	-	-	-	-	-
SVP	LUG	SEU	empty	+	++	++	-	-	-	-	-	-
SVP	LUG	empty	BPC2	+/-	+	+	-	-	-	-	-	-
SVP	empty	SEU	BPC2	+	+	++	-	-	-	-	-	-
empty	LUG	SEU	BPC2	+	+	+	-	-	-	-	-	-
SVP	**LUG**	**SEU**	**BPC3**	++	++	++	+	+	+	-	-	-
SVP	LUG	SEU	empty	+	+	++	-	-	-	-	-	-
SVP	LUG	empty	BPC3	+/-	+/-	-/+	-	-	-	-	-	-
SVP	empty	SEU	BPC3	++	++	++	+	++	++	+	++	++
empty	LUG	SEU	BPC3	+	+	+	-	-	-	-	-	-

Supplemental table 2. Each interaction has been tested on media lacking Histidine and/or supplemented with different concentration of 3-AT. For each interaction, three independent replicates were tested. SVP/AP1 were cloned in the pGADT7 vector whereas BPC1-3 in the pGBKT7 vector. SEU and LUG were used always as bridge vectors and are expressed in frame with the Nulcear Localization Signal, already present in the pTFT and pRED vectors .

++ = strong interaction; + = intermediate interaction; +/- = weak interaction; - = no interaction.

CHAPTER 3

BPC proteins regulate HOMEOBOX transcription factors involved in meristem size maintenance

Simonini Sara [a] **and Martin M. Kater** [a]

[a] Department of Biomolecular Sciences and Biotechnology, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy.

INTRODUCTION

The *Arabidopsis* genome contains more than 1900 genes encoding transcription factors. Based on sequence homology, function and activity these factors can be subdivided into 64 transcription factor families (Guo *et al*., 2005). The BARLEY B RECOMBINANT/BASIC PENTACYSTEINE (BBR/BPC) family is a poorly understood plant-specific transcription factor family and member genes have been found in different plant species, such as *Glycine max* (soybean), *Hordeum vulgare* (barley), *Oryza sativa* (rice) and *Arabidopsis thaliana* (Sangwan and O'Brian, 2002; Santi *et al*., 2003; Meister *et al*., 2004; Kooiker *et al*., 2005). The BBR/BPC family members are characterized by the ability to bind the DNA at GA-rich sites: the GAGA BINDING PROTEIN (GBP) of soybean specifically binds a $(GA)_9$ repeat sequence contained in the Glutamate 1-Semialdehyde Aminotransferase (Gsa1) promoter (Sangwan and O'Brian, 2002), BARLEY B RECOMBINANT (BBR) of barley binds a $(GA)_8$ sequence in vitro (Santi *et al*., 2003). The *Arabidopsis* BPC proteins specifically bind $(GA)_6$ and $(GA)_9$ repeats in vitro and *in vivo* (Meister *et al*., 2004; Kooiker *et al*., 2005; Simonini *et al*., 2012).

There are 7 *BPC* codifying genes in the *Arabidopsis* genome and on the basis of sequence homology they were divided into three classes. Class 1 (*BPC1-3*), class 2 composed by *BPC4-6* and class 3 containing only *BPC7* (Meister *et al*., 2004). They can be activators and repressors of transcription and all of them, except for *BPC5,* are expressed ubiquitously suggesting that BPC function is not specific to one developmental process and/or tissue. Indeed, more than 3000 genes in the *Arabidopsis* genome contain at least one GA-rich sequence in their regulatory region and combining multiple *bpc* mutant alleles together results in a broad range of developmental defects (Meister *et al*., 2004; Monfared *et al*., 2011).

The BPC proteins share functional similarities with the Trithorax-like protein GAGA Associated Factor (GAF) of *Drosophila melanogaster*, which is a transcription factor involved in regulation of homeotic *HOX* genes (Botas, 1993; Graba *et al*., 1997; Lehmann, 2004; Berger *et al*., 2012). Moreover, GAF is a structural protein because of its ability in establishing contacts with subunits belonging to NURF and FACT complexes which are involved in nucleosome spacing processes (Orphanides *et al*., 1998, Xiao *et al*., 2001; Shimojima *et al*., 2003). Similarly BPC1, is able to loop the DNA (Kooiker *et al*., 2005) causing conformational changes in the promoter of the ovule identity gene *SEEDSTICK* (*STK*; Pinyopich *et al*., 2003; Favaro *et al*., 2003). Recent experiments revealed that BPCs binding to the *STK* promoter is essential for its ovule specific expression since mutation of BPC binding sites in this promoter caused ectopic *STK* expression throughout the flower (Simonini *et al*., 2012) due to the inability to recruit a MADS domain transcription factor containing repressor complex. This data suggest that BPCs are essential to recruit this repressor complex to specific binding sites and thereby reveals a mechanism by which BPCs are involved in gene regulation (Simonini *et al*., 2012).

It has already been published that the BBR factor of barley binds and regulates the *BKN3* gene, the homologue of *Arabidopsis SHOOTMERISTEMLESS* (*STM*), a KNOX transcription factor (Santi *et al*., 2003) suggesting a possible connection among the BPCs and the control of meristem formation. *STM* tightly regulates the cytokinin pathway, which is involved in the

maintenance of the undifferentiated state of the cells making up the apical meristem (Yanai *et al*., 2005; Bartrina *et al*., 2011). Plants with hyper-production or slow degradation of cytokinin display compact inflorescences, extra floral organs and altered phyllotaxis caused by enlarged meristems (Venglat *et al*., 1996; Bartrina *et al*., 2011).

Here we unravel the role of BPC in inflorescence development and show their involvement in the regulation of *HOMEOBOX* genes and the hormone cytokinin.

RESULTS

Absence of BPCs proteins cause defects in inflorescence development

The *bpc1*, *bpc2* and *bpc3* single or double mutants do not have obvious phenotypes, probably due to functional redundancy; however the *bpc1 bpc2 bpc3* triple mutant displays interesting inflorescence phenotypes. This triple mutant produces more flowers than normal plants. A wild-type flower is composed of four sepals, four sepals, six stamens and a pistil composed by two fused carpels. In the *bpc1 bpc2 bpc3* triple mutant more than 90% of the flowers are composed of five or more sepals, often fused along their margins, five or more petals, eight or more stamens and up to four carpels (Figure 1A-D). The flower is also partially sterile since ovule development is also affected (Monfared *et al*., 2011). This partial sterility probably causes also defects in fruit development (Figure 1E).

Since the number of flowers and floral organs is a direct consequence of meristem size and activity, the *bpc1 bpc2 bpc3* inflorescence meristem (IM) was analysed by DAPI staining. The inflorescence meristem is composed by three different cell layers where the upper one, called L1, is composed 12-15 adjacent cells. In the *bpc1 bpc2 bpc3* triple mutant the L1 layer

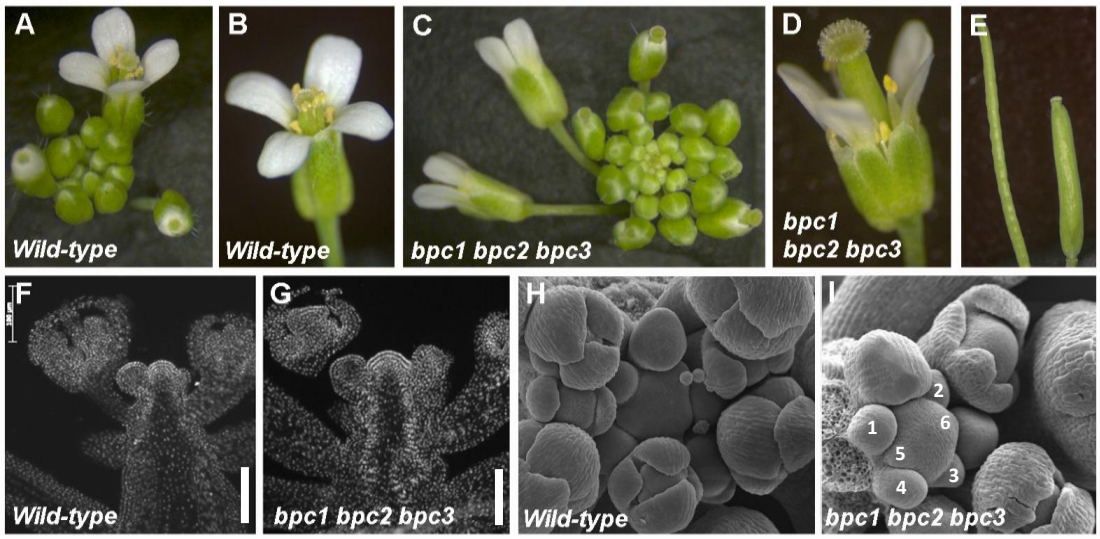


Figure 1. Inflorescence phenotype of the *bpc1 bpc2 bpc3* triple mutant.
(A-B) Wild-type inflorescence and flower. **(C-D)** *bpc1 bpc2 bpc3* triple mutant inflorescence and flower. **(E)** Wild-type fruit (left) and *bpc1 bpc2 bpc3* fruit (right). **(F)** DAPI sytaining on wild-type inflorescence. **(G)** DAPI staining on *bpc1 bpc2 bpc3* triple mutant. **(H)** SEM analyses on wild-type inflorescence. **(I)** SEM analyses on *bpc1 bpc2 bpc3* triple mutant.
Bars in (F) and (G): 100µn.

was composed of more than 20 cells and it was clearly enlarged when compared to wild-type (Figure 1F and 1G). Moreover, the IM produces more floral primordia (n>4) than wild-type (n=3), which probably due to steric hinderance develop randomly from the IM (Figure 1H and 1I).

Taken together, these phenotypical observations this suggests that BPC proteins of class I might be involved in meristem size and the maintenance of its activity.

BPC1 is involved in many aspects of plant development

BPCs can be both activators and repressors of transcription (Meister et al., 2004, Kooiker et al., 2005, Simonini et al., 2012). To investigate this further, we investigated the role BPC1 by adding to its coding sequence the constitutive EAR repressor domain. This chimeric protein was placed under the control of the 35S promoter and used to transform wild-type plants. Out of 270 selected plants, 90% (n= 239) had no phenotype, being completely indistinguishable from wild-type (data not shown) whereas the remaining plants (10%, n=31) showed a strong phenotype (Figure 2A and 2B). These plants had severe defects in vegetative and reproductive development with few small curved leaves, which lost adaxal- abaxial identity (Figure 2A). Although several flowers were produced, the inflorescence remained attached to the rosette due to the inability to develop a stem (Figure 2A and 2B). The flowers were abnormal, sterile also if pollinated with wild-type pollen.

To investigate the role of BPC1 focusing on flower development and to prevent pleitropic effects of the expression of BPC1-EAR during the vegetative phase, the chimeric BPC1-EAR motif was placed under the control of the pALC

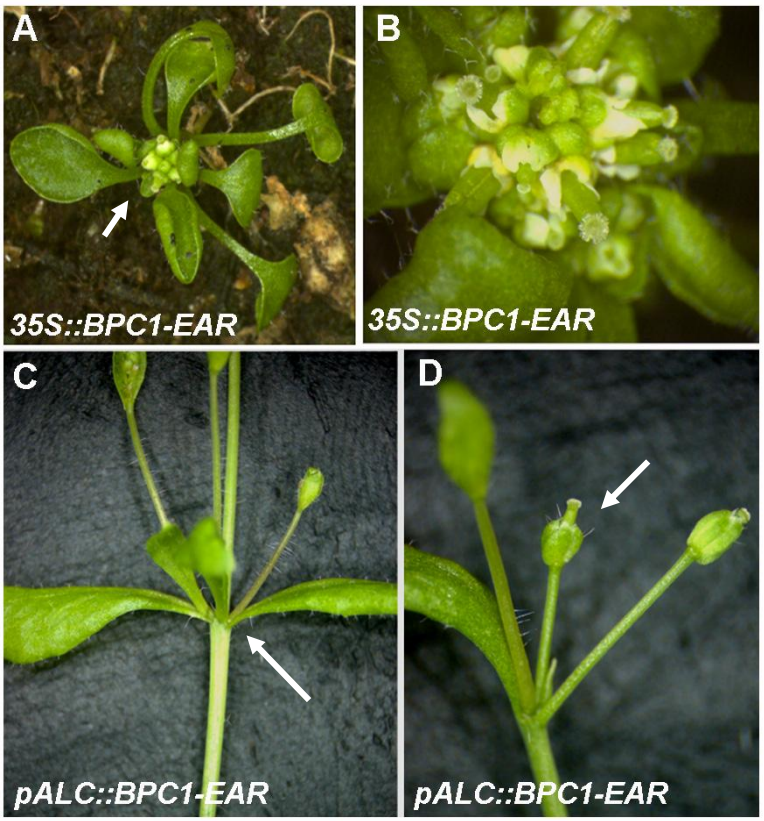
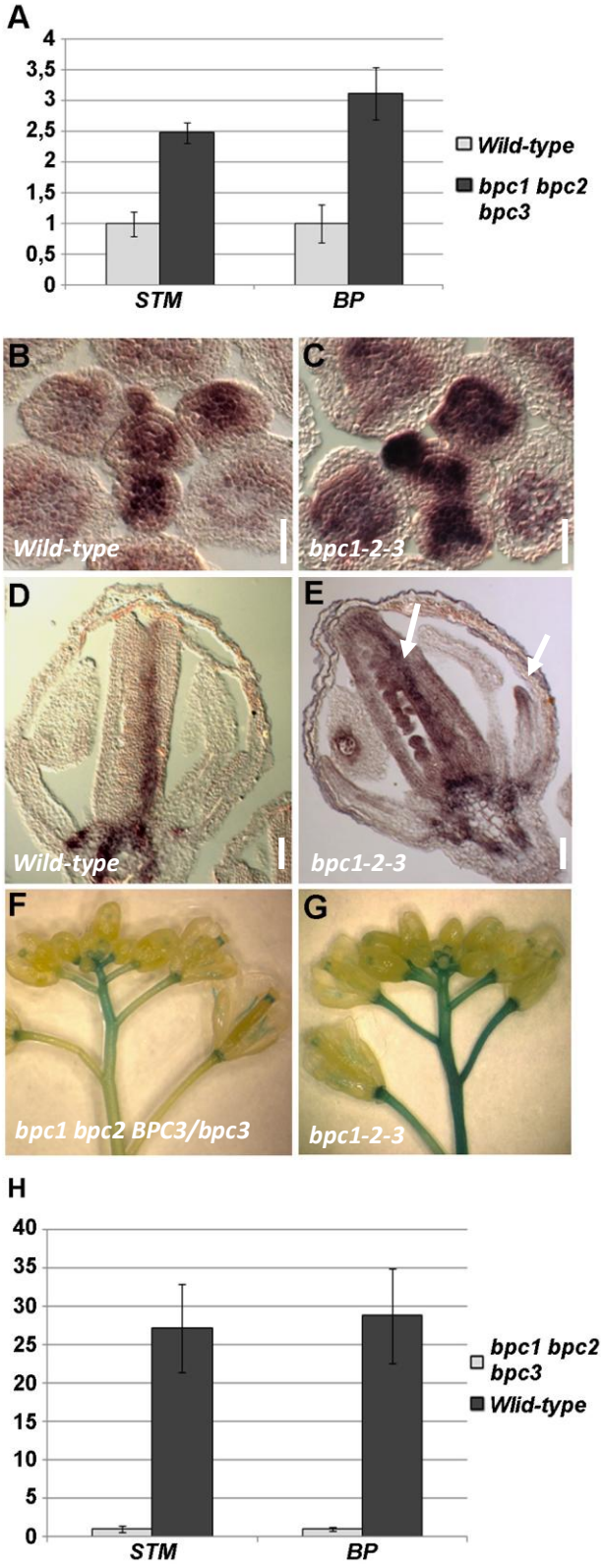


Figure 2. BPC1-EAR Motif plants.
(A-B) Plants transformed with the 35S::BOC1-EAR Motif
(C-D) Plants transformed with the pALC-BPC1-EAR Motif after 6 days of induction.



inducible promoter. The induction was applied for 4 to 6 days (8 hours per day) after bolting. In all the inducted plants, a strong phenotype was detected (Figure 2C and 2D). The inflorescence was developing only a few flowers due premature arrest of meristem activity. Morevoer, the flowers were sterile and composed by fused sepals, sometimes extranumerary.

This data suggest that BPC1 might be involved in many developmental processes as activator of the transcription.

HOMEOBOX KNOX genes are direct targets of class I BPCs

The *HOMEOBOX* gene family in *Arabidopsis* is composed of different classes, in particular, the KNOX class includes several members essential for meristem and organ development. Members that belong to this family are for istance *SHOOTMERISTEMLESS (STM)*, *BREVIPEDICELLUS* (*BP*) and the *KNOTTED-LIKE* genes (*KNAT* genes). The phenotypes of mutants in which one or more *KNOX* genes are

Figure 3. *STM* and *BP* are deregulated in the *bcp1 bpc2 bpc3* mutant background. **(A)** Expression analyses of *STM* and *BP* transcripts level in the *bpc1-2-3* mutant. **(B-E)** In situ hybridization with STM probe in wild-type (B-D) and *bpc1-2-3* (C-E) inflorescence meristems and flowers. **(F-G)** *BP::GUS* in *bpc1 bpc2 BPC3/bpc3* (F) and *bpc1-2-3* (E) backgrounds. (H) ChIP experiment on *STM* and *BP* promoters. Bars: 50µn

misregulated (REF) resembles the *bpc1 bpc2 bpc3* triple mutant inflorescence phenotype and the BPC1-Ear Motif transgenic lines. For this reason, the expression level of *STM* and *BP* has been investigated by quantitative real-time PCR in the *bpc1 bpc2 bpc3* triple mutant. This revealed that both genes were upregulated of 2.5 fold and 3.2 fold respectively in this background compared to wild-type (Figure 3A). *In-situ* hybridization using a *STM* antisense probe revelead that the domain of *STM* expression in *the bpc1 bpc2 bpc3* triple mutant inflorescence meristem is stronger than in wild-type, probably due to the enlarged meristem size, peculiar of the *bpc1 bpc2 bpc3* triple mutant (Figure 3B and 3C). Interestingly, *STM* expression was also detected to be ectopically present in petals and ovules (Figure 3D and 3E), suggesting that *STM* is repressed by BPCs in these tissues.

We also introduced by crossing the *BP::GUS* reporter contruct in the *bpc1 bpc2 bpc3* triple mutant background. Analysis of GUS expression revealed that the BP expression was stronger than in wild-type and persistent in the stem and the pedicel (Figure 3F and 3G) suggesting that, as what we also observed for *STM*, BPCs of class I are repressors of *BP* expression.

In order to verify if BPCs directly regulate *STM* and *BP* expression, three independent ChIP experiments on wild-type inflorescences using a polyclonal antibody raised against the BPC of class I (BPC1-BPC2-BPC3; Simonini *et al.,* 2012) was performed. As negative control and positive control to check for the presence of immuniprecipitated chromatin, the *bpc1 bpc2 bpc3* triple mutant and the *STK* promoter have been tested (data not shown), respectively (Simonini *et al.,* 2012). In all the ChIP experiments, a high enrichment has been detected

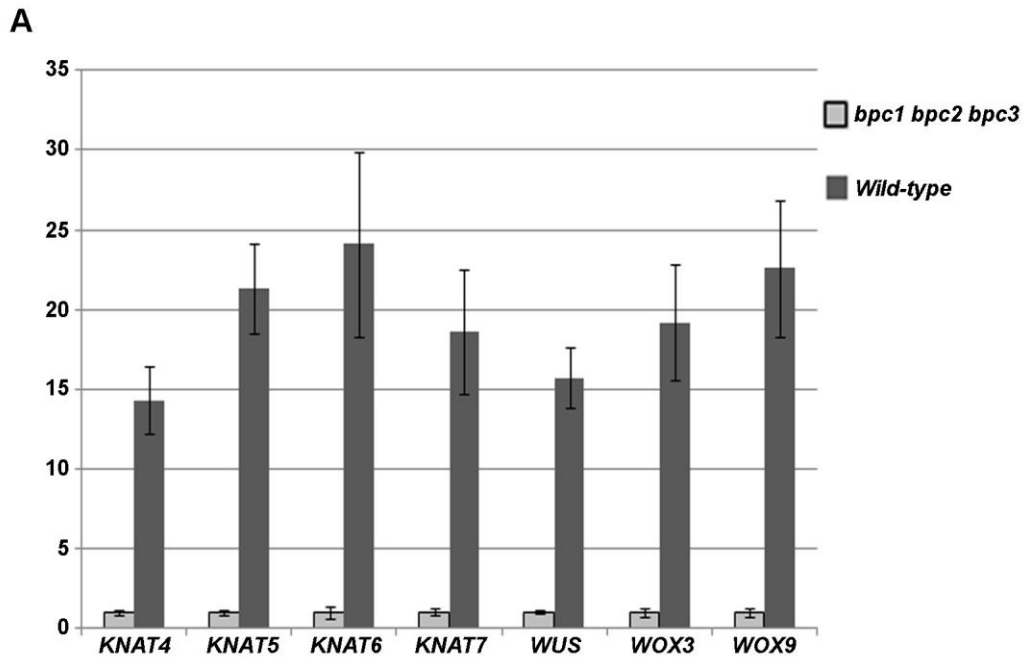


Figure 4. ChIP experiments on several HOMEOBOX promoters using a BPC1-2-3 specific antibody.

when *STM* and BP promoter were tested (Figure 3H), confirming that *STM* and *BP* are direct targets of BPC proteins of class I.

HOMEOBOX genes contains GA-rich stretches in their promoter

It has been recently published that BPCs bind the promoter of MADS-box transcription factor *SEEDSTICK* at GA-rich sites (Kooiker *et al.,* 2005; Simonini *et al.,* 2012): there are seven, relatively short sequences (9-15 bp) which are spread along 2900 bp of the *STK* regulatory region. These kinds of GA-sites are also located in the promoter of other MADS-box transcription factors, such as *SEPALLATA3* and *AGAMOUS*, which are also bound by BPCs *in vivo*. Interestingly, the GA-rich sites contained in the *STM* and *BP* promoter are extremely long (up to 50bp), unique and located within 500bp from the transcription start site.

In order to verify if this might be a common feature shared with other *HOMEOBOX* genes, we searched for the presence of GA-rich sites in 500bp upstream the transcription start site of different genes *HOMEOBOX* genes belonging also to different clades. Interestingly, GA-rich sites can be found in the promoter of many *HOMEOBOX* genes such as *KNOTTED-LIKE 4* (*KNAT4*), *KNAT5*, *KNAT6*, *KNAT7*, *WUSCHEL* (*WUS*), *WUSCHEL RELATED HOMEOBOX 3* (*WOX3*) and *WOX9.* Moreover, ChIP assays using the antibodies against the class I BPC proteins revealed that these sites were bound by BPCs *in vivo* (Figure 4). This suggests that BPCs are common regulators of the expression of *HOMEOBOX* transcription factors involved in many and different developmental processes.

BPCs are responsive to cytokinin

Few *KNOX* genes, such as *STM* and *BP*, are known to be involved in the cytokinin (CK) pathway (Bartrina *et al.,* 2011; Yanai *et al.,* 2005), a class of hormones active in the maintenance of meristem size and activity. Mutants with overproduction or slow degradation of CK display enlarged inflorescence meristems and flowers composed of more floral organs than wild-type flowers (Bartrina *et al.,* 2011, Venglat *et al.,* 1996). Whereas in mutants with impaired CK synthesis or CK perception the meristem terminates prematurely due to precocious cell differentiation (Bartrina *et al.,* 2011).

To check if the BPCs are involved in the CK pathways, we treated the *BPC1::GUS*, *BPC2::GUS*, *BPC4::GUS*, *BPC6::GUS*, *BPC7::GUS* marker lines (Monfared *et al.,* 2011) with exogenous N6-Benzylaminopurine (BAP, a synthetic cytokinin; Venglat *et al*., 1996; Bencivenga *et al.,* 2012). In all the lines, the expression of the GUS reporter gene was different in respect to the MOCK treatment (Figure 5A-J): *BPC1*, *BPC6* and *BPC7* were repressed by the increase in CK concentration (Figure 5A-B, 5G-J), whereas *BPC2* and *BPC4* expression was strongly stimulated by CK (Figure 5C-F) supporting the idea that BPCs might be involved in the cytokinin signaling pathway.

To support this hypothesis, we checked the expression pattern of *ARABIDOPSIS RESPONSE REGULATOR 7* (*ARR7*), which transcription is induced by cytokinin (Buechel *et al*., 2010) and its domain is enlarged in meristems with higher concentration of CK.

In the *bpc1 bpc2 bpc3* triple mutant, *ARR7* was strongly expressed in the meristem (Figure 6C), which is in contrast to wild-type plants where its expression is broad and weak (Figure 6B; Zhao *et al.*,2010).

Moreover, ChIP experiments confirmed that BPCs of class I strongly bind the ARR7 promoter (Figure 6A), suggesting that BPCs may act on the CK pathway also at this level.

All together our data assigns to the BPCs a role as sensors of hormonal homeostasis changes (i.e. cytokinin), and to convert this information into a specific regulation of transcription of its target genes.

DISCUSSION AND FUTURE PERSPECTIVES

The *Arabidopsis* genome encode for many different transcription factor families and for many of them a detailed charachterization has not been reported yet. The BBR/BPC factors are GAGA binding proteins belonging to a poorly characterized transcription factor family and in the *Arabidopsis* genome there are 7 BPCs enconding genes. The BPCs are known to be general regulators of the transcription indicated by their broad expression pattern and the fact that thousands of genes contain GA-rich stretches in the regulatory region, suggest that BPCs might be involved in many and different developmental processes. Since *bpc* single mutants don't display any phenotype, whereas combining multiple *bpc* mutants together results in plants with many pleiotropic defects, supports the idea that BPCs are involved in many aspect of plant development (Monfared *et al.,* 2011). For istance, the BPCs are known to be

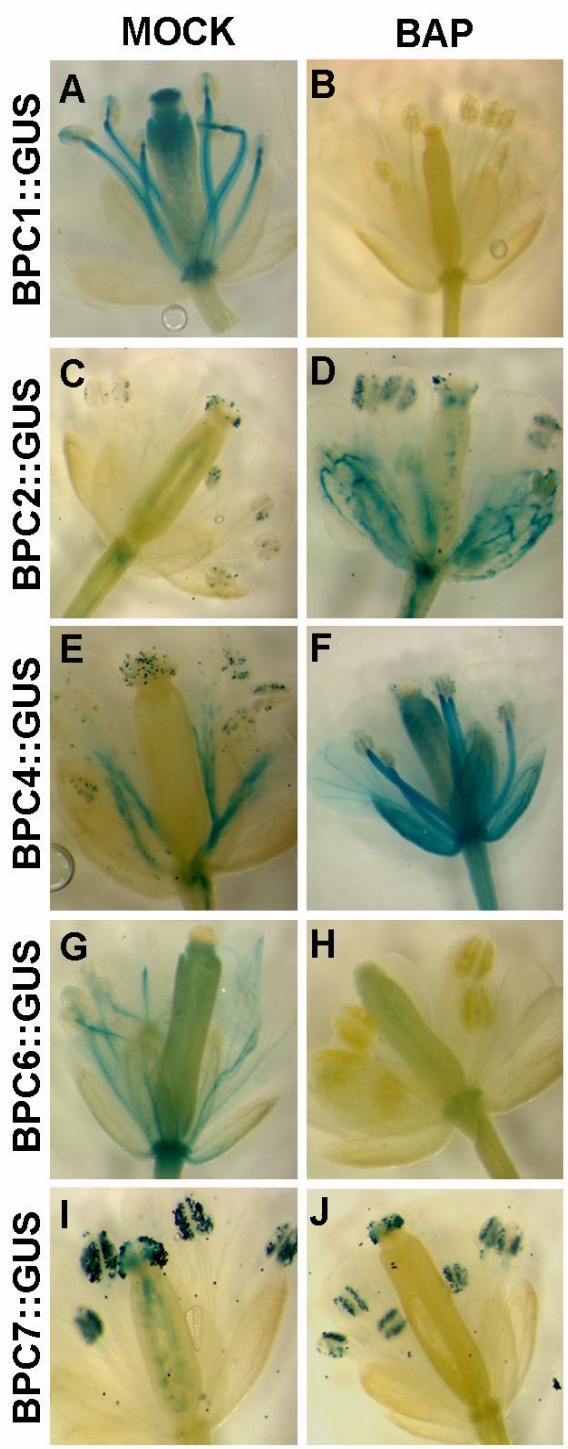


Figure 5. BPCs are responsive to CK.
BAP treatment on BPC1, BPC2, BPC4, BPC6 and BPC7 marker lines. Left panel: MOCK; Right panel: BAP.

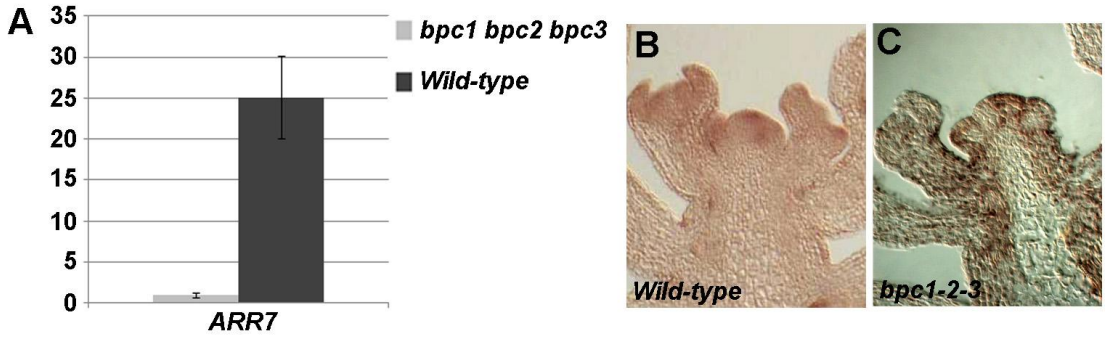


Figure 6. ARR7 is a BPCs target.
(A) Enrichment of the *ARR7* promoter detected throught ChIP experiment using antibody against BPC1-2-3. (B-C) In sity hybridization with *ARR7* specific probe in wild-type (B) and *bpc1-2-3* backgrounds.
Bars 50µm.

regulators of the YABBI transcription factors, such as *INNER NO OUTER*, a gene involved in ovule development (Meister *et al.,* 2004). Moreover the BPCs regulate the expression of the MADS-box transcription factor *SEEDSTICK* (Kooiker *et al.,* 2005; Simonini *et al.,* 2012), which is involved in ovule development and identity. The BPCs are also involved in seed development, being regulators of the *LEAFY COTYLEDON 2* genes (Berger *et al.,* 2011).

Here we assing another role to BPCs as common direct regulators of many *HOMEOBOX* gene family members. In our manuscript, we focused on the two KNOX genes *STM* and *BP*, but, since BPCs strongly bind many other promoter regions of *HOMEOBOX* genes', they might represent an evolutionary conserved class of regulators of these transcription factors .

STM and *BP* are expressed in the inflorescence and floral meristems especially in undifferentiated cells promoting their meristematic indentity and activity. Loss of function alleles of *STM* display precocious differentiation and consequent a loss of the meristematic tissues of the inflorescence meristem. Therefore these plants produce only a few flowers with less floral organs (Durbak and Tax, 2011). On the other hand, upregulation of *STM* expression levels leads to an inflorescence meristem enlargement connected to an increase of meristematic activity; these meristems produce more floral primordia with more floral organs (Yanai *et al.,* 2005).

STM and *BP* are upregulated in the *bpc1 bpc2 bpc3* triple mutant background and it is consistent with the observation that the *bpc1-2-3* triple mutant has an enlarged inflorescence meristem.

To deeply characterize this aspect also from a morphological point of view, we plan to analyze in detail the size and the activity of the *bpc1 bpc2 bpc3* triple mutant inflorescence meristem through confocal microscopy using *in vivo* live imaging. This aspect can be developed using both marker lines (e. g. *CLV3::mCherry:NLS*; *WUS::GFP:NLS*) and different staining methods.

STM and *BP* are also involved in the cytokinin pathway, a class of hormone tightly linked to meristem activity. Interestingly, expression of all the *BPCs* that we tested (*BPC1-2-4-6-7*)

showed to be responsive to the treatment with exogenous CK, some were represses others activated. The expression profile of *ARR7* (Zhao *et al.,* 2010) was also investigated in the *bpc1-2-3* triple mutant background since *ARR7* is a CK responsive gene which transcription is stimulated by increased CK concentration (Zhao *et al.,* 2010; Buechel *et al*., 2010). In this background *ARR7* expression was stronger than wild type and it is consistent with the role of *ARR7* as gene which transcription is induced by CK. Moreover, this data supports the hypothesis that the *bpc1 bpc2 bpc3* triple mutant inflorescence meristem is more active due to an overproduction of CK, which is consequence of the upregulation of meristematic genes such as *STM*.

With the data obtained until now, we can speculate that BPCs are common regulators of HOMEOBOX transcription factors.

The fact that the sestuple *bpc* mutant (Meister *et al.,* 2011) was able to grow even if with many phenotipic defects, suggest that BPCs might not be essential for plant growth. We might consider them to be as modulator of transcription helping higher order complexes to reach the DNA or keeping the chromatin packaged to maintain a region in a repressed state. However, this hypothesis is still to be considered with caution since the sestuple *bpc* mutant does not represent a full knock-out for all *BPC* genes. For instance the *bpc1-1* allele that was used to create this higher order mutant is not a complete knock-out (Monfared *et al.,* 2011). Therefore, it might well be that a complete knock-out of all genes results in more severe or lethal phenotypes.

BPCs also seem to be responsive to increased hormone concentrations, which is a fundamental aspect of any developmental process. To better investigate this aspect, the *bpc1 bpc2 bpc3* triple mutant will be crossed with different marker lines of interest known to be involved in hormone signaling: *PIN1-GFP* and *DR5::GFP*, which important to study auxin fluxes, and *TCS::GFP* and *ARR5::GFP* which are responsive to cytokinin. This analysis should provide a better insight in the link between BPC and the hormonal pathways.

In conclusion, our data show the importance of BPCs in the regulation of meristem development through their direct regulation of *HOMEOBOX* genes.

MATERIAL AND METHOD

Plant material and growth conditions

The Arabidopsis thaliana ecotype used in this work are Columbia and Landsberg; the plants were sow directly on soil and kept under short day conditions for two weeks (22°C, 8 h light and 16 h dark) and then moved under long-day conditions (22°C, 16 h light and 8 h dark). *The bpc1 bpc2 bpc3* triple mutant, *BPC1::GUS*, *BPC2::GUS*, *BPC3::GUS*, *BPC4::GUS*, *BPC6::GUS*, *BPC7::GUS* were kindly provided by prof. C. Gasser. The *BP::GUS* lines was obtained from NASC.

GUS staining and In Situ hybridization

GUS assays were performed overnight as described previously (Liljegren *et al.,* 2000). The samples were mounted in 5% glycerol and subsequently observed using a Zeiss Axiophot D1 microscope equipped with DIC optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

In situ hybridization experiments were performed as previously described in Dreni *et al.,* 2011; the STM antisense probe has been already published in Long *et al.,* 1996.

Ethanol induction experiment and BAP treatments

The plants were inducted for 4 to 6 days for 8h per day. Few open eppendorf full of ethanol 100% were stucked into the soil and the plants covered with a plastic bell without any hole. The ethanol vapor was applied at the bolting. Inflorescence has been collected at 4 and 6 days.

BAP treatments were conducted as previously described in Bencivenga *et al.,* 2012.

RNA isolation, Reverse Transcription-PCR and quantitative Real-Time (qRT-PCR) analysis

Total RNA was extracted using the LiCl method (Verwoerd *et al.,* 1989) for all the expression analyses. Total RNA was treated with the Ambion TURBO DNA-free DNase kit and then retro-transcribed using the ImProm-II™ Reverse Transcription System (Promega). The cDNAs were standardized relative to UBIQUITIN10 (UBI10) and PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2A-At1g13320) transcripts and the gene expression analyses was performed using the iQ5 Multi Colour Real-Time PCR detection system (Bio-Rad) with a SYBR Green PCR Master Mix (Biorad). Baseline and threshold levels were set according to the manufacturer's instructions.

For RT-PCR and quantitative real-time RT-PCR primers, see Table S1 in the supplementary material.

Chomatin immunoprecipitation (ChIP) assays

ChIP experiments were performed as previously reported by Gregis *et al.,* 2008 using a policlonal antibody raised against the entire BPC1 protein (Simonini *et al.,* 2012). Chromatin was extracted from whole wild type plants (Col0) inflorescences and from the *bpc1 bpc2 bpc3* triple mutant used as negative control. The DNA fragments obtained from the immune-precipitated chromatin were amplified by qRT-PCR using specific primers (see table S1). Three real-time PCR amplifications on three independent chromatin extractions were performed. For the complete primer sets see (see table S1) in the supplementary material. Enrichment of the target region was determined using the iQ5 Multi Colour Real-Time PCR detection system (Bio-Rad) with a SYBR Green PCR Master Mix (Biorad). The qRT PCR assays and the following fold enrichment calculation were performed as previously described (Matias-Hernandez *et al.,* 2010).

REFERENCES

Bartrina I., Otto E., Strnad M., Werner T., and Schmülling T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in Arabidopsis thaliana. Plant Cell 23: 69-80.

Berger N., and Dubreucq B. (2012). Evolution goes GAGA: GAGA binding proteins across kingdoms. BBA - Gene Regulatory Mechanisms, 1–6.

Berger N., Dubreucq B., Roudier F., Dubos C. and Lepiniec L. (2011). Transcriptional regulation of Arabidopsis LEAFY COTYLEDON2 involves RLE, a cis-element that regulates trimethylation of histone H3 at lysine-27. Plant Cell 23: 4065-4078.

Botas J. (1993). Control of morphogenesis and differentiation by HOM/Hox genes. Curr Opin Cell Biol. 5: 1015-1022.

Buechel, S., Leibfried, A., To, J.P., Zhao, Z., Andersen, S.U., Kieber, J.J., and Lohmann J.U. (2010). Role of A-type ARABIDOPSIS RESPONSE REGULATORS in meristem maintenance and regeneration. Eur J Cell Biol. 89:279-284.

Favaro R., Pinyopich A., Battaglia R., Kooiker M., Borghi L., Ditta G., Yanofsky M.F., Kater M.M. and Colombo L. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell 15: 2603-2611.

Graba Y., Aragnol D. and Pradel J. (1997). Drosophila Hox complex downstream targets and the function of homeotic genes. Bioessays 19: 379-388.

Guo A., He K., Liu D., Bai S., Gu X., Wei L. and Luo J. (2005) DATF: a database of Arabidopsis transcription factors. Bioinformatics 21: 2568–2569.

Kooiker M., Airoldi C.A., losa A., Finzi L., Kater M.M. and Colombo L. (2005). BASIC PENTACYSTEINE1 a GA-binding protein that induces conformational changes in the regulatory region of the homeotic Arabidopsis gene SEEDSTICK. The Plant Cell 17:722-729.

Lehmann M. (2004). Anything else but GAGA: a nonhistone protein complex reshapes chromatin structure. Trends Genet. 20: 15-22

Meister R.J., Williamsa L.A., Monfared M.M., Gallagher T.L., Kraft E.A., Nelson C.G. and Gasser C.S. (2004). Definition and interaction of a positive regulatory element of the Arabidopsis INNER NO OUTER promoter. The Plant Journal 37: 426-438.

Monfared M.M., Simon M.K., Meister R.J., Roig-Villanova I., Kooiker M., Colombo L., Fletcher J.C. and Gasser C.S. (2011). Overlapping and antagonistic activities of BASIC PENTACYSTEINE genes affect a range of developmental processes in Arabidopsis. Plant J. 66: 1020-1031.

Orphanides G., LeRoy G., Chang C.H., Luse D.S. and Reinberg D. (1998). FACT, a factor that facilitates transcript elongation through nucleosomes. Cell 92: 105-116.

Pinyopich A., Ditta G.S., Savidge B., Liljegren S.J., Baumann E., Wisman E. and Yanofsky, M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424: 85-88.

Sangwan I. and O'Brian M.R. (2002). Identification of a soybean protein that interacts with GAGA element dinucleotide repeat DNA. Plant Physiol. 129: 1788-1794.

Santi L., Wang Y., Stile M.R., Berendzen K., Wanke D., Roig C., Pozzi C., Müller K., Müller J., Rohde W. and Salamini F. (2003). The GA octodinucleotide repeat binding factor BBR participates in the transcriptional regulation of the homeobox gene Bkn3. Plant J. 34: 813-826.

Shimojima T., Okada M., Nakayama T., Ueda H., Okawa K., Iwamatsu A., Handa H. and Hirose S. (2003). Drosophila FACT contributes to Hox gene expression through physical and functional interactions with GAGA factor. Genes Dev. 17: 1605-1616.

Venglat S.P. and Sawhney V.K. (1996). Benzylaminopurine induces phenocopies of floral meristem and organ identity mutants in wild-type Arabidopsis plants. Planta 198: 480-487.

Wanke D., Hohenstatt M.L., Dynowski M., Bloss U., Hecker A., Elgass K., Hummel S., Hahn A., Caesar K., Schleifenbaum F., Harter K. and Berendzen K.W. (2011). Alanine zipper-like coiled-coil domains are necessary for homotypic dimerization of plant GAGA-factors in the nucleus and nucleolus. PLoS One 10;6: e16070.

Xiao H., Sandaltzopoulos R., Wang H.M., Hamiche A., Ranallo R., Lee K.M., Fu D. and Wu C. (2001). Dual functions of largest NURF subunit NURF301 in nucleosome sliding and transcription factor interactions. Mol Cell 8: 531-543.

Yanai O., Shani E., Dolezal K., Tarkowski P., Sablowski R., Sandberg G., Samach A. and Ori N. (2005) Arabidopsis KNOXI proteins activate cytokinin biosynthesis. Curr Biol. 15: 1566-1571.

Zhao Z, Andersen SU, Ljung K, Dolezal K, Miotk A, Schultheiss SJ, Lohmann JU. (2010) Hormonal control of the shoot stem-cell niche. Nature. 2010 Jun 24;465(7301):1089-92.

CHAPTER 4

The Transcription Factors BEL1 and SPL Are Required for Cytokinin and Auxin Signaling During Ovule Development in *Arabidopsis*

Stefano Bencivenga,[a] Sara Simonini,[a] Eva Benková,[b,c] and Lucia Colomboa,[d]

[a] Dipartimento di Bioscienze, Università degli Studi di Milano, 20133 Milan, Italy

[b] Department of Plant Systems Biology, Flanders Institute for Biotechnology, Universiteit Gent, 9052 Ghent, Belgium

[c] Department of Functional Genomics and Proteomics, Faculty of Science and Central European Institute of Technology, Masaryk University, 62500 Brno, Czech Republic

d Istituto di Biofisica, Consiglio Nazionale delle Ricerche, 20133 Milan, Italy

Plant Cell. 2012 Jul 10

ABSTRACT

Hormones, such as auxin and cytokinin, are involved in the complex molecular network that regulates the coordinated development of plant organs. Genes controlling ovule patterning have been identified and studied in detail; however, the roles of auxin and cytokinin in ovule development are largely unknown. Here we show that key cytokinin pathway genes, such as i*sopentenyt-ransferase* and cytokinin receptors, are expressed during ovule development. Also, in a *cre1-12 ahk2-2 ahk3-3* triple mutant with severely reduced cytokinin perception, expression of the auxin efflux facilitator *PIN-FORMED 1* (*PIN1*) was severely reduced. In sporocyteless/nozzle (spl/nzz) mutants, which show a similar phenotype to the *cre1-12 ahk2-2 ahk3-3* triple mutant, *PIN1* expression is also reduced. Treatment with the exogenous cytokinin N6-benzylaminopurine also altered both auxin distribution and patterning of the ovule; this process required the homeodomain transcription factor BELL1 (BEL1). Thus, this article shows that cytokinin regulates ovule development through the regulation of *PIN1*. Furthermore, the transcription factors BEL1 and SPL/NZZ, previously described as key regulators of ovule development, are needed for the auxin and cytokinin signaling pathways for the correct patterning of the ovule.

INTRODUCTION

The plant hormone cytokinin acts in concert with auxin, and the different accumulation of these two hormones is known to be important for the development of plant organs (Skoog and Miller, 1957). Despite increasing evidence for the importance of hormonal networks in the regulation of plant development, the role of auxin and cytokinin in ovule patterning is still unknown. There is evidence that both hormones play important functions in ovule primordia formation and female fertility. Plants with reduced cytokinin production or perception show a drastic reduction in ovule numbers and female fertility (Werner *et al.*, 2003; Hutchison *et al.*, 2006; Miyawaki *et al.*, 2006; Riefler *et al.*, 2006; Kinoshita-Tsujimura and Kakimoto, 2011). CYTOKININ INDE-PENDENT1 (CKI1) is known to be involved in cytokinin signaling, and the cki1 mutant shows female gametophyte defects (Kakimoto, 1996; Pischke *et al.*, 2002). When the amount of cytokinin increases, like in the *ckx3 ckx5* double mutant, the number of ovule primordia increases, confirming a clear correlation between cytokinin levels and ovule numbers (Bartrina *et al.*, 2011). Effects on ovule development have also been reported in plants treated with auxin efflux inhibitors, which develop a naked placenta (Nemhauser *et al.*, 2000). Furthermore, female gametophyte cell identity seems to be compromised when the expression of auxin synthesis or auxin response genes are modified (Pagnussat *et al.*, 2007). Although the role of hormones in ovule formation has been understudied, the genetic network controlling ovule development has been investigated for many years, and several key factors have been identified and characterized (reviewed in Colombo *et al.*, 2008). Among them, BELL1 (BEL1), a homeodomain transcription factor, has been reported to be one of the major factors controlling ovule pattering, in particular determining identity and development of the

integuments. In the *bel1* mutant, ovules deve-lop a single integument-like structure, which expresses carpel-specific genes (Robinson-Beers *et al.*, 1992; Reiser *et al.*, 1995; Brambilla *et al.*, 2007). It has been reported that the right balance between BEL1 and the MADS domain transcription factors AGAMOUS (AG) and SEEDSTICK (*STK*) is needed for the correct determination of integument identity (Brambilla *et al.*, 2007). Another important factor regulating ovule pattering is SPOROCYTELESS/ NOZZLE (SPL/NZZ), which is required for the development of the mega-sporocyte, from which the female gametophyte develops (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). Furthermore, SPL together with BEL1 has been shown to control chalaza development, because, in the *bel1* spl double mutant, the ovules develop as finger-like structures without integuments (Balasubramanian and Schneitz, 2002). Here we analyze the role of cytokinin in ovule development and show that an increase in cytokinin levels influences ovule patterning. These phenotypes are a consequence of a chan-ge in *PIN-FORMED 1* expression. *PIN1* is one of the best-studied auxin efflux facilitators, and recently it has been reported that, at least in roots, cytokinin negatively controls secondary root formation by regulating *PIN1* expression and consequently changing the auxin pattern along the root (Laplaze *et al.*, 2007; Dello Ioio *et al.*, 2008; Ruzicka *et al.*, 2009). The link between *PIN1* expression and cytokinin was further evidenced by the fact that in plants defective for the cytokinin receptors *ARABIDOPSIS* HISTIDINE KINASE4/CYTOKININ RESPONSE1 (AHK4/CRE1), AHK2, and AHK3, the expression of *PIN1* was compromised. The data we present here show an important role for the transcription factors BEL1 and SPL in the cytokinin-dependent regulation of *PIN1*, which is important for the correct development of the chalaza region in the ovule.

RESULTS

Analysis of the Cytokinin Pathway during Ovule Development

Recent studies indicated the involvement of auxin in controlling ovule development, including the formation of the megagametophyte (Benková *et al.*, 2003; Pagnussat *et al.*, 2009). However, so far little is known about the role of other plant hormones, such as cytokinin, in this process. A first step to investigate the possible role of cytokinin in ovule development was the analysis of the expression of genes involved in the cytokinin signaling pathway (Figure 1A). Among these, the genes encoding i*sopentenyl-transferases* (*IPT*), which are the principal enzymes responsible for cytokinin synthesis, were selected (Kakimoto, 2001; Sun *et al.*, 2003). Previously, it has been reported that *Arabidopsis thaliana IPT1* is the only *isopente-nyltransferase*–encoding gene that is expressed in ovules (Miyawaki *et al.*, 2004). We have analyzed in detail *IPT1* expression using 20 pistils at different stages of development from eight *IPT1pro:bglucuronidase* (*GUS*) plants (Miyawaki *et al.*, 2004). GUS expression was observed in all these plants in the whole ovule starting from stage 2-III (Figure 1B). During the following stages, GUS activity was detected in the funiculus and in the developing female gametophyte (Figures 1C to 1E). To detect the cytokinin signaling output (Figure 1A), we analyzed ovules at different stages of development in eight *Arabidopsis* plants (20 pistils each) containing the *TCSpro:green fluorescent protein (GFP)*

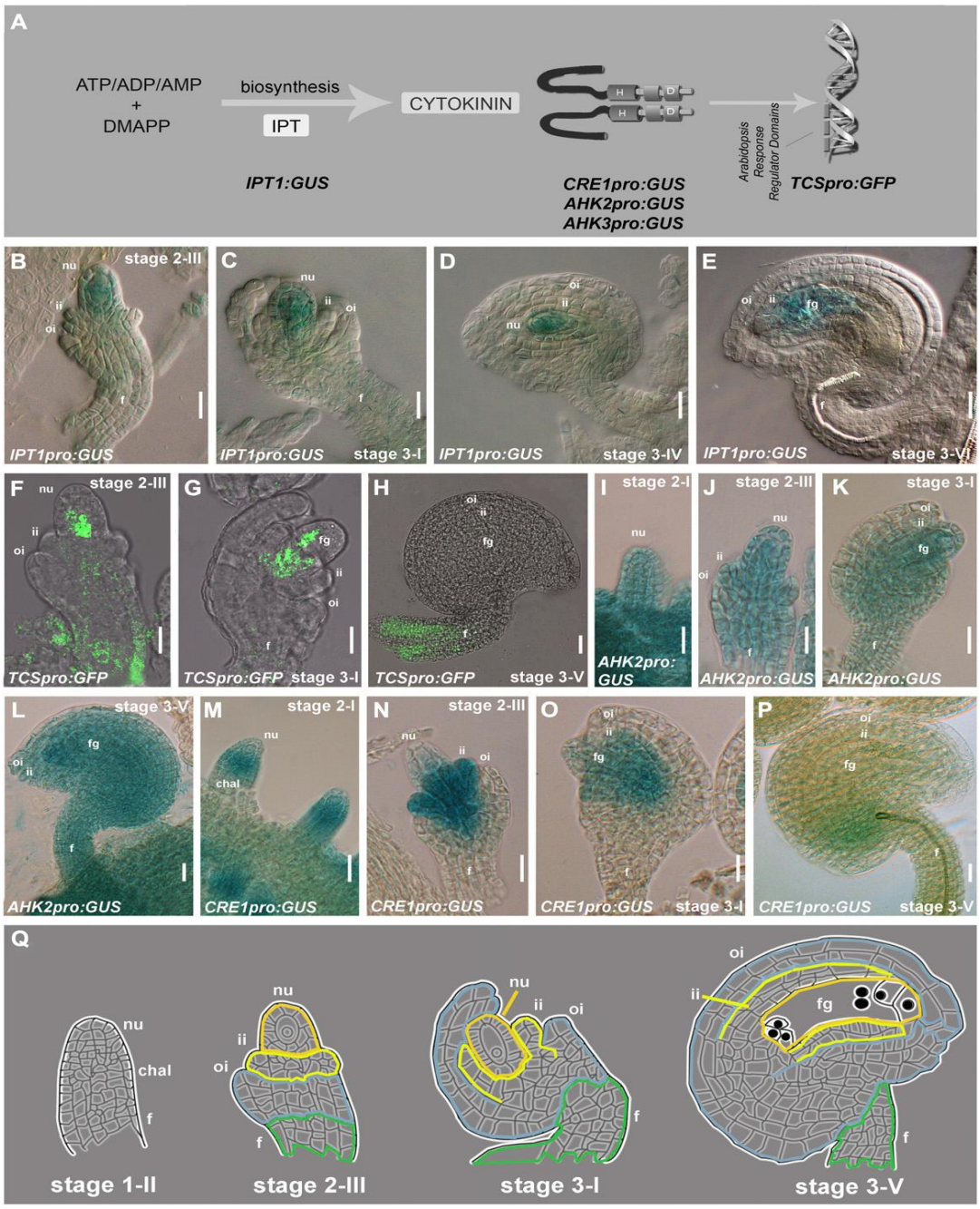


Figure 1. Analysis of the Cytokinin Pathway during Ovule Development.
Ovule stages as in Schneitz *et al.* (1995).
(A) Schematic representation of the cytokinin pathway and the genes analyzed in this article. **(B)** to **(E)** GUS expression in *IPT1pro:GUS* ovules from stage 2-III to stage 3-VI. **(F)** to **(H)** GFP expression in *TCSpro:GFP* ovules from stage 2-III to ovule stage 3-V. **(I)** to **(L)** GUS expression in *AHK2pro:GUS* ovules from stage 1-I to stage 3-V. **(M)** to **(P)** GUS expression *CRE1pro:GUS* ovules from stage 1-I to stage 3-V. **(Q)** Scheme of ovule development from stage 1-II to stage 3-V.
chal, chalaza; f, funiculus; fg, female gametophyte; ii, inner integument; oi, outer integument; n, n nucellus. Bars = 20 mm.

construct. TCS is a synthetic promoter, containing the B-type *Arabidopsis* response regulator binding motifs and the minimal 35S promoter (Müller and Sheen, 2008). The GFP signal was detected in the basal part of the nucellus and in the funiculus starting from stage 2-III (Figures 1F and 1G). At stage 3-V, the GFP signal was drastically reduced and was hardly visible except for the funiculus, where GFP expression remained detectable (Figure 1H). The receptors AHK2, AHK3, and AHK4/CRE1 are important components of the cytokinin signaling pathway and are needed for cytokinin signal transduction (Figure 1A).

These proteins are known to interact with cytokinins to start the multistep twocomponent

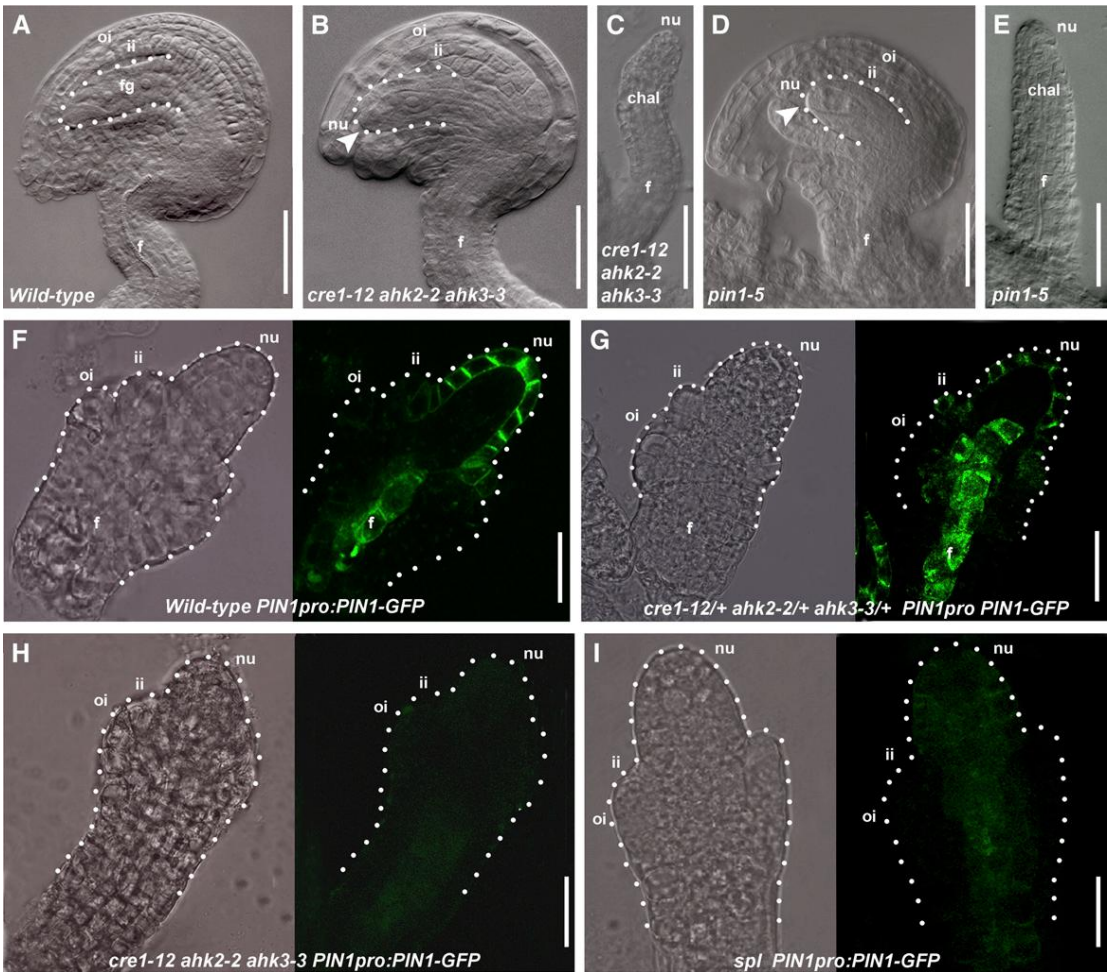


Figure 2. Role of Cytokinin in Ovule Development.

(A) Wild-type ovule at stage 3-V. The dotted line indicates the female gametophyte. **(B)** *cre1-12 ahk2-2 ahk3-3* ovule at stage 3-V. The female gametophyte arrested at stage FG1 (arrowhead). **(C)** cre1-*12 ahk2-2 ahk3-3* finger-like structure. **(D)** *pin1-5* ovule at stage 3-V. The female gametophyte arrested at stage FG1 (arrowhead). **(E)** *pin1-5* finger-like structure. **(F)** Wild-type ovule expressing *PIN1pro:PIN1-GFP*. **(G)** *cre1-12/+ ahk2-2/+ ahk3-3/+* ovule expressing *PIN1pro:PIN1-GFP.* **(H)** *cre1-12 ahk2-2 ahk3-3* triple mutant ovule expressing *PIN1pro:PIN1-GFP.* **(I)** spl ovule expressing *PIN1pro:PIN1-GFP*. **(F)** to **(I)** Pictures taken using the bright field (left) and the dark field (right). The dotted line shows the ovule profile.

chal, chalaza; f, funiculus; fg, female gametophyte; ii, inner integument; nu, nucellus; oi, outer integument. Bars = 20 mm.

signaling pathway (Inoue *et al.*, 2001). To study the expression pattern of these three genes during ovule development, we analyzed transgenic plants containing the *CRE1pro:GUS*, *AHK2pro:GUS*, and *AHK3pro:GUS* constructs (Nishimura *et al.*, 2004). All three GUS lines showed activity in developing ovules. GUS expression driven by the *AHK2* regulatory region was observed during all stages of ovule development, starting from the early primordia stage (Figure 1I) until the ovule reached maturity (stage 3-V; Figures 1J to 1L). The same GUS activity was observed in *AHK3pro:GUS* lines (see Supplemental Figure 1 online). Transgenic plants containing the *CRE1pro:GUS* construct showed GUS expression in the chalaza region of the developing ovule primordia (Figure 1M).

Subsequently, the *CRE1* promoter maintains its activity in the chalaza and in the inner integuments until stage 3-I of ovule development (Figures 1N to 1O). After stage 3-I, the GUS signal drastically decreased (Figure 1P). This analysis showed that important components of the cytokinin pathway are expressed during ovule development.

Cytokinin Perception Is Required for *PIN1* Expression in Ovules

Because important genes for the cytokinin signaling pathway are expressed during *Arabidopsis* ovule development, we were interested to investigate the role of cytokinin during this process. Therefore, we analyzed the ovules of the c*re1-12 ahk2-2 ahk3-3* triple mutant, which is considered to have a dramatic reduction in cytokinin responses, including cytokinin primary-response gene induction (Higuchi *et al.*, 2004). As reported previously, the single and double mutants do not present a phenotype at the level of the ovule (Kinoshita- Tsujimura and Kakimoto, 2011), whereas the *cre1-12 ahk2-2 ahk3-3* triple mutant showed defects in the formation of the female gametophyte, which arrested at stage FG1-FG2 (Figure 2B) (Higuchi *et al.*, 2004). We analyzed two pistils of five *cre1-12 ahk2-2 ahk3-3* triple mutant plants and noticed a severe reduction in ovule number with respect to the wild type (see Supplemental Table 1 and Supplemental Figure 2 online). Furthermore, 10% of these ovules (50 out of 530) developed as finger-like structures (Figure 2C); in wild-type plants, this phenotypic defect was never observed. The *cre1-12 ahk2-2 ahk3-3* triple mutant phenotype is very similar, if not identical, to the weak *pin1-5* mutant phenotype. It is important to note that the weak *pin1-5* mutant does develop flowers with ovule-bearing carpels (Bennett *et al.*, 1996; Sohlberg *et al.*, 2006). We analyzed in detail ovule development in the *pin1-5* mutant and observed a reduction in ovule number with respect to the wild type (see Supplemental Table 1 and Supplemental Figure 2 online). Furthermore, in this mutant, 10% of the ovules (17 out of 184 analyzed) developed as finger-like structures (Figure 2E). A few ovules developed normally (37 out of 184 analyzed), whereas most of them (130 out of 184) (Figure 2D) showed an arrest in gametophyte development at stage FG1. It has been reported that cytokinin regulates *PIN1* expression in roots (Dello Ioio *et al.*, 2008; Ruzicka *et al.*, 2009); thus, we investigated whether cytokinin controls PIN1 expression in ovules as well and whether this regulation can explain the similarity in ovule phenotype between *pin1-5* and the *cre1-12 ahk2-2 ahk3-3* triple mutant. Therefore, we crossed the *PIN1pro:PIN1-GFP* marker line with the *cre1-12 ahk2-2 ahk3-*

3/AHK3 mutant. Eight (F3) plants with the *PIN1pro:PIN1-GFP* construct in the *cre1-12 ahk2-2 ahk3-3* triple mutant background were analyzed by confocal microscopy. Two *cre1-12/CRE1 ahk2-2/AHK2 ahk3-3/AHK3* plant *PIN1pro:PIN1-GFP* identified in the F1 generation were used as a control. We examined the ovules of 10 pistils in each of the two *cre1-12/CRE1 ahk2-2/AHK2 ahk3-3/AHK3* plants (Figure 2G), showing that *PIN1-GFP* is expressed in the funiculus, in the nucellus, and in the inner integument primordium at stage 2-III as in wild-type ovules (Benková *et al.*, 2003) (Figure 2F). In *PIN1pro:PIN1-GFP cre1-12 ahk2-2 ahk3-3* plants, PIN1-GFP was undetectable in the ovule of the 10 pistils of each of the 10 plants analyzed (Figure 2H). This strongly suggests that cytokinin is indeed important for the correct activation of *PIN1* expression in ovules.

The Transcription Factor SPL Is Required for *PIN1* Expression

To identify putative targets of the cytokinin signaling pathway that could be involved in the regulation of PIN1 expression, ovule phenotypes of the *cre1-12 ahk2-2 ahk3-3* triple mutant were compared with those of previously described mutants. Among them, the

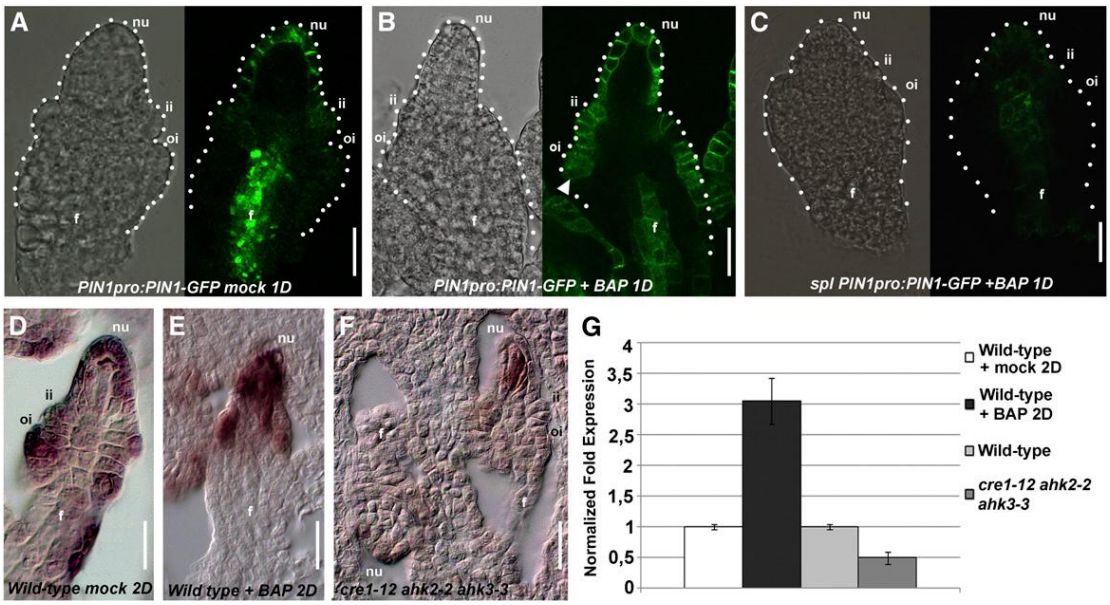


Figure 3. Analysis of BAP-Treated Ovules.

(A) Wild-type ovule at stage 2-III mock-treated, expressing *PIN1pro:PIN1-GFP* 1 d after treatment (1D). **(B)** Wild-type ovule at stage 2-III BAP-treated, expressing *PIN1pro:PIN1-GFP* 1 d after treatment. Arrow indicates ectopic *PIN1* expression. **(C)** *spl* ovule at stage 2-III BAP-treated, expressing *PIN1pro:PIN1-GFP* 1 d after treatment. **(D)** In situ hybridization with *SPL/NZZ* probe, on wild-type mock-treated ovule (2D, 2 d after treatment). **(E)** In situ hybridization with *SPL/NZZ* probe on wild-type BAP-treated ovule 2 d after treatment. **(F)** In situ hybridization with *SPL/NZZ* probe on *cre1-12 ahk2-2 ahk3-3* triple mutant ovule. **(G)** Quantitative *SPL/NZZ* expression analysis in wild-type BAP-treated plants and *cre1-12 ahk2-2 ahk3-3* triple mutant flowers by real-time RT-PCR. **(A)** to **(C)** Pictures were taken using bright field (left) and dark field (right). The dotted line shows the ovule's profile.

f, funiculus; fg, female gametophyte; ii, inner integument; nu, nucellus; oi, outer integument.

Bars = 20 mm.

spl/nzz mutant captured our attention. *SPL* is a gene encoding a putative transcription factor (Yang *et al.*, 1999), which is expressed throughout the ovule during its development (Schiefthaler *et al.*, 1999; Balasubramanian and Schneitz, 2000; Ito *et al.*, 2004; Sieber *et al.*, 2004). Although *spl* single mutant ovules have normal integuments, they do not develop the megaspore mother cell (only 5% of the ovules at stage 2-III showed a megaspore mother cell) (Balasubramanian and Schneitz, 2000). To analyze the *spl-1* mutant in more detail, we crossed *PIN1pro:PIN1-GFP* and *DR5rev-pro:GFP* reporter lines with plants heterozygous for the *spl-1* mutation. Analysis of GFP expression in homozygous *spl-1* mutant plants showed that the GFP signal driven by the *PIN1* promoter in the nucellus, the inner integument and the funiculus was very weak (Figure 2I) when compared with *spl-1/SPL* control plants (see Supplemental Figure 2 online) that segregated from the same F2 population. Furthermore, *DR5rev-pro:GFP spl-1* plants did not show a GFP signal at stage 2-III, although at early stages (stage 1-II) of development, the GFP signal was detected in fewer ovules (53 out of 494 ovules analyzed) (see Supplemental Figure 2 online). By contrast, the GFP signal was clearly visible in all ovules of *spl-1/SPL* control plants (see Supplemental Figure 2 online). Taken together, these results suggest that in ovules SPL seems to be required for *PIN1* expression.

SPL Is Required for Cytokinin-Induced *PIN1* Expression in Ovules

Because our results showed that *PIN1* expression in ovules was dependent on the cytokinin signaling pathway, we analyzed the effects of an increase in cytokinin levels in ovules by treating *Arabidopsis* flowers with the exogenous cytokinin N6-benzylaminopurine (BAP). BAP treatment has already been successfully used for flower meristem studies (Venglat and Sawhney, 1996; D'Aloia *et al.*, 2011). First, to investigate the effects of BAP application on cytokinin pathway activity, 10 transgenic plants containing the *TCSpro: GFP* construct (Müller and Sheen, 2008) were treated with BAP, and the ovules were analyzed 1 d after treatment. As a control, five *TCSpro:GFP* plants were treated with water only as a mock control. One d after the BAP treatment, the plants showed a general increase in the GFP signal in ovules, in particular at the level of the chalaza, suggesting a good penetrance of BAP (see Supplemental Figure 3 online). However, 1 d after the BAP treatment, the ovules still looked normal from a morphological point of view (see Supplemental Figure 3 online). Interestingly, the BAP treatment resulted in the formation of new primordia (in average 20 6 3 primordia in each of the 20 pistils that were analyzed) positioned among the ovules formed before the treatment (see Supplemental Figure 3 online). We have verified the identity of these new primordia by treating two *STKpro:GUS* plants with BAP. The ovule-specific *STK* promoter (Kooiker *et al.*, 2005) was shown to be active in these new primordia, indicating that these primordia have ovule identity (see Supplemental Figure 3 online). An increase in ovule number was also reported in the cytokinin oxidase *ckx5 ckx6* double mutant, which has increased endogenous cytokinin levels caused by absence of these oxidases (Bartrina *et al.*, 2011). To study the effect of increased levels of cytokinin on the regulation of *PIN1* expression, flowers of *PIN1pro:PIN1-GFP* lines were treated with BAP, and GFP expression in the ovules was analyzed by confocal microscopy. In *PIN1pro:PIN1-*

GFP plants 1 d after BAP treatment, the *PIN1-GFP* signal was present in 293 ovules out of 300 analyzed not restricted to the nucellus, the inner integument, and the inner region of the funiculus as was observed in the mock-treated control plants (300 ovules analyzed; Figure 3A) but was also detected in the outer integument and in the epidermal layer of the funiculus (Figure 3B). This suggests that cytokinin is able to trigger ectopic *PIN1* expression. We also treated 10 plants having the *PIN1pro:PIN1-GFP* construct in the *spl-1* mutant background and found that, in ovules of 10 pistils for each of the 10 plants that were analyzed, the *PIN1-GFP* signal was not induced by the BAP treatment (Figure 3C). This observation further strengthened our hypothesis that SPL is needed for *PIN1* expression. Furthermore, these data also suggest that cytokinin induced the expression of SPL. To investigate this in more detail, we studied the expression of *SPL* by *in situ* hybridization analysis using ovules treated with BAP and *cre1-12 ahk2-2 ahk3-3* triple mutant ovules. As shown in Figure 3D, *SPL* is expressed in ovule tissues at stage 2-III, as was reported previously (Balasubramanian and Schneitz, 2000; Sieber *et al.*, 2004). In BAP-treated plants, the SPL expression seemed to increase (Figure 3E), whereas in the *cre1-12 ahk2-2 ahk3-3* triple mutant, SPL transcripts were drastically reduced and only detectable in the nucellus (Figure 3F). To quantify the changes in *SPL* expression in BAP-treated pistils and in *cre1-12 ahk2-2 ahk3-3* triple mutant pistils, we performed real-time PCR analysis (Figure 3G). In BAP-treated pistils, SPL was upregulated with respect to mock-treated plants, whereas it was downregulated in *cre1-12 ahk2-2 ahk3-3* triple mutant ovules (Figure 3G), confirming the *in situ* hybridization analysis.

High Cytokinin Levels Modify Ovule Patterning

Interestingly, 2 d after BAP treatment, all ovules at stage 2-III developed instead of two integuments a single structure (which we named Cytokinin-Induced Structure [CK-IS]) (Figure 4A). The cytokinin receptors CRE1, AHK2, and AHK3 are important for ovule development, as reported here and by Higuchi *et al.* (2004). To understand whether the observed BAP-induced cytokinin receptors, five plants for each c*re1-12, ahk2-2, and ahk3-3* single mutant were treated with BAP. After 2 d, the effect of the BAP treatment was

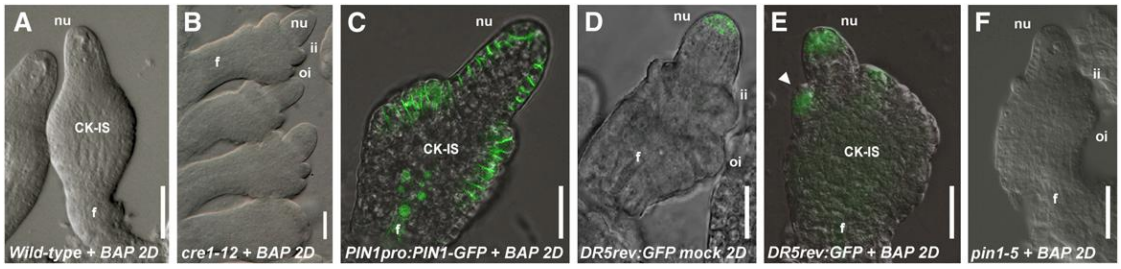


Figure 4. Effect on Ovule Development after 2 d of BAP Treatment.

(A) Wild-type ovule 2 d (2D) after BAP treatment. **(B)** *cre1-12* ovule 2 d after BAP treatment. **(C)** Wild-type ovule of *PIN1pro:PIN1-GFP* plants 2 d after BAP treatment. **(D)** and **(E)** *DR5rev-pro:GFP* ovule 2 d after the mock treatment **(D)** or BAP treatment **(E)**. Arrow indicates ectopic *DR5rev:GFP* signal in the CK-IS. **(F)** *pin1-5* ovule 2 d after BAP treatment.

f, funiculus; ii, inner integument; nu, nucellus; oi, outer integument. Bars = 20 mm.

evaluated in terms of the number of ovules that formed the CK-IS instead of developing two integuments, like in mock-treated control plants (see Supplemental Table 2 online). Interestingly, only *cre1-12* mutant plants treated with BAP developed two integuments, whereas in the other single mutants, integument development was affected (Figure 4B), suggesting that the CRE1 receptor has a major role in the response to cytokinin in the chalaza region. The analysis of the *PIN1pro:PIN1-GFP* plants treated with BAP showed that, 2 d after BAP treatment, *PIN1-GFP* expression was observed in the epidermal layer of the CK-IS that developed from the chalaza (Figure 4C). Considering that the *PIN1-GFP* ectopic expression was seen before CK-IS formation (Figure 3B), this suggests that the phenotype of the BAP-treated ovules is a consequence of ectopic *PIN1-GFP* expression. To monitor the effects of the ectopic *PIN1* expression on the formation of auxin maxima, the same BAP treatment experiments were done using plants containing the GFP reporter gene driven by the auxin-induced DR5 promoter construct. In two mock-treated *DR5rev-pro:GFP* control plants, the GFP signal was detected in the nucellus of all 30 ovules that we analyzed (Figure 4D), confirming the auxin pattern that was reported by Benková *et al.* (2003). In BAP-treated *DR5rev-pro:GFP* plants, the GFP signal was also detected inside the CK-IS structures that developed from the chalaza (Figure 4E). These auxin maxima in the CK-IS structures are in agreement with the observed *PIN1-GFP* localization in BAP-treated plants (Figure 4C). Interestingly, *pin1-5* mutant plants were insensitive to the BAP treatment (Figure 4G); the ovules developed integuments as in *pin1-5* mock-treated plants. These results show that high cytokinin levels resulted in a deregulation of *PIN1* expression causing severe defects in ovule development. All together, these results corroborate the hypothesis that the role of cytokinin in ovule development is mediated by the PIN1-dependent auxin distribution.

The Homeodomain Transcription Factor BEL1 Is Involved in *PIN1* Regulation

It has been reported that one of the major players in chalaza development is the homeodomain transcription factor BEL1 (Robinson-Beers *et al.*, 1992; Reiser *et al.*, 1995). *BEL1* is expressed in the chalaza of ovules starting from stage 1-II of development. The bel1-1 mutant shows interesting similarities with the ovule phenotype obtained by BAP treatment, because in *bel1-1*, the two integuments (Figure 5A) are replaced by a single structure (Figure 5B) that resembles the CK-IS structure we observed in the BAP-treated plants (Figure 5C). Moreover, it has been published that, in the *bel1-1* mutant, this structure is at later developmental stages converted into a carpel-like structure (Robinson-Beers *et al.*, 1992; Brambilla *et al.*, 2007), as has been reported to happen after BAP treatment (Venglat and Sawhney, 1996). These data suggest a possible interaction between BEL1 and cytokinin signaling in the ovule. To understand whether cytokinin controls BEL1 expression, we performed *in situ* hybridization using wild-type ovules mock-treated (control plants) or treated with BAP. In control plants, *BEL1* expression was observed in the chalaza and in the developing integuments, which is similar to wild-type plants (Figure 5D). In BAP-treated plants, BEL1 expression was restricted to a small group of cells at the basal part of the CK-IS (Figure 5E). Furthermore, *BEL1* was expressed similar to the wild type in *cre1-12 ahk2-2*

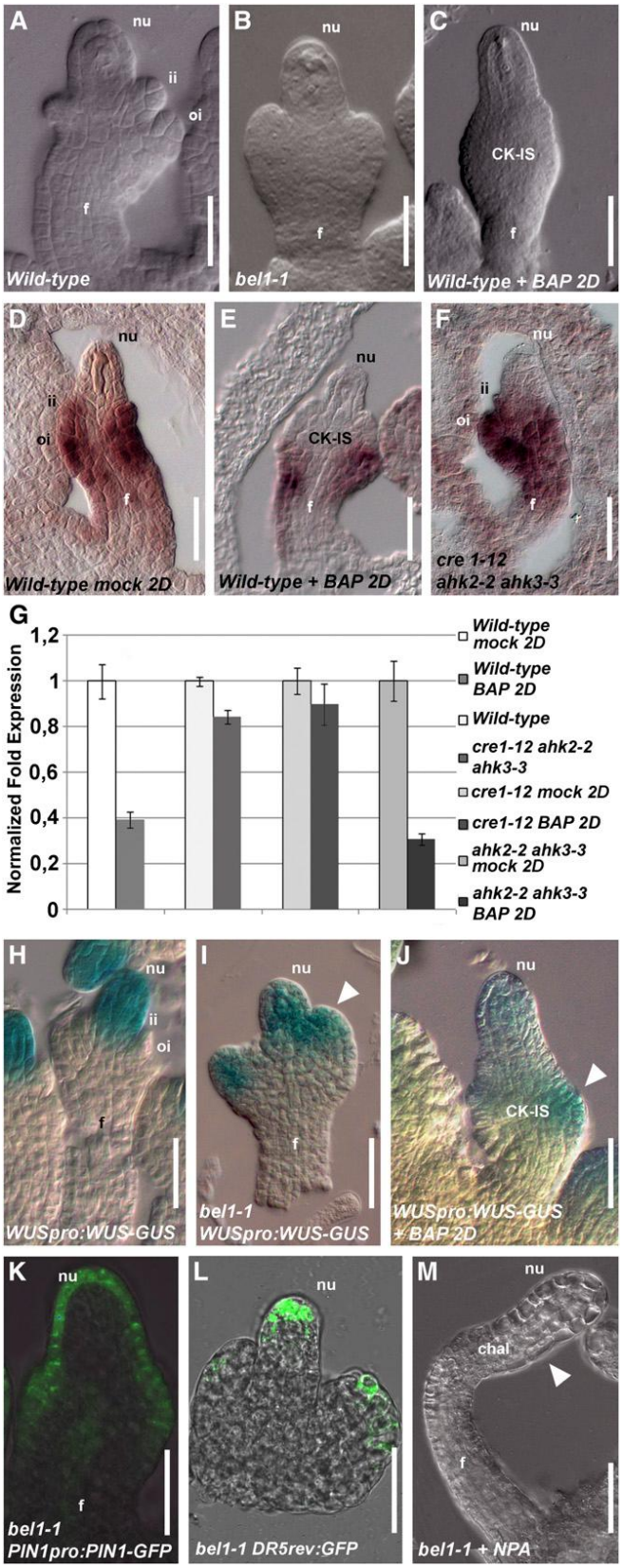


Figure 5. *BEL1* Expression Is Regulated by Cytokinin

(A) Wild-type ovule, stage 2-III. **(B)** bel1-1 ovule, stage 2-III. **(C)** Wild-type ovule, stage 2-III, 2 d (2D) after BAP treatment. **(D)** In situ hybridization on wild-type ovule with *BEL1* probe. **(E)** In situ hybridization on wild-type ovule treated with BAP using the *BEL1* probe 2 d after treatment. **(F)** In situ hybridization on *cre1-12 ahk2-2 ahk3-3* triple mutant ovule with *BEL1* probe. **(G)** Quantitative *BEL1* expression analysis by real-time RT-PCR. Wildtype mock-treated or BAP-treated 2 d after treatment, wild-type and *cre1-12 ahk2-2 ahk3-3* triple mutant flowers, *cre1-12* single mutant, and *ahk2-2 ahk3-3* double mutant 2 d after mock treatment or BAP treatment. **(H)** to **(J)** *WUSpro:WUS-GUS* activity in wild-type ovule **(H)**, *bel1-1* ovule **(I)**, and in a wild-type ovule 2 d after BAP treatment **(J)**. The ovules are at stage 2-III/3-I. The white arrowhead indicates ectopic *WUSpro:WUS-GUS* expression in the aberrant structures of the ovules ([I] to [J]). **(K)** *PIN1pro:PIN1-GFP* in *bel1-1* ovule. **(L)** *DR5rev-pro:GFP* in *bel1-1* ovule. The ovule is at stage 2-III. **(M)** *bel1-1* ovule treated with NPA. The arrowhead indicates the region where the *bel1-1* structure is formed.

chal, chalaza; f, funiculus; ii, inner integument; nu, nucellus; oi, outer integument.

Bars = 20 mm.

ahk3-3 triple mutant ovules (Figure 5F). To quantify *BEL1* expression, we performed real-time PCR, confirming that in BAP-treated plants, *BEL1* was downregulated, whereas in *cre1-12 ahk2-2 ahk3-3* triple mutant ovules, *BEL1* was expressed similar to the wild type (Figure 5G). We also quantified BEL expression in the BAP-treated *cre1-12* mutant and in the *ahk2-2 ahk3-3* double mutant (Figure 5G), which confirmed that the cytokinin regulation of *BEL1* expression in the chalaza is mediated by the CRE receptor. These data are consistent with the observed phenotypes and suggest that cytokinin might control *BEL1* expression. To corroborate this conclusion, we analyzed the regulation of *WUSCHEL* (*WUS*) expression. WUS is expressed in the nucellus (Figure 5H; Gross-Hardt *et al.*, 2002), but in the *bel1-1* mutant, *WUS* is ectopically expressed in the chalaza (Figure 5I; Brambilla *et al.*, 2007). Based on this observation, it has been suggested previously that BEL1 negatively regulates *WUS* expression (Brambilla *et al.*, 2007). We analyzed the ovules (two pistils for each of three BAP treated plants) (Figure 5J) containing a *WUSpro:WUS-GUS* construct and showed that GUS in these plants is ectopically expressed in the chalaza, as observed in bel1-1 ovules (Figure 5I), supporting the observed downregulation of *BEL1* expression after BAP treatment. To understand the role of BEL1 in *PIN1* regulation in ovules*, PIN1pro:PIN1-GFP* and *DR5rev-pro:GFP* constructs were introduced in the bel1-1 mutant background. As shown in Figure 5K, in the *bel1-1* mutant, the *PIN1-GFP* expression profile was similar to the profile that was observed in the *PIN1pro:PIN1-GFP* plants treated with BAP (Figures 3B and 4C), suggesting that BEL1 is important for the correct expression of *PIN1*.

Because *BEL1* expression was deregulated on application of exogenous cytokinin, we were curious whether correct auxin fluxes were dependent on *BEL1* activity. This would suggest that the *bel1-1* mutant phenotype is caused by changes in auxin fluxes, as we showed for the BAP-treated plants (Figure 5L). To investigate this, we treated *bel1-1* mutant plants with the auxin transport inhibitor N-1-naphthylphthala-mic acid (NPA). Analysis of these plants showed that after 2 d of treatment, finger-like ovules were obtained (Figure 5M), suggesting that formation of the abnormal structures in the *bel1-1* mutant is mediated, as in BAP-treated plants, by *PIN1* ectopic expression. In conclusion, we found that cytokinin is involved in ovule development by modulating auxin fluxes through the control of *PIN1* expression. Furthermore, our data suggest that the transcription factors NZZ/SPL and BEL1 play an important role in this hormonal network in ovules.

DISCUSSION

Regulation of *PIN1* Expression Requires SPL and BEL1 in Ovules

To integrate the known molecular network controlling ovule patterning with the hormonal regulation of this process, we have selected well-characterized transcription factor mutants with ovule phenotypes that resemble those obtained by the increase in cytokinin levels or mutations in cytokinin receptors. SPL/NZZ is a transcription factor expressed throughout the ovule and is needed for correct nucellus development and together with *BEL1* for chalaza formation (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999; Balasubramanian and Schneitz, 2002). Previously it was suggested that SPL is involved in auxin homeostasis (Li *et al.*, 2008). The

activation-tagged mutant *spl-D* showed an auxin-related defective phenotype, such as reduced apical dominance and a reduced number of lateral roots. Furthermore, the ARF, *YUC2*, and *YUC6* genes were downregulated in this mutant (Li *et al.*, 2008). We found that in the *spl* mutant, *PIN1* expression was compromised, suggesting that *SPL* is important for *PIN1* expression. Interestingly, an increase in exogenous cytokinin levels in the *spl* mutant background did not result in a change in *PIN1* expression, whereas in wild-type flowers treated with cytokinin (BAP), *PIN1* expression was strongly increased. This clearly indicates that for cytokinin-mediated *PIN1* expression, the SPL function is required (Figure 6). All together, these findings attribute to SPL a master role in auxin-dependent ovule developmental processes. As mentioned previously, Balasubramanian and Schneitz (2002) proposed that BEL1 works together with NZZ/SPL for the proper formation of the chalaza, because in *nzz/spl bel1* double mutant ovules, no chalaza structures developed, and finger-shaped organs formed instead. The mechanism behind the redundant function of these two different transcription factors involved in ovule development remained unclear. Our findings suggest a scenario in which the bel1 phenotype is caused by an ectopic expression of *PIN1* and that NZZ/SPL is essential for *PIN1* expression in the ovules. We therefore propose that the transcription factor SPL is necessary for the ectopic expression of *PIN1* in the *bel1* mutant. If the NZZ/SPL function is missing in the *bel1* mutant, the ectopic expression of *PIN1* is not possible, and for this reason a *bel1 nzz/spl* double mutant phenotype is similar to the *bel1* mutant treated with the auxin flux inhibitor NPA (Figure 6).

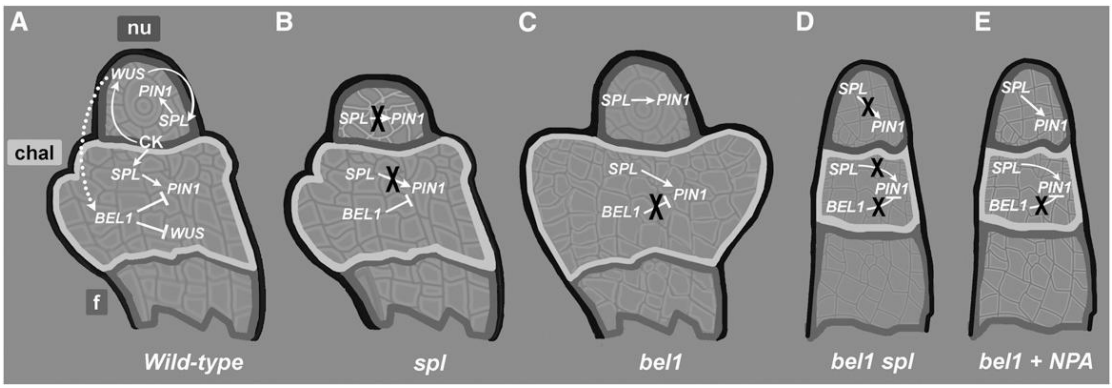


Figure 6. BEL1 and SPL Integrate Hormonal Signaling in Ovules.
(A) In wild-type ovules, cytokinin activates WUS, which promotes the expression of *SPL* in the nucellus (nu) (Sieber *et al.,* 2004) and BEL1 in the chalaza (chal). *PIN1* is activated in the nucellus by *SPL* and repressed in the chalaza by BEL1, which in turn represses WUS (Brambilla *et al.,* 2007). f, funiculus. **(B)** In *spl* mutant ovules, *PIN1* is not expressed, leading to a premature block of female gametophyte development and phenocopies the *pin1-5* mutant. **(C)** In the *bel1* mutant, *PIN1* is upregulated and is also expressed in the chalaza region, where normally it is not present. **(D)** In finger-like *bel1 spl* double mutant ovules, *PIN1* is not expressed in the ovule. **(E)** The application of exogenous NPA to the *bel1* mutant triggers the formation of finger-like ovules, because the inhibition of the auxin flux by NPA treatment avoids the formation of the aberrant structures typical for bel1 ovules.

Similarities in the WUS Regulatory Networks in Ovules and the Shoot Apical Meristem

The *bel1 nzz/spl* double mutant phenotype is similar to the phenotype previously described for *wus* mutant ovules (Gross-Hardt *et al.*, 2002). In the *bel1* mutant, WUS is ectopically expressed in the chalaza (Brambilla *et al.*, 2007). Confirming this, *WUS* ectopic expression was also observed in wild-type plants treated with exogenous cytokinin (BAP), showing that the downregulation of *BEL1* caused by the increase of cytokinin levels caused the same effect on *WUS* expression (Figure 6). Interestingly, regulation of *WUS* expression in ovules seems to be similar to the regulation of this gene in the shoot apical meristem. For instance, in the shoot apical meristem, cytokinin is important for *WUS* expression (Gordon *et al.*, 2009), and we have shown that SPL/NZZ might be involved in cytokininmediated *WUS* expression (Figure 6). WUS and WUSCHEL RELATED HOMEOBOX are known to act in a non–cell-autonomous manner for the maintenance of stem cells both in shoot apical and root apical meristems (Brand *et al.*, 2000; Schoof *et al.*, 2000). This stem cell maintenance depends on a negative feedback loop between *WUS* and *CLAVATA3* (*CLV3*) (Brand *et al.*, 2000; Schoof *et al.*, 2000). In ovules, *WUS* is expressed in the nucellus (Figure 6) and plays an important role in the chalaza, promoting integument development (Gross-Hardt *et al.*, 2002). Furthermore, WUS might promote in a non–cellautonomous manner the expression of *BEL1* in the chalaza, which as already proposed, negatively regulates *WUS* expression (Brambilla *et al.*, 2007).

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (ecotype Columbia) plants were grown at 22°C under long-day (16-h light/8-h dark) conditions. The *Arabidopsis* lines that were obtained from the European *Arabidopsis* Stock Centre collection are *spl-1* (N6586), *bel1-1* (N3090). *TCSpro* (two-component-output-sensor):*GFP*, *AHK2pro:GUS*, *AHK3pro:GUS*, *CRE1/AHK4pro:GUS*, *DR5rev-pro:GFP*, *PIN1pro:PIN1:GFP*, *cre1-12*, *ahk2-2*, and *ahk3-3* seeds were provided by Jirí Friml (Ghent University). *WUSpro:WUS-GUS* seeds were provided by Thomas Laux (University of Freiburg). *IPT1pro:GUS* seeds were provided by Tatsuo Kakimoto (Osaka University).

The observed ovule phenotypes were consistent in the F2, F3, and F4 segregating populations and the backcross population, which was made to introduce a reporter gene construct. This indicates that the observed phenotypic effects are not caused by differences in the ecotype background of our mutants.

The *cre1-12 ahk2-2 ahk3-3 PIN1pro:PIN1-GFP* lines were obtained by crossing *PIN1pro:PIN1-GFP* plants with *cre1-12 ahk2-2 ahk3-3/AHK3* plants. F3 *cre1-12 ahk2-2 ahk3-3* plants homozygous for *PIN1pro:PIN1-GFP* were selected. The *PIN1pro:PIN1-GFP* plants were crossed with the *spl/SPL* mutant. F3 *spl/nzz* plants homozygous for *PIN1pro:PIN1-GFP* were selected, and GFP expression was analyzed in the root as positive control (Benková *et al.*, 2003).

Genotyping

To genotype for the spl allele, the following primers were used: SPL-F (59-GGCGAGATCCGGACAGAGAC) and SPL-R (59-AGAAGCGTTAAACATTTGAGGATT) and Ds primers DS 3-3A (59-TCGTTTCCGTCCCGCAAGT) Roles of Auxin and Cytokinin in Ovules 9 of 12 or DS 5 to 3A (59-CGGTCGGTACGGGATTTTCC). The bel1-1 allele contains a C-to-T transition at nucleotide 497, which

introduces a BsaAI restriction site. The *bel1-1* allele was identified by BsaAI digestion of PCR products amplified with the primers 59-GAGAG ACATGGCAAGAGATCAG and 59- GAGCATGGAGAGCAACTTGG. To identify the presence of the T-DNA encoding PIN1pro:PIN1-GFP, the following primers were used: PIN1-RP (59-CCAGTACGTGGAGAGGGAAG) and GFP-LP (59-GAAAGTAGTGACAAGTGTTGGC).

BAP Treatment

BAP was obtained from Sigma-Aldrich and was used at a concentration of 10-3 M. Plants were treated once with 30 mL of a BAP solution or a solution of distilled water for mock-treated controls (both in 0.05% Tween 20). Solutions were applied directly onto the inflorescences, and then the plants were covered with a plastic transparent bag for 1 d. NPA was used at a concentration of 1 mMand was applied as described for the BAP treatment.

Microscopy

To analyze ovule development, flowers at different developmental stages were cleared and analyzed as described previously (Brambilla *et al.*, 2007). All GUS assays were performed overnight as described previously (Liljegren *et al.*, 2000) or with a different clearing method according to Jones-Rhoades *et al.* (2007). Samples were incubated in clearing solution, dissected, and observed using a Zeiss Axiophot D1 microscope equipped with differential interference contrast optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1) For confocal laser scanning microscopy, dissected ovules were mounted in water and observed with a SP2 Leica confocal microscope and SPE Leica confocal with a 488-nm argon laser line for excitation of GFP fluorescence. Emissions were detected between 505 and 580 nm. Using a 633 water-immersion objective (numerical aperture (NA) = 1.25, pinhole), confocal scans were performed with the pinhole at 1 airy unit.

***In situ* Hybridization and Real-Time PCR**

In situ hybridization was performed as described by Dreni *et al.* (2011). The *SPL/NZZ* and *BEL1* specific antisense probes were amplified according to Balasubramanian and Schneitz (2000). For expression analysis, total RNA was extracted using NucleoSpin RNA Plant KIT (Macherrey-Nagel) and was then subjected to reverse transcription using the ImProm-II Reverse Transcription System (Promega). The cDNAs were standardized relative to *UBIQUITIN10* (*UBI10*), *ACTIN8* (*ACT8*), *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*PP2A* [At1g13320]) transcripts, and gene expression analysis was performed using the iQ5 Multi Color Real-Time PCR detection system (Bio-Rad) with a SYBR Green PCR Master Mix (Bio-Rad). Baseline and threshold levels were set according to the manufacturer's instructions.

Accession Numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: *STK* (At4G09960), *BEL1* (At5G41410), *SPL/NZZ* (At4G27330), *WUS* (At2G17950), *PIN1* (At1G73590), *AHK2* (At5G35750), *AHK3* (At1G27320), *AHK4/CRE1* (At2G01830), *IPT1* (At1G68460), *CKX5* (At1g75450), and *CKX6* (At3g63440).

SUPPLEMENTAL DATA

Supplemental Figure 1. GUS Expression in *AHK3pro:GUS* Ovules from Stage 1-II to Stage 3-IV.

Supplemental Figure 2. Ovules of *cre1-12 ahk2-2 ahk3-3* and *pin1-5* Mutants; *PIN1pro:PIN1-GFP* and *DR5rev-pro:GFP* Analyses in *spl-1* and *spl-1/SPL* Plants.

Supplemental Figure 3. Ovule Development after BAP Treatment.
Supplemental Table 1. Ovule Number in the *cre1-12 ahk2-2 ahk3-3* and *pin1-5* Mutants.
Supplemental Table 2. The Effect of BAP Treatment on the Cytokinin Receptor Mutants.

ACKNOWLEDGMENTS

We thank Tatsuo Kakimoto for providing the *IPT1pro:GUS* line, Agnieszka Bielach for technical help, and Simona Masiero and Martin M. Kater for their comments and critical reading of the article. This research was supported by Fondazione Cariplo and by a Starting Independent Research grant from the European Research Council (ERC-2007-Stg-207362-HCPO to E.B.) and the project CZ.1.07/2.3.00/ 20.0043 (to CEITEC, Central European Institute of Technology).

REFERENCES

Balasubramanian, S., and Schneitz, K. (2000). NOZZLE regulates proximal-distal pattern formation, cell proliferation and early sporogenesis during ovule formation in *Arabidopsis thaliana*. Development 127: 4227–4238.

Balasubramanian, S., and Schneitz, K. (2002). NOZZLE links proximaldistal and adaxial-abaxial pattern formation during ovule development in *Arabidopsis thaliana*. Development 129: 4291–4300.

Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmülling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. Plant Cell 23: 69–80.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, effluxdependent auxin gradients as a common module for plant organ formation. Cell 115: 591–602.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A. (1996). *Arabidopsis* AUX1 gene: A permease-like regulator of root gravitropism. Science 273: 948–950.

Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S., Kater, M.M., and Colombo, L. (2007). Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in *Arabidopsis*. Plant Cell 19: 2544–2556.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by CLV3 activity. Science 289: 617–619. 10 of 12 The Plant Cell

Colombo, L., Battaglia, R., and Kater, M.M. (2008). *Arabidopsis* ovule development and its evolutionary conservation. Trends Plant Sci. 13: 444–450.

D'Aloia, M., Bonhomme, D., Bouché, F., Tamseddak, K., Ormenese, S., Torti, S., Coupland, G., and Périlleux, C. (2011). Cytokinin promotes flowering of *Arabidopsis* via transcriptional activation of the FT paralogue TSF. Plant J. 65: 972–979.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. Science 322: 1380–1384.

Dreni, L., Pilatone, A., Yun, D., Erreni, S., Pajoro, A., Caporali, E., Zhang, D., and Kater, M.M. (2011). Functional analysis of all AGAMOUS subfamily members in rice reveals their roles in reproductive organ identity determination and meristem determinacy. Plant Cell 23: 2850–2863.

Gordon, S.P., Chickarmane, V.S., Ohno, C., and Meyerowitz, E.M. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. Proc. Natl. Acad. Sci. USA 106: 16529–16534.

Gross-Hardt, R., Lenhard, M., and Laux, T. (2002). WUSCHEL signaling functions in interregional communication during *Arabidopsis* ovule development. Genes Dev. 16: 1129–1138.

Higuchi, M. *et al.* (2004). In planta functions of the *Arabidopsis* cytokinin receptor family. Proc. Natl. Acad. Sci. USA 101: 8821–8826.

Hutchison, C.E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M.W., Maxwell, B.B., Perdue, T.D., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2006). The *Arabidopsis* histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. Plant Cell 18: 3073–3087.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature 409: 1060–1063.

Ito, T., Wellmer, F., Yu, H., Das, P., Ito, N., Alves-Ferreira, M., Riechmann, J.L., and Meyerowitz, E.M. (2004). The homeotic protein AGAMOUS controls microsporogenesis by regulation of SPOROCYTELESS. Nature 430: 356–360. **Kakimoto, T.** (1996). CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science 274: 982–985.

Kakimoto, T. (2001). Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. Plant Cell Physiol. 42: 677–685.

Kinoshita-Tsujimura, K., and Kakimoto, T. (2011). Cytokinin receptors in sporophytes are essential for male and female functions in *Arabidopsis thaliana*. Plant Signal. Behav. 6: 66–71.

Kooiker, M., Airoldi, C.A., Losa, A., Manzotti, P.S., Finzi, L., Kater, M.M., and Colombo, L. (2005). BASIC PENTACYSTEINE1, a GA binding protein that induces conformational changes in the regulatory region of the homeotic *Arabidopsis* gene SEEDSTICK. Plant Cell 17: 722–729.

Jones-Rhoades, M.W., Borevitz, J.O., and Preuss, D. (2007). Genome- wide expression profiling of the *Arabidopsis* female gametophyte identifies families of small, secreted proteins. PLoS Genet. 3: 1848–1861.

Laplaze, L. *et al.* (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell 19: 3889–3900.

Li, L.-C., Qin, G.-J., Tsuge, T., Hou, X.-H., Ding, M.-Y., Aoyama, T., Oka, A., Chen, Z., Gu, H., Zhao, Y., and Qu, L.-J. (2008). SPOROCYTELESS modulates YUCCA expression to regulate the development of lateral organs in *Arabidopsis*. New Phytol. 179: 751–764.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L., and Yanofsky, M.F. (2000). SHATTERPROOF MADS-box genes
control seed dispersal in *Arabidopsis*. Nature 404: 766–770.

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: Tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. 37: 128–138.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G., and Kakimoto, T. (2006). Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA 103: 16598–16603.

Müller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature 453: 1094–1097.

Nemhauser, J.L., Feldman, L.J., and Zambryski, P.C. (2000). Auxin and ETTIN in *Arabidopsis* gynoecium morphogenesis. Development 127: 3877–3888.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. Plant Cell 16: 1365–1377.

Pagnussat, G.C., Alandete-Saez, M., Bowman, J.L., and Sundaresan, V. (2009). Auxin-dependent patterning and gamete specification in the *Arabidopsis* female gametophyte. Science 324: 1684–1689.

Pagnussat, G.C., Yu, H.J., and Sundaresan, V. (2007). Cell-fate switch of synergid to egg cell in *Arabidopsis* eostre mutant embryo sacs arises from misexpression of the BEL1-like homeodomain gene BLH1. Plant Cell 19: 3578–3592.

Pischke, M.S., Jones, L.G., Otsuga, D., Fernandez, D.E., Drews, G. N., and Sussman, M.R. (2002). An *Arabidopsis* histidine kinase is essential for megagametogenesis. Proc. Natl. Acad. Sci. USA 99: 15800–15805.

Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G.W., and Fischer, R.L. (1995). The BELL1 gene encodes a homeodomain protein involved in pattern formation in the *Arabidopsis* ovule primordium. Cell 83: 735–742.

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell 18: 40–54.

Robinson-Beers, K., Pruitt, R.E., and Gasser, C.S. (1992). Ovule development in wild-type *Arabidopsis* and two female-sterile mutants. Plant Cell 4: 1237–1249.

Ruzicka, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., Van Montagu, M.C., and Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc. Natl. Acad. Sci. USA 106: 4284–4289.

Schiefthaler, U., Balasubramanian, S., Sieber, P., Chevalier, D., Wisman, E., and Schneitz, K. (1999). Molecular analysis of NOZZLE, a gene involved in pattern formation and early sporogenesis during sex organ development in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA
96: 11664–11669.

Schneitz, K., Hülskamp, M., and Pruitt, R.E. (1995). Wild-type ovule development in *Arabidopsis thaliana*: A light microscope study of cleared whole-mount tissue. Plant J. 7: 731–749.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F., Jürgens, G., and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100: 635–644.

Sieber, P., Gheyselinck, J., Gross-Hardt, R., Laux, T., Grossniklaus, U., and Schneitz, K. (2004). Pattern formation during early ovule development in *Arabidopsis thaliana*. Dev. Biol. 273: 321–334.

Skoog, F., and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11: 118–130.

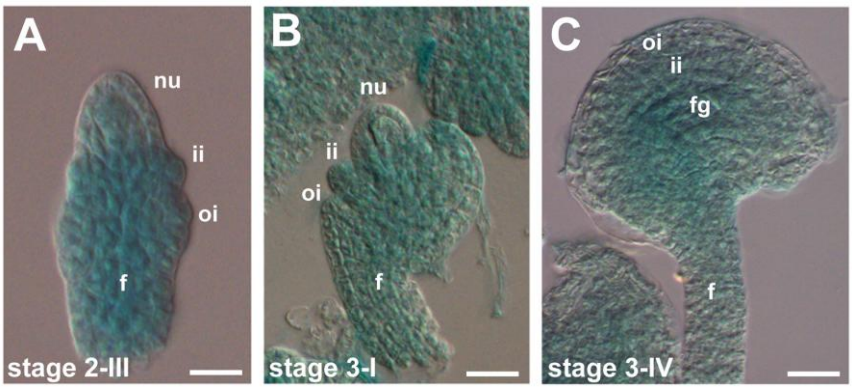
Sohlberg, J.J., Myrenås, M., Kuusk, S., Lagercrantz, U., Kowalczyk, M., Sandberg, G., and Sundberg, E. (2006). STY1 regulates auxin homeostasis and affects apical-basal patterning of the *Arabidopsis* gynoecium. Plant J. 47: 112–123.

Sun, J.Q., Niu, Q.W., Tarkowski, P., Zheng, B.L., Tarkowska, D., Sandberg, G., Chua, N.H., and Zuo, J.R. (2003). The *Arabidopsis* AtIPT8/PGA22 gene encodes an isopentenyl transferase that is involved in de novo cytokinin biosynthesis. Plant Physiol. 131: 167–176.

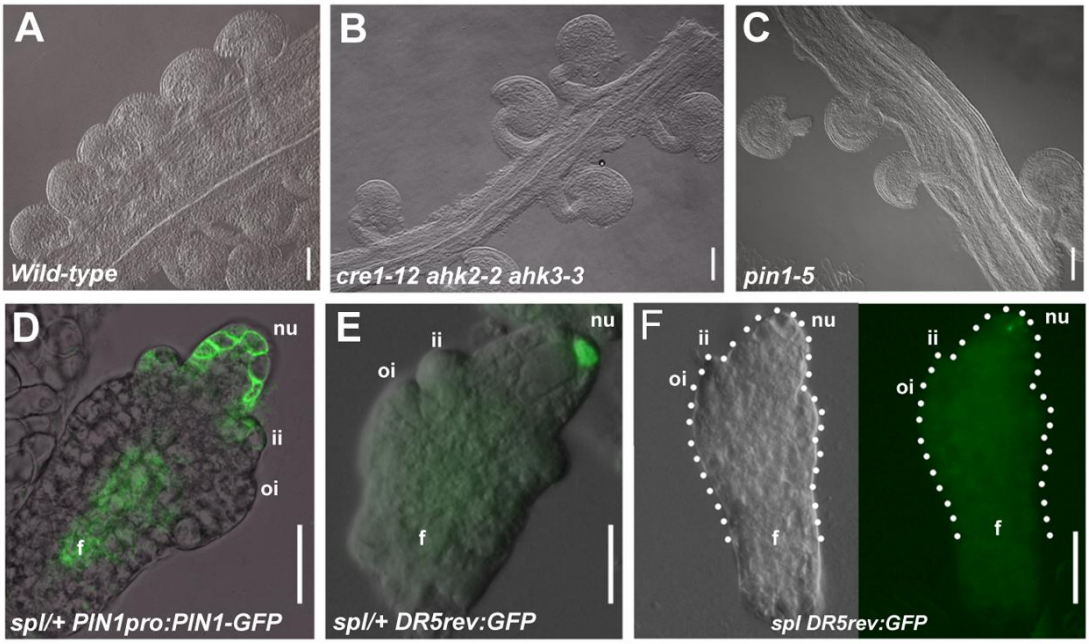
Venglat, S.P., and Sawhney, V.K. (1996). Benzylaminopurine induces phenocopies of floral meristem and organ identity mutants in wildtype *Arabidopsis* plants. Planta 198: 480–487.

Werner, T., Hanus, J., Holub, J., Schmülling, T., Van Onckelen, H., and Strnad, M. (2003). New cytokinin metabolites in IPT transgenic *Arabidopsis thaliana* plants. Physiol. Plant. 118: 127–137.

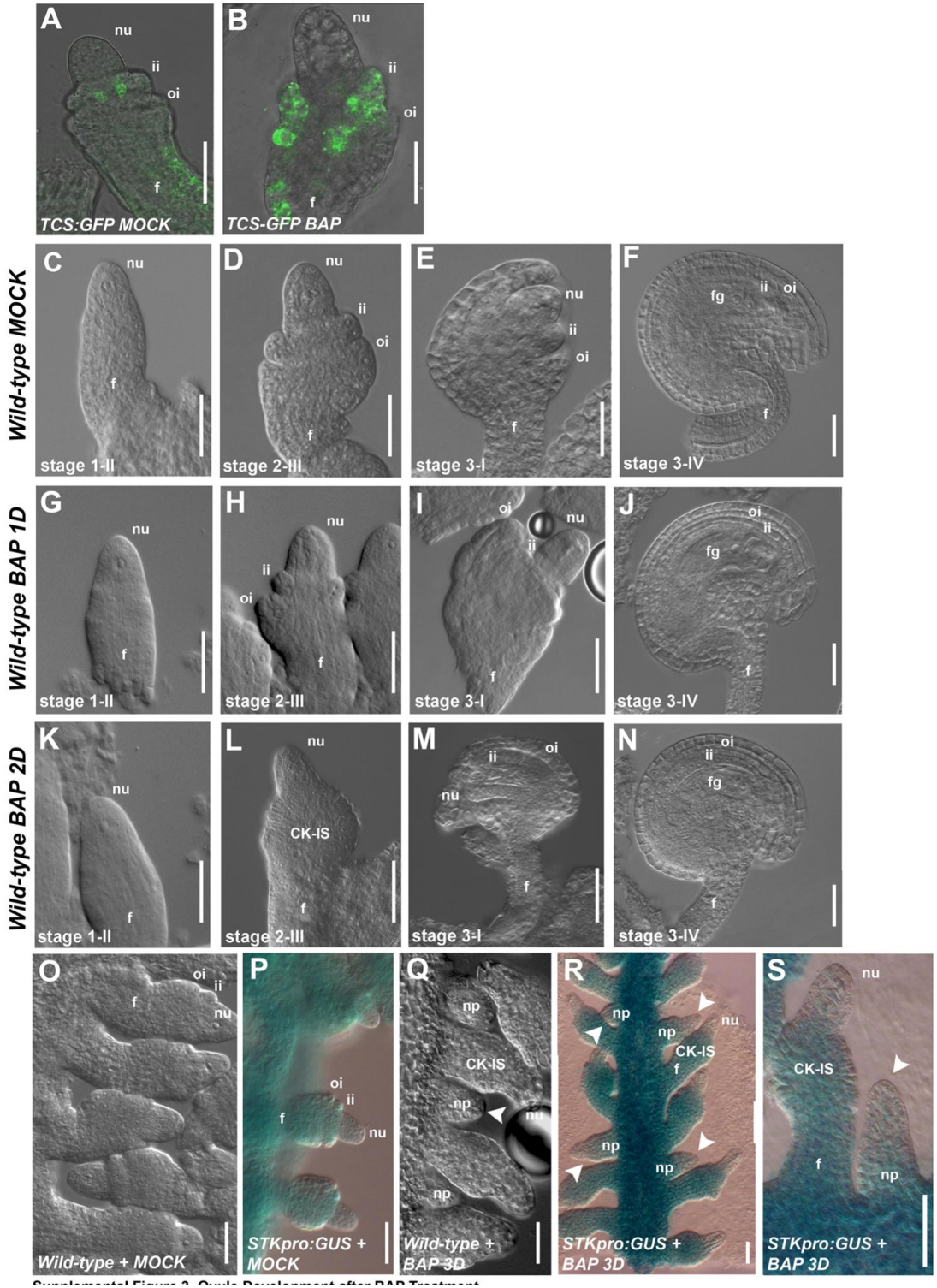
Yang, W.C., Ye, D., Xu, J., and Sundaresan, V. (1999). The SPOROCYTELESS gene of *Arabidopsis* is required for initiation of sporogenesis and encodes a novel nuclear protein. Genes Dev. 13: 2108–2117.



Supplemental Figure 1. GUS Expression in *AHK3pro:GUS* Ovules from Stage 1-II to Stage 3-IV.



Supplemental Figure 2. Ovules of *cre1-12 ahk2-2 ahk3-3* and *pin1-5* Mutants; *PIN1pro:PIN1-GFP* and *DR5rev-pro:GFP* Analyses in *spl-1* and *spl-1/SPL* Plants.



Supplemental Figure 3. Ovule Development after BAP Treatment.

Supplemental Table 1. Ovule number in *cre1 ahk2 ahk3* and *pin1-5*

	Carpel analysed	Number of ovules
wt	10	480
cre1 ahk2 ahk3	10	53
pin1-5	20	187

Supplemental Table 2. BAP effect on cytokinin receptor mutants

	Observed 2-IV stage	Ovules with CK-IS	Percentage CK-IS
wt	612	603	98.5%
cre	550	10	1.8%
ahk2	470	465	98%
ahk3	500	465	93%
ahk2\ahk3	456	412	90%

CHAPTER 5

Identification of Pathways Directly Regulated by SHORT VEGETATIVE PHASE During Vegetative and Reproductive Development in *Arabidopsis*

Veronica Gregis*[,a], Fernando Andrés*[,b], Alice Sessa*[,a], Rosalinda F. Guerra[c], Sara Simonini[a], Julieta L. Mateos[b], Stefano Torti[b], Federico Zambelli[a], Gian Marco Prazzoli[a], Giulio Pavesi[a], Lucia Colombo[c,d], George Coupland [b] and Martin M. Kater [a,e]

[a] Department of Biomolecular Sciences and Biotechnology, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy.
[b] Max Planck Institute for Plant Breeding Research, D–50829 Cologne, Germany
[c] Dipartimento di Biologia, Università degli Studi di Milano, via Celoria 26 20133 Milan, Italy.
[d] Consiglio Nazionale delle Ricerche Istituto di Biofisica, 20133 Milan, Italy

*These authors contributed equally to this work

SUMMARY

MADS-domain transcription factors play important roles during plant development. The *Arabidopsis* MADS-box gene SVP is a key regulator of two developmental phases. It functions as a repressor of the floral transition during the vegetative phase and later it contributes to the specification of the floral meristems. How these distinct activities are conferred by a single transcription factor is unclear, but interactions with other MADS domain proteins which specify binding to different genomic regions is likely one mechanism. To compare the genome wide DNA binding profile of SVP during vegetative and reproductive development we combined chromatin immunoprecipitation with high throughput DNA Sequencing (ChIP-seq). These ChIP-seq data were combined with Tiling Array expression analysis and qRT-PCR to identify biologically relevant binding sites. In addition, we compared genome-wide target genes of SVP with those of the MADS domain transcription factors FLC and AP1, which interact with SVP during the vegetative and reproductive phases, respectively.

Our analyses resulted in the identification of pathways that are regulated by the SVP which include those regulating meristem development during vegetative growth and flower development whereas floral transition pathways and hormonal signaling were regulated predominantly during the vegetative phase. Thus, SVP regulates many developmental pathways, some of which are common to both of its developmental roles whereas others are specific to only one of them.

My contribution to this work was the validation through ChIP experiments and the expression analyses by means of Real-Time PCR and *in situ* hybridization of five selected genes expressed also in the floral meristem: *CLV1*, *KAN1*, *ARF3*, *PHB* and *PIN1*. These genes are SVP direct targets and their expression is higer in the *svp agl24 ap1-12* triple mutant background, suggesting that SVP is a repressor of their transcription. Moreover, analyses to better characterize the size of the floral meristem in the *svp sgl24 ap1-12* mutant has been conducted through both SEM microscopy and *in situ* hybridization with *WUS* specific antisense probe.

CHAPTER 6

MONOPTEROS (MP) is required as central integrator of auxin and cytokinin pathways for ovule primordia formation.

Dola Sihna Roy[1#], Francesca Galbiati[1#], Sara Simonini[1], Mara Cucinotta[1], Dolf Weijers[2], Rüdiger Simon[3], Martin M. Kater[1], Simona Masiero[1] and Lucia Colombo[1, 4*]

[1], Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

[2], Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703HA Wageningen, The Netherlands

[3], Institut für Entwicklungsgenetik, Heinrich Heine Universität, D-40225 Düsseldorf, Germany

[4], CNR, Istituto di Biofisica, Università di Milano, Via Celoria 26, 20133 Milano, Italy

# equal contribution

SUMMARY

A crucial question in developmental biology is how molecular pathways control the formation of well-defined organ primordia from undifferentiated cells.
The placenta is a meristematic tissue that upon hormonal signaling gives rise to ovules as lateral organs. Ovule numbers ultimately determine the number of seeds that can develop and, thereby, control the final seed yield in crop plants. Previously it was reported that the number of ovules is controlled by CUP-SHAPED COTYLEDON (CUC1), CUC2 and AINTEGUMENTA (ANT), that we show to have an additive role in ovule number determination. Furthermore, we assign a key role to the auxin response factor MONOPTEROS (MP/ARF5) in the direct regulation of *CUC1*, *CUC2* and *ANT* expression during ovule development. We show that the expression of *CUC1* and *CUC2* genes is needed to redundantly regulate cytokinin dependent PIN1 expression and PIN1 protein localization required for ovule primordia formation.
Therefore we propose a model in which MP plays a crucial role in the integration of the auxin and cytokinin pathways controlling ovule primordia formation.

My contribution to this work was the analyses of the expression profile of *CUC1*, *CUC2*, *ANT* and *MP* in wild-type and in different mutant backgrounf (*yuc1 yuc4; mp weak*) through *in situ* hybridization.

CHAPTER 7

TAF13 Interacts with PRC2 Members and Is Essential for *Arabidopsis* Seed Development

Matias Lindner[a], Sara Simonini[a], Maarten Kooiker[a,†], Valeria Gagliardini[b], Marc Somssich[c], Mareike Hohenstatt[c], Rüdiger Simon[c], Ueli Grossniklaus[b] and Martin M. Kater[a,d]

[a] Dipartimento di BioScienze, Università degli Studi di Milano, Milan, Italy
[b] Institute of Plant Biology & Zürich-Basel Plant Science Center, University of Zürich, Zürich, Switzerland
[c] Institut für Genetik, Heinrich-Heine-Universität, Universitätstr. 1, 40225, Düsseldorf, Germany.
[†]Present address: CSIRO Plant Industry, 306 Carmody Road, St Lucia, QLD, Australia.

SUMMARY

TBP-Associated Factors (TAFs) are components of complexes like TFIID, TFTC, SAGA/STAGA and SMAT that are important for the activation of transcription, either by establishing the basic transcription machinery or by facilitating histone acetylation. However, in *Drosophila* embryos several TAFs were shown to be associated with the Polycomb Repressive Complex 1 (PRC1), even though the role of this interaction remains unclear. Here we show that in *Arabidopsis* TAF13 interacts with MEDEA and SWINGER, both members of a plant variant of Polycomb Repressive Complex 2 (PRC2). PRC2 variants play important roles during the plant life cycle, including seed development. The *taf13* mutation causes seed defects, showing embryo arrest at the 8-16 cell stage and over-proliferation of the endosperm in the chalazal region, which is typical for *Arabidopsis* PRC2 mutants. Our data suggest that TAF13 functions together with PRC2 in transcriptional regulation during seed development.

My contribution to this work was the pull-down assay through which the interaction between MEA and TAF13 has been proved.

Discussion

BPC proteins and their role in inflorescence and flower development

The regulation of transcription is a fascinating topic in molecular biology. In *Arabidopsis*, an important model organism for plant research, several classes of transcription factors have been discovered but many of them have not been characterized yet.

An example of a poorly characterized plant specific transcription factor family is the BBR/BPC family, which encodes transcriptional regulators able to bind the DNA at GA-rich sites (Meister et al., 2004; Kooiker et al., 2005; Santi et al., 2003; Sangwan'o brian et al., 2001; Simonini et al., 2012). In *Arabidopsis* there are seven BPC encoding genes, of which *BPC5* is probably a pseudogene (Meister *et al.,* 2004). The BPCs seem to have similar functions as the GAGA Associated Factor (GAF) of *Drosophila melanogaster*, which plays important roles in the regulation of the chromatin state and in the expression of the *HOX* genes (Berger and Dubreuq, 2012). Moreover, the BPCs seems to act in a redundant way; indeed, neither single nor double mutant combinations display aberrant phenotypes (Monfared *et al.,* 2011). The sestuple *bpc1-2-3-4-6-7* mutant is able to germinate and to produce leaves and flowers although these have severe defects (Monfared *et al.,* 2011). However, the *bpc1-1* allele that was used to generate this higher order mutant is not a full knock out making it very likely that the sestuple mutant described by Monfared *et al.* (2011) is not representing the phenotype when all 6 active *BPC* genes are knocked out. At the moment in our laboratory we are creating a new sestuple mutant with the *bpc1-3* allele, which is a complete loss-of-function allele. It will be interesting to see if this mutant is viable and if it shows more severe defects.

Since the BPCs are able to autoregulate themselves (data not published), it could be possible that in the *bpc1-2-3-4-6-7* sestuple mutant reported by Monfared *et al.* (2011), the expression level of *BPC1* is high enough to partially compensate the absence of the other BPCs. Nevertheless, its contribution is not sufficient to overcome all the defects, suggesting that, therefore although BPCs share high redundancy, there are different developmental processes, which require the presence of specific BPC members.

The homology with the BBR factor of barley (Santi *et al.,* 2003) and the phenotype of the *bpc1-3 bpc2 bpc3* triple mutant inflorescence, led us to hypothesize a role for BPCs in the regulation of *HOMEOBOX* genes (Chapter 3). The first analyses conducted were focused on the two KNOX genes *STM* and *BP*, which are involved in the control of inflorescence meristem size and activity. Indeed, the inflorescence meristem of the *bpc1-2-3* triple mutant is bigger and more active than wild-type, and it is consistent with the up regulation of *STM* and *BP* expression as we detected in this triple mutant. Moreover, the *STM* and *BP* promoter are highly enriched in ChIP experiments using antibody against BPC1-2-3, strongly suggesting a direct link between BPCs and these meristem genes. However, the regulation of *HOMEOBOX* genes seems not to be restricted to *STM* and *BP*, but it seems to be a more

general regulatory mechanism which includes several HOMEOBOX transcription factors. Moreover the BPCs seem to mediate the effects of hormonal signaling, being responsive to changes in cytokinin concentration. A possible scenario assigns to the BPCs the role of sensor of hormone homeostasis in the inflorescence meristem working in the STM-Cytokinin-WUS regulatory loop, which has already been well characterized (Bartrina *et al.,* 2010). Further analyses are in progress to better characterize the role of BPCs in the maintenance of meristem size and activity.

The information we have until now about BPCs suggests that they might not be essential for the plant. They most probably act as cofactors in transcription stabilizing complexes and or facilitate recruitment of transcription factors to the DNA. Furthermore, they seem to act as both transcriptional repressors and activators. This is further strengthened by the regulatory mechanism which regulates the *STK* expression through the interaction between BPCs and the repressor complex made by SVP in order to repress *STK* expression at early stages of flower development (Simonini *et al.,* 2012). The BPC binding sites contained in the *STK* regulatory region are necessary for its correct transcriptional regulation, indeed mutagenesis experiments that abolished BPC binding sites in the *STK* promoter resulted in *STK* deregulation (Chapter 2). This aspect suggests another possible regulatory mechanism in which the BPCs, associating with a MADS-box transcription factor, regulate gene expression. The research described in chapter 2 shows that BPC binding sites are essential for binding of the SVP containing repressor complex to the *STK* promoter and for *STK* transcriptional regulation. Although this is a strong indication that BPC binding to the *STK* promoter is needed for proper expression it does not provide the final proof that BPC proteins are needed for this. This proof should come from experiments in which we show deregulation of *STK* in a *bpc* higher order mutant. We already analysed the triple mutant but did not observe deregulation of *STK* suggesting that there is also redundancy between class I BPCs and other classes in the regulation of *STK*. Another open question is whether BPCs bind first to the DNA and then recruit the MADS-domain factors to the DNA by for instance opening up the chromatin and exposing the CArG boxes or that they first interact with MADS-domain factors (or other transcription factors) and then bind the DNA. At the moment we are setting up experiments to answer these questions, which should provide a deeper mechanistic insight in how BPCs control gene expression.

Hormonal control of ovule development

Hormones are small non-peptide molecules involved in many developmental processes. They are normally synthesized in a few cells and then transported by different mechanism to all parts of the plant to activate their signaling.

Auxin and cytokinin are the most important hormones whose potential was already clear half a century ago (Skooge and Miller, 1957) and the ratio between the two hormones is necessary and sufficient to determine the fate of plant organ cells.

Recently several evidences have indicated that in ovules both hormones are important for proper ovule development. In particular modulation of the auxin concentration which results in the formation of auxin maxima, is important for ovule initiation like this is important for any other lateral organ (Reinhardt *et al.,* 2000; Reinhardt *et al.,* 2003; Laskowski *et al.,* 1995; Benkova *et al.,* 2003).

Auxin accumulates first at the tip of the ovule primordium, later on accumulation was observed in the nucellus, and inside the funiculus (Ceccato *et al.,* unpublished). Thus, ovule primordia formation and the following steps of ovule development are tightly linked to the auxin concentration. Moreover, also the right balance in cytokinin (CK) concentration is required for proper ovule primordia outgrowth. In fact, in the *cre1 ahk2 ahk3* triple mutant, in which the perception of the CK is strongly decreased, the pistils contain only a few ovules (Chapter 4). Interestingly, the absence of CK causes also down-regulaton of *PIN1* expression and vice versa, high CK concentration leads to a PIN1 mislocalization. In this scenario, the cytokinin seems to play a synergic role in respect to auxin, since the CK concentration triggers the correct PIN1 localization to form the auxin peek necessary for promordium outgrowth. This is further evidenced by the role of CK during ovule development. Once the primordium arises, the expression of CK receptors and synthesis encoding genes become restricted from the whole primordia to the inner integument and the gametophyte, suggesting that at later stages the CK have a different but important role in proper ovule development. The auxin-cytokinin cross-talk in ovule development is mediated by two transcription factors involved in ovule development, SPL and BEL1. Through them and their responsiveness to the two hormones, *PIN1* expression and localization is maintained in the nucella and correct ovule development can proceed.

This aspect further strengthens the evidence that auxin and cytokinin (but probably also other hormones) act in concert to precisely regulate ovule development, and it could be a general concept that governs different developmental processes.

Bartrina I., Otto E., Strnad M., Werner T., and Schmülling T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in Arabidopsis thaliana. Plant Cell 23: 69-80.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115: 591-602.

Berger, N., and Dubreucq, B. (2012). Evolution goes GAGA: GAGA binding proteins across kingdoms. BBA - Gene Regulatory Mechanisms, 1–6.

Kooiker, M., Airoldi, C.A., Losa, A., Finzi, L., Kater, M.M. and Colombo, L. (2005). BASIC PENTACYSTEINE1 a GA-binding protein that induces conformational changes in the regulatory region of the homeotic *Arabidopsis* gene *SEEDSTICK*. The Plant Cell 17:722-729.

Laskowski, M. J., Williams, M. E., Nusbaum, H. C., and Sussex, I. M. (1995). Formation of lateral root meristems is a two-stage process. Development 121: 3303-3310.

Meister, R.J., Williams, L.A., Monfared, M.M., Gallagher, T.L., Kraft, E.A., Nelson, C.G. and Gasser C.S. (2004). Definition and interaction of a positive regulatory element of the *Arabidopsis INNER NO OUTER* promoter. The Plant Journal 37: 426-438.

Monfared M.M., Simon M.K., Meister R.J., Roig-Villanova I., Kooiker M., Colombo L., Fletcher J.C. and Gasser C.S. (2011). Overlapping and antagonistic activities of *BASIC PENTACYSTEINE* genes affect a range of developmental processes in Arabidopsis. Plant J. 66: 1020-1031.

Reinhardt, D., Mandel, T., and Kuhlemeier, C. (2000). Auxin regulates the initiation and radial position of plant lateral organs. Plant Cell 12:507-18

Reinhardt, D., Pesce, E. R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. Nature 426: 255-260.

Sangwan I. and O'Brian M.R. (2002). Identification of a soybean protein that interacts with GAGA element dinucleotide repeat DNA. Plant Physiol. 129: 1788-1794.

Santi L., Wang Y., Stile M.R., Berendzen K., Wanke D., Roig C., Pozzi C., Müller K., Müller J., Rohde W. and Salamini F. (2003). The GA octodinucleotide repeat binding factor BBR participates in the transcriptional regulation of the homeobox gene *Bkn3*. Plant J. 34: 813-826.

Simonini S., Roig-Villanova I., Gregis V., Colombo B., Colombo L., Kater M.M. (2012). BASIC PENTACYSTEINE Proteins Mediate MADS Domain Complex Binding to the DNA for Tissue-Specific Expression of Target Genes in Arabidopsis thaliana. Plant Cell. Oct 9.

Skoog F, Miller C. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol. 11:118-30.

Ringraziamenti

A ***Martin*** e ***Lucia***

per avermi sempre dato fiducia e spunti su cui lavorare sodo.

A ***Simo***

per avermi insegnato tantissimo, dall'amore per la scienza all'amore per sé stessi.

A ***Ludo***

per i preziosi consigli e per tutti gli insegnamenti, la pazienza e le castagne.

A ***Stefano***

per l'aiuto, i consigli, le idee, il supporto e l'oasi.