

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

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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 isopentenyltransferase 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 INDEPENDENT1 (CK1)* 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 patterning, in particular determining identity and development of the integuments. In the *bel1* mutant, ovules develop 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 patterning is SPOROCTELESS/NOZZLE (SPL/NZZ), which is required for the development of the megasporocyte, 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).

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^WOnline version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.112.100164

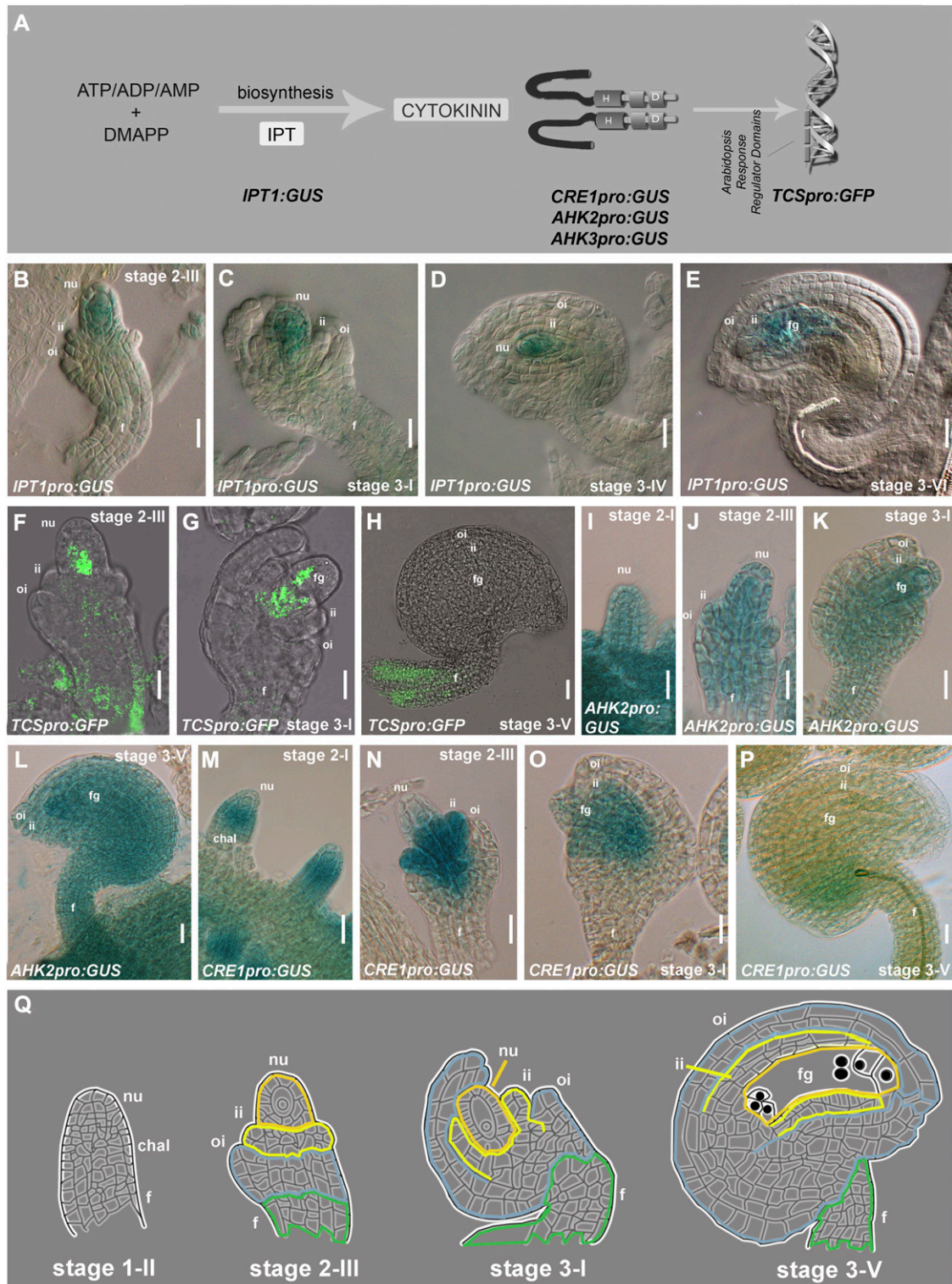


Figure 1. Analysis of the Cytokinin Pathway during Ovule Development.

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.

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 change 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 Iorio 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 isopentenyltransferases (*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 isopentenyltransferase-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:β-glucuronidase (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)* 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 two-component 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 *cre1-12 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%

Figure 1. (continued).

(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, nucellus.

Bars = 20 μm.

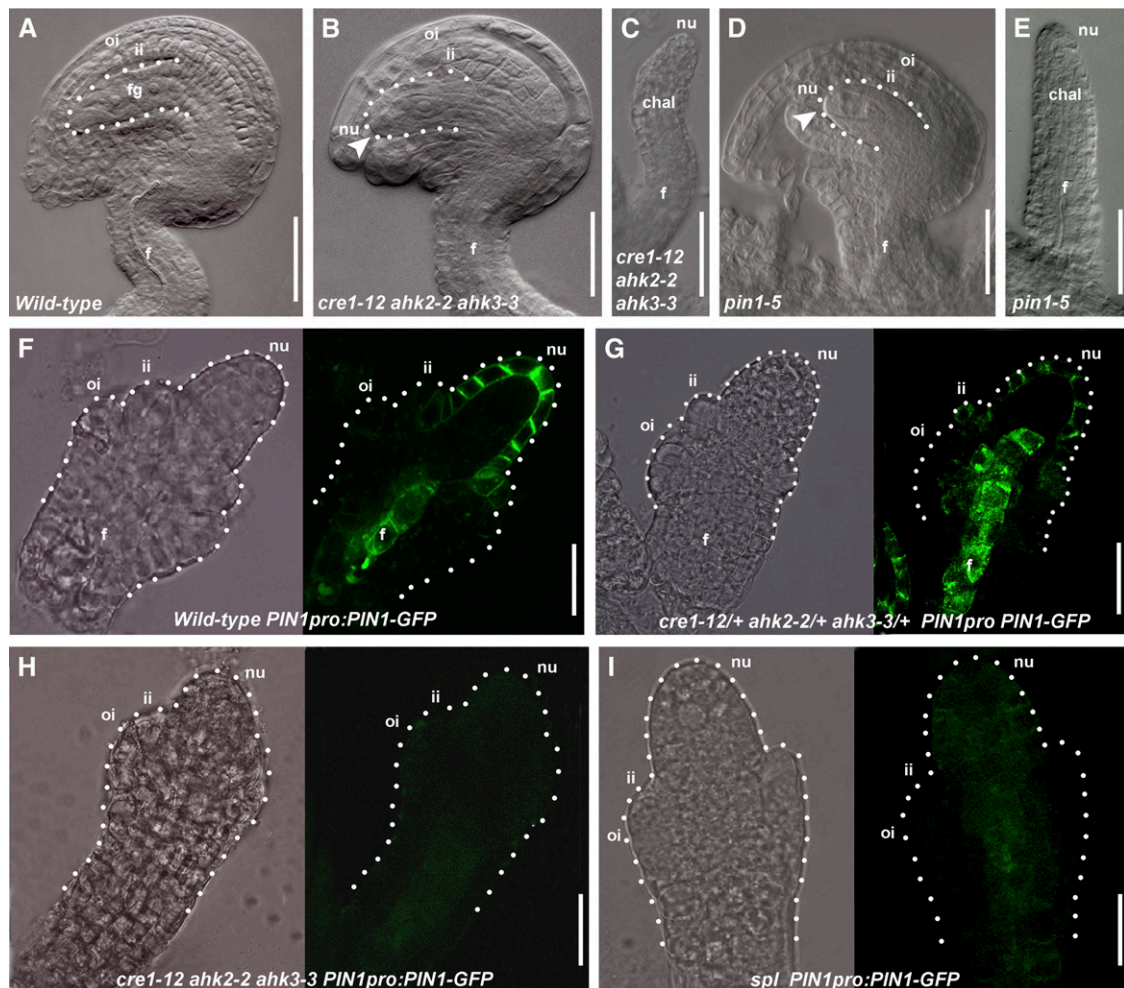


Figure 2. Role of Cytokinin in Ovule Development.

(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 μ m.

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 Iorio 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

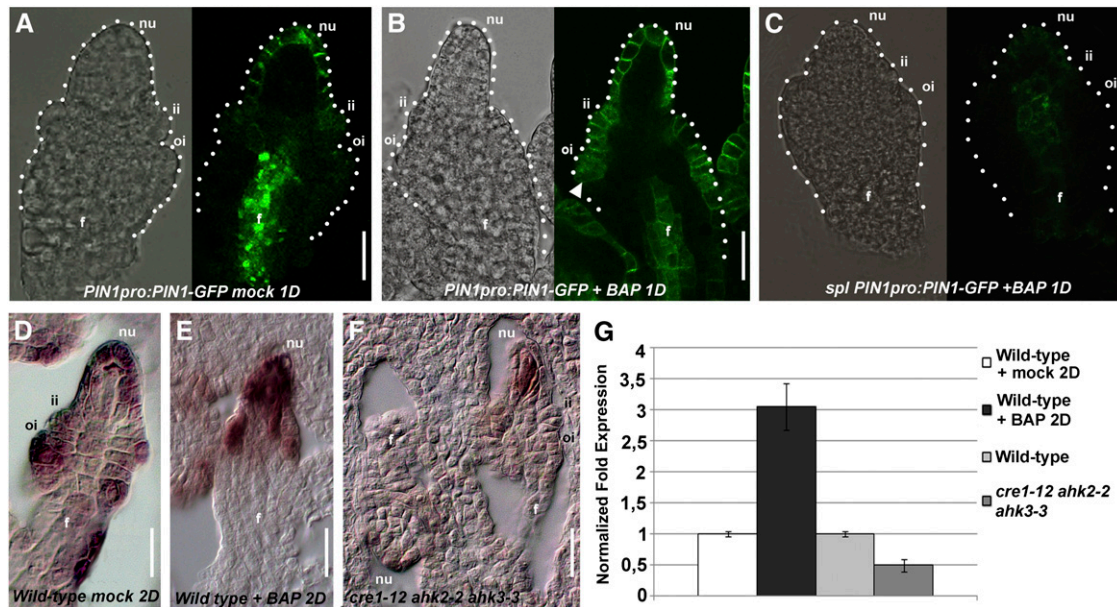


Figure 3. Analysis of BAP-Treated Ovules.

- (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 μ m.

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. 8 (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* plants containing *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 *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).

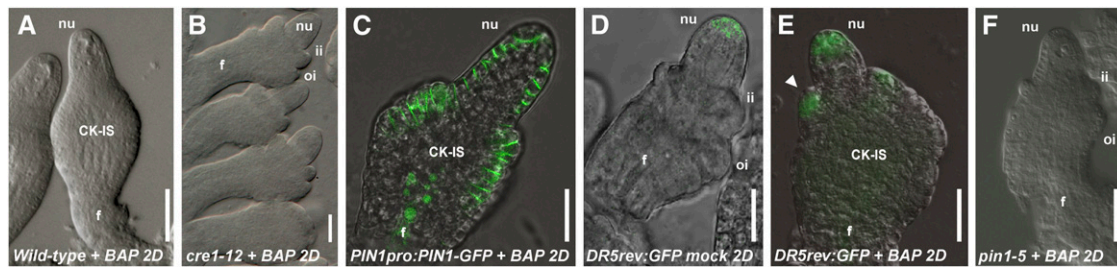


Figure 4. Effect on Ovule Development after 2 d of BAP Treatment.

(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 μ m.

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 ± 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 *cxk5 cxk6* 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

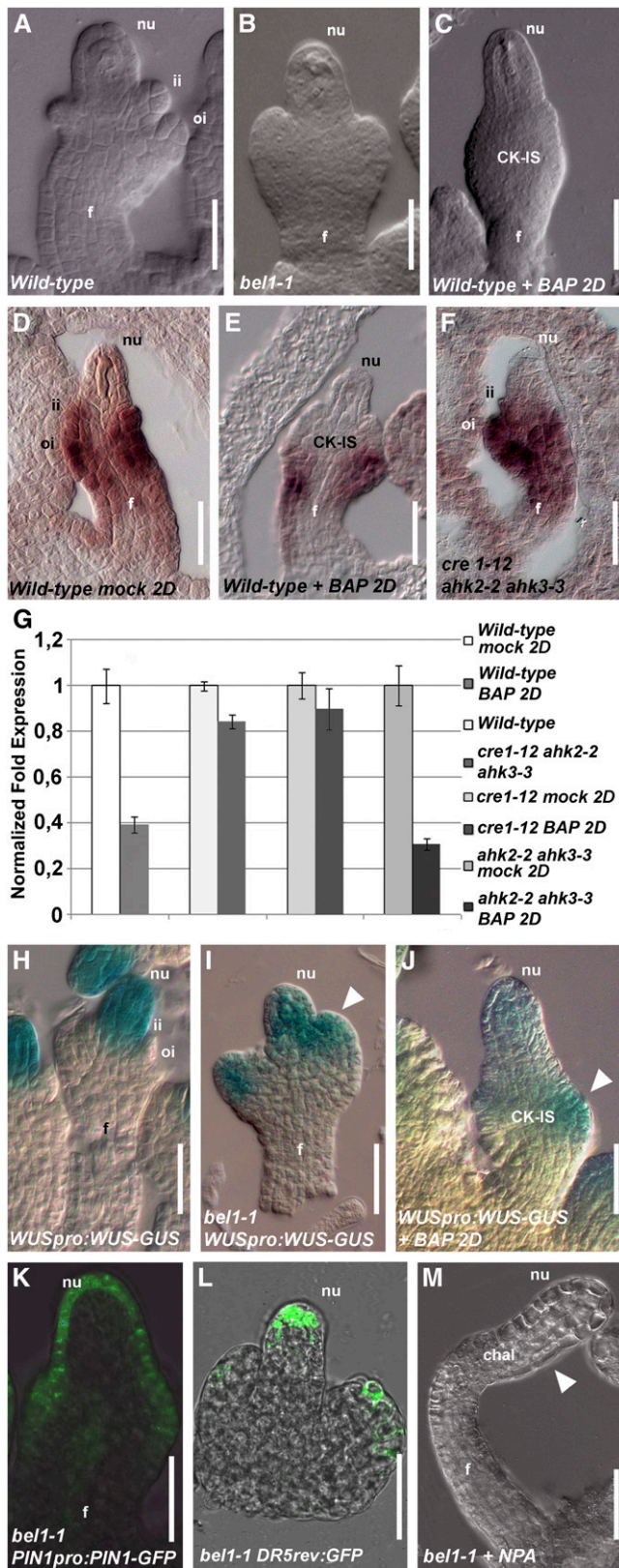


Figure 5. *BEL1* Expression Is Regulated by Cytokinin.

et al. (2004). To understand whether the observed BAP-induced ovule phenotype was mediated by these three cytokinin receptors, five plants for each *cre1-12*, *ahk2-2*, and *ahk3-3* single mutant were treated with BAP. After 2 d, the effect of the BAP treatment was 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

(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. Wild-type 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 μ m.

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 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

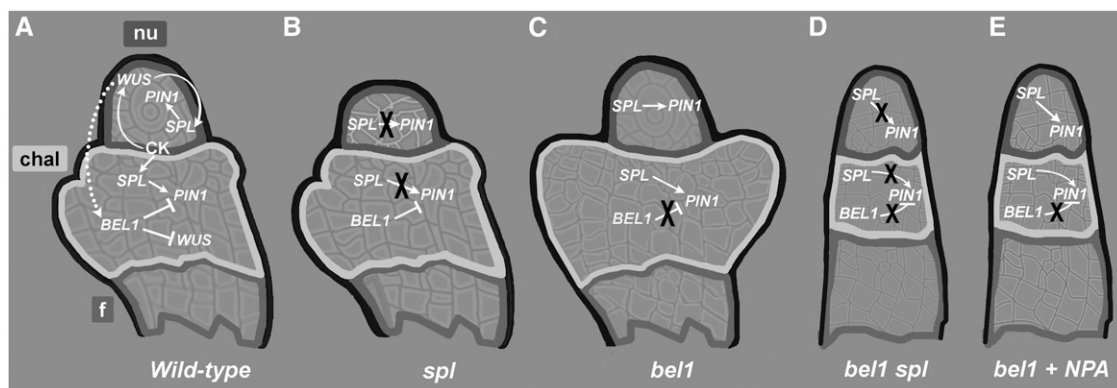


Figure 6. *BEL1* and *SPL* Integrate Hormonal Signaling in Ovules.

- (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.

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-naphthylphthalamic 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 (Schieffhale 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 down-regulated 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).

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 down-regulation 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 cytokinin-mediated *WUS* expression (Figure 6).

WUS and *WUSCHEL RELATED HOMEBOX* 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-cell-autonomous 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 Jiří 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 (5'-GGCGAGATCCGGACAGAGAC) and SPL-R (5'-AGAAGCGTTAAACAT-TTGAGGATT) and Ds primers DS 3-3A (5'-TCGTTCCGTCGCCAAGT)

or DS 5 to 3A (5'-CGTCCGGTACGGGATTTCC). The *bel1-1* allele contains a C-to-T transition at nucleotide 497, which introduces a *Bsa*I restriction site. The *bel1-1* allele was identified by *Bsa*I digestion of PCR products amplified with the primers 5'-GAGAG ACATGGCAAGAGATCAG and 5'-GAGCATGGAGAGCAACTTGG. To identify the presence of the T-DNA encoding *PIN1pro:PIN1-GFP*, the following primers were used: PIN1-RP (5'-CCAGTACGTGGAGAGGGAAG) and GFP-LP (5'-GAAAGTAGTGA-CAAGTGTGGC).

BAP Treatment

BAP was obtained from Sigma-Aldrich and was used at a concentration of 10^{-3} M. Plants were treated once with 30 μ L 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 μ M and 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 63 \times water-immersion objective (numerical aperture = 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

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

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 the Central European Institute of Technology).

AUTHOR CONTRIBUTIONS

S.B., E.B., and L.C. designed the research. S.B. and S.S. performed the experiments. All authors analyzed and discussed the data and the article. S.B. and L.C. wrote the article.

Received May 2, 2012; revised June 12, 2012; accepted June 25, 2012; published July 10, 2012.

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

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Plant Cell 2012;24;2886-2897; originally published online July 10, 2012;
DOI 10.1105/tpc.112.100164

This information is current as of October 29, 2012

Supplemental Data	http://www.plantcell.org/content/suppl/2012/07/03/tpc.112.100164.DC1.html
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