# The Mobile *bypass* Signal Arrests Shoot Growth by Disrupting Shoot Apical Meristem Maintenance, Cytokinin Signaling, and *WUS* Transcription Factor Expression[1][OPEN]

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The *bypass1* (*bps1*) mutant of Arabidopsis (*Arabidopsis thaliana*) produces a root-sourced compound (the *bps* signal) that moves to the shoot and is sufficient to arrest growth of a wild-type shoot; however, the mechanism of growth arrest is not understood. Here, we show that the earliest shoot defect arises during germination and is a failure of *bps1* mutants to maintain their shoot apical meristem (SAM). This finding suggested that the *bps* signal might affect expression or function of SAM regulatory genes, and we found *WUSCHEL* (*WUS*) expression to be repressed in *bps1* mutants. Repression appears to arise from the mobile *bps* signal, as the *bps1* root was sufficient to rapidly down-regulate *WUS* expression in wild-type shoots. Normally, *WUS* is regulated by a balance between positive regulation by cytokinin (CK) and negative regulation by CLAVATA (CLV). In *bps1*, repression of *WUS* was independent of CLV, and, instead, the *bps* signal down-regulates CK responses. Cytokinin treatment of *bps1* mutants restored both *WUS* expression and activity, but only in the rib meristem. How the *bps* signal down-regulates CK remains unknown, though the *bps* signal was sufficient to repress expression of one CK receptor (*AHK4*) and one response regulator (*AHP6*). Together, these data suggest that the *bps* signal pathway has the potential for long-distance regulation through modification of CK signaling and altering gene expression.

Leaves and other lateral organs arise from a stem cell population called the shoot apical meristem (SAM). In Arabidopsis (*Arabidopsis thaliana*), the SAM initiates during embryogenesis in a rectangular area between the two cotyledons (Medford et al., 1992; Barton and Poethig, 1993). Recruitment of leaf founder cells from the SAM periphery causes a temporary reduction of SAM size, which is rapidly restored by homeostatic mechanisms; thereafter, the SAM is maintained at a stereotypical size. The SAM is divided into domains based on function and gene expression patterns. The central zone (CZ) is a narrow domain at the SAM's apex and is the expression domain of *CLAVATA3* (*CLV3*), which encodes a signaling peptide (Clark et al., 1995; Fletcher et al., 1999). The CLV3 peptide is perceived by a set of partially redundant receptors, encoded by *CLV1*, *CLV2*, *CORYNE*, and *RECEPTOR-LIKE KINASE2* (Clark et al., 1997; Jeong et al., 1999; Miwa et al., 2008; Müller et al., 2008; Kinoshita et al., 2010), and functions to constrain cell proliferation. Another important SAM domain is the organizing center (OC), a small set of cells located just below the CZ that expresses *WUSCHEL* (*WUS*), a homeodomain protein that promotes SAM cell proliferation and specifies stem cell identity in adjacent cells (Laux et al., 1996; Mayer et al., 1998). Subjacent to the OC is the rib meristem (RM), whose activity contributes to stem growth. An elegant model for SAM homeostasis features interactions between WUS, which activates *CLV3* expression, and the CLV pathway, which is proposed to repress *WUS* expression (Brand et al., 2000; Schoof et al., 2000). *WUSCHEL-LIKE HOMEOBOX* (*WOX*) genes appear to confer stem cell identity in other plant stem cell niches; e.g. WOX5 regulates activity of the root meristem, WOX4 regulates the activity of the vascular cambium, and in Arabidopsis, WOX1 and WOX3 together promote leaf blade outgrowth (Sarkar et al., 2007; Hirakawa et al., 2010; Nakata et al., 2012). What is less understood is how signaling originating from other organs regulates the SAM.

Coordination of SAM homeostasis relies in part on mobile signaling molecules. An important mobile signal for SAM homeostasis is cytokinin (CK). This compound is a potent activator of *WUS* expression that is normally produced in the upper tiers of the SAM's CZ

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(Gordon et al., 2009; Zhao et al., 2010; Chickarmane et al., 2012). This localized production of active CK is important for SAM maintenance, as rice (*Oryza sativa*) mutants defective in its production have SAM maintenance defects (Kurakawa et al., 2007). Equally important for *WUS* expression is the localized perception of CK, which is carried out by two partially redundant CK receptors, *ARABIDOPSIS HIS KINASE2* (*AHK2*) and *AHK4* (Gordon et al., 2009; Chickarmane et al., 2012). Another mobile signal that is important for SAM homeostasis is the active *CLV3* gene product, which is derived from a precursor protein that is processed into a secreted 12-amino acid glycopeptide (Fletcher et al., 1999; Kondo et al., 2006). The WUS transcription factor itself is also mobile, but instead of apoplastic movement, it travels through plasmodesmata (Yadav et al., 2011; Daum et al., 2014). Plasmodesmata are critical for SAM maintenance, presumably because they transport essential signaling molecules, such as WUS, and their aperture is also developmentally regulated to define symplastic domains (Rinne and van der Schoot, 1998; Kim et al., 2005; Benitez-Alfonso et al., 2009). However, how plasmodesmata gating regulation might play into stem cell homeostasis is unknown.

While plant stem cell populations typically function as intrinsically regulated drivers of growth, their activity can also be affected by environmental conditions (Xiong et al., 2002; Wolters and Jürgens, 2009). A large variety of environmental conditions lead to temporary growth reduction, including shoot arrest following drought perception by roots (Gowing et al., 1990; Mulholland et al., 1996; Jackson, 2002; Schachtman and Goodger, 2008). These observations suggest the existence of a mobile compound that is produced at sites where environmental stress is perceived, moves to the shoot, and regulates growth. Recently, translational regulation emerged as a possible mechanism coordinating SAM homeostasis with stress responses; an internal ribosome entry site in the *WUS* 5′ untranslated region appears to be used to restore normal SAM size as plants transition back to growth following stress perception (Cui et al., 2015). However, how stress perception leads to the initial growth arrest is not understood.

One key for understanding growth arrest is to identify the causal mobile signaling molecules. Both abscisic acid (ABA) and reactive oxygen species are candidates for the mobile stress-induced signaling compound (Munns and Sharp, 1993; Ishitani et al., 1997; Zhu, 2002; Miller et al., 2009); however, it is unclear whether either of these induces growth arrest. Another candidate for the growth-arrest pathway was discovered when analyzing the *bypass1* (*bps1*) mutant (At1g01550) of Arabidopsis. Shortly after germination, *bps1* mutants arrest shoot growth due to a mobile root-synthesized compound, which we call the *bps* signal (Van Norman et al., 2004). Because *bps1* mutants are recessive, and *BPS1* overexpression rescues *bps1* mutants but only to a wild-type phenotype, we proposed that BPS1 is a negative regulator whose normal function is to prevent (or modulate) synthesis of the *bps* signal. The predicted BPS1 protein has no functionally characterized domains, but it does contain a large uncharacterized plant-specific domain of unknown function. Genetic analysis showed that synthesis of the *bps* signal occurs across multiple cell types in the root, requires an intact carotenoid biosynthesis pathway, and requires active root growth (Van Norman and Sieburth, 2007; Van Norman et al., 2011). Although the link between a carotenoid biosynthesis requirement and root-to-shoot signaling made it appealing to consider the possibility that the *bps* signal was ABA, double mutants that combine *bps1* with ABA biosynthesis deficiency show the same severe *bps1* phenotype, indicating that the *bps* signal continues to be synthesized, even in mutants not producing ABA. Because the BPS1 protein appears to negatively regulate *bps* signal synthesis, we suspect that the very severe *bps1* phenotype arises because this mobile compound is produced in vast excess. We are pursuing characterization of this pathway to determine whether wild-type plants use the *bps* signal as part of a normal physiological response. For example, the *bps* signal might be used for growth arrest of salt-stressed plants, as both salt stress and the *bps* signal halt cell cycle progression (Burssens et al., 2000; Van Norman et al., 2011; Adhikari et al., 2013).

Here, we undertook a detailed analysis of shoot responses to the *bps* signal. We identified SAM maintenance as the earliest developmental defect in shoots and showed that the *bps* signal leads to both repression of *WUS* expression and diminished CK responses, even in wild-type shoots that were transiently grafted to the *bps1* root. These data lead to an attractive model of long-distance growth control by interfering with stem cell homeostasis through diminished CK responses.

## RESULTS

### *bps1* Mutants Show SAM Maintenance Defects

Previously, we showed that the growth arrest phenotype in *bps1* shoots arises from a non-cell-autonomous activity derived from the root and that this activity is sufficient to repress growth of wild-type shoots (Van Norman et al., 2004, 2011). Those studies revealed that an early developmental defect was leaf blade outgrowth, which we detected in 5 d postimbibition (dpi) seedlings. Here, we sought to uncover the molecular and genetic basis for shoot arrest, and so reexamined very young *bps1* seedlings for possible earlier shoot development phenotypes (Supplemental Fig. S1A). We found that *bps1* mutants show defects in SAM maintenance. The normal SAM is a dome-shaped structure with distinct cell layers; by contrast, the 3 dpi *bps1* SAM was small and flat, and it remained small at day five (Fig. 1, A and B; Supplemental Fig. S1B). We also examined SAM cell sizes; in the wild-type SAM, cells showed a cross-sectional area of 4 to 4.8 $\mu m^2$, while the cells in nearby differentiating organs were larger (6 to 6.5 $\mu m^2$). The *bps1* SAM cell sizes were

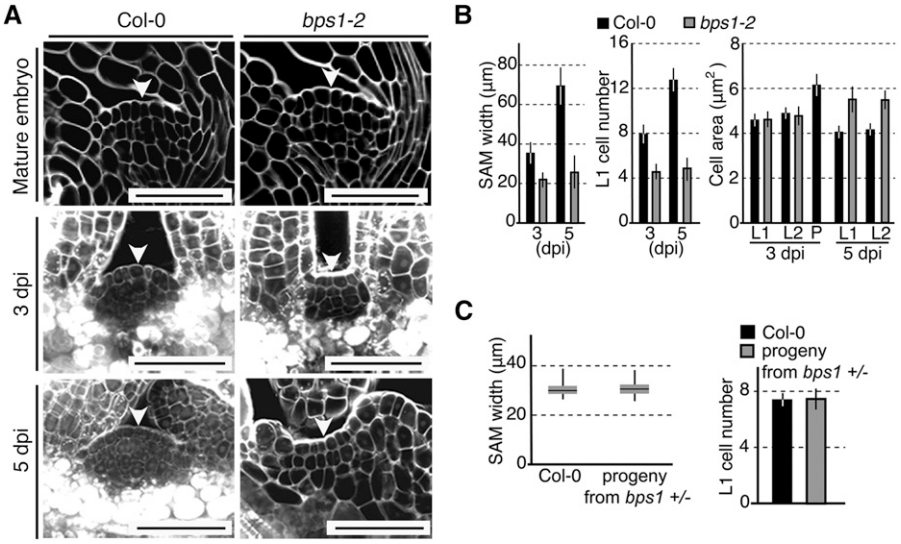


**Figure 1.** BPS1 is required for postembryonic shoot apical meristem maintenance. A, Confocal images of the SAM of the wild type (Col-0) and *bps1-2* mutants. White arrowheads indicate the center of the SAM, the CZ. For mature embryos and 5 dpi seedlings, the images show frontal longitudinal sections, while for 3 dpi seedlings, the images show sagittal longitudinal sections. Bars = 50 $\mu$m. B, Quantitative analysis of SAM sizes in 3 and 5 dpi seedlings. Between 16 and 24 SAMs were analyzed for each developmental stage and for each genotype. A diagram depicting the measurements is provided in Supplemental Figure S1B. Data are shown as the mean ± SD. C, Quantitative analysis of SAM size in mature embryos from the wild type and from the progeny of a self-pollinated *bps1-2* heterozygote. The box and whisker plots depict the SAM width, with the box showing the sizes ranging from the 25th to the 75th quartile, and the whiskers showing the data extremes. L1 cell numbers show the mean ± SD.

indistinguishable from those of the wild type at 3 dpi, but showed a modest size increase at 5 dpi (Fig. 1, A and B; Supplemental Fig. S1B). These observations suggested that *bps1* mutants might have defects in stem cell maintenance. Consistent with this, we were unable to find the SAM in the 7 dpi *bps1* seedlings (Supplemental Fig. S1D).

We examined SAM morphology in mature embryos to determine whether the *bps1* SAM defect arose during embryogenesis or during germination. We dissected mature embryos from seeds of a self-pollinated *bps1-2* heterozygote and compared their SAM morphology to that of mature wild-type embryos. If the *bps1* SAM defect arose during embryogenesis, we expected to find SAM defects in 22 to 23 of the 90 embryos examined. However, we found no embryonic SAM defects (Fig. 1, A and C), indicating that the *bps1* SAM is established normally, and because defects arose after germination, that the *bps1* SAM size defect is due to a lack of SAM maintenance. This timing of SAM defects is consistent with a previous study that found the *bps* signal to be largely produced post embryogenesis (Van Norman et al., 2011).

### *WUS* Expression Is Lost in the *bps1* SAM

Maintenance of the SAM is largely governed by three well-established activities (Supplemental Fig. S1C). The first is *SHOOTMERISTEMLESS* (*STM*), which encodes a knotted-like homeobox gene that is required for establishment of the SAM during embryogenesis and for SAM maintenance after germination (Long et al., 1996; Kirch et al., 2003). We analyzed *STM* expression using *pSTM::GUS*, which in the wild type reveals the normal pattern of *STM* expression across the SAM (Fig. 2A). In *bps1* mutants, *pSTM::GUS* expression looked normal in 2 and 3 dpi seedlings, though the expression area was smaller, consistent with the small and flat SAM in these mutants. However, in 4 dpi seedlings, *pSTM::GUS* expression was discontinuous. Staining appeared normal around the perimeter of the SAM but was lost from the central region. This change in expression occurred later than the appearance of SAM morphological defects, and so it is unlikely to explain the *bps1* SAM maintenance defects.

A second activity that is important in regulation of SAM maintenance is the CLV3 signaling peptide pathway. Perception of the CLV3 peptide by its receptors leads to repression of *WUS* expression (Brand et al., 2000; Schoof et al., 2000), and we reasoned that SAM arrest might be brought about by CLV3 overexpression. We used *pCLV3::GUS* to compare expression in wild-type and *bps1* seedlings. In both the wild type and *bps1* mutants, *pCLV3::GUS* expression was found in its normal CZ expression domain (Mayer et al., 1998; Fig. 2B). To more carefully visualize these GUS staining patterns, we also compared a GUS incubation time course (Supplemental Fig. S1E); this revealed that while the *pCLV3::GUS* expression domain was smaller in *bps1*, the staining intensity in the two genotypes was very similar, indicating that shoot arrest in *bps1* is unlikely to be a consequence of the CLV pathway.

A third important activity that governs normal SAM maintenance is *WUS*, which encodes a transcription factor that is expressed in the OC, a small domain at the

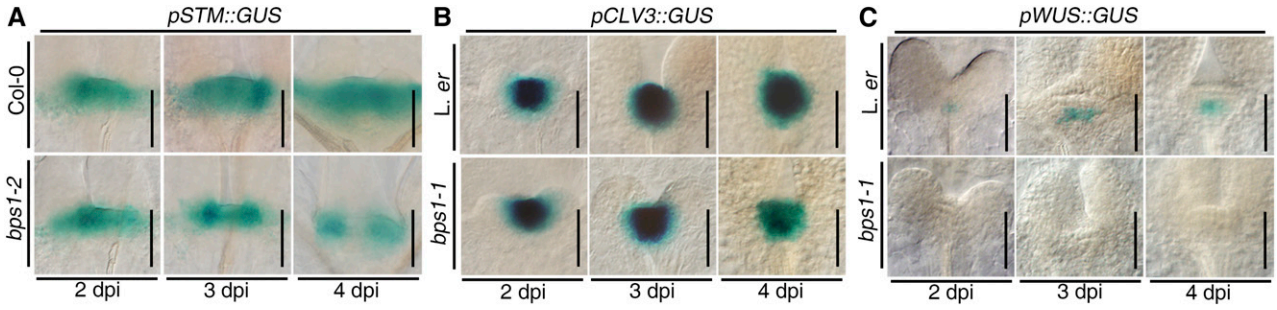


**Figure 2.** The *bps1* SAM fails to express *WUS*. A to C, GUS staining in the SAM of 2, 3, and 4 dpi seedlings. In each, the top row shows the pattern in the wild type (Col-0 or L*er*), and the bottom row shows the pattern in *bps1* mutants. A, *pSTM::GUS*; B, *pCLV3::GUS*; C, *pWUS::GUS*. Bars = 50 $\mu$m.

center of the SAM whose function is to promote SAM identity in nearby cells (Laux et al., 1996; Mayer et al., 1998). We characterized *WUS* expression using *pWUS::GUS* (Bäurle and Laux, 2005). Expression in the wild type matched its normal embryonic expression in 2, 3, and 4 dpi seedlings, namely, in the top central L3 as well as the OC (Fig. 2C; Supplemental Fig. S1G). By 5 dpi, *WUS* expression matched the adult pattern, in the OC below L3 and at the top central regions of the RM (Supplemental Fig. S1G). By contrast, *pWUS::GUS* expression was not detectable in *bps1* mutants at any of the time points examined (Fig. 2C; Supplemental Fig. S1F). A loss of *WUS* expression is an attractive explanation for the *bps1* shoot phenotype, as it is consistent with the observed loss of SAM maintenance.

### The *bps* Signal Is Sufficient to Reduce *WUS* Expression

Previously, we used traditional micrografts to show that the *bps1* root was sufficient to arrest shoot development in the wild type (Van Norman et al., 2004). Here, we extended the grafting approach to test whether the *bps1* root was also sufficient to repress *pWUS::GUS* expression. We used a transient micrograft assay (Adhikari et al., 2013), which, by providing an aqueous cushion between the scion and rootstock (agarose in water, ~0.3 mm; Supplemental Fig. S2A), allows the transfer of hydrophilic molecules between scion and rootstock without the delay from tissue repair that occurs with traditional grafting. The 4 dpi wild-type scions carrying SAM markers were coupled to either wild-type or *bps1* roots or applied to just agarose (no root) and GUS stained 24 h after coupling. Scions carrying *pSTM::GUS* and *pCLV3::GUS* produced GUS staining patterns that were identical, regardless of whether they had been transiently grafted to *bps1* (Fig. 3A). However, scions carrying *pWUS::GUS* showed a root genotype-dependent change in GUS expression. *pWUS::GUS* expression was undetectable in wild-type shoots coupled to the *bps1* root, whereas in transient micrograft controls (no root or wild-type root), we found normal *pWUS::GUS* expression (Fig. 3A; Supplemental Fig. S2B). This specific response to the *bps1* root indicated that the mobile root-sourced *bps* signal affected SAM maintenance by interfering with *WUS* expression.

Because these analyses relied on *pWUS::GUS*, a transgenic marker, we addressed whether the *bps1* root response was specific for *WUS* or whether it might be associated with the *pWUS::GUS* transgene, e.g. because of its insertion site. We tested two independently produced *pWUS::GUS* markers, *FspBst* and *ApaBst* (Bäurle and Laux, 2005). Both of these lines showed identical robust and specific *bps1* root-induced repression in transient graft assays (Supplemental Fig. S2C), which ruled out insertion site as a confounding influence. We also tested whether the *bps1* root affected expression from the endogenous *WUS* locus. We generated transient micrografts that coupled wild-type scions to either *bps1* or wild-type roots for 24 h, isolated RNA from the wild-type scions, and used qRT-PCR to analyze transcript levels for *STM*, *WUS*, and *CLV3*. Only *WUS* showed a significant change in expression (−5.3-fold; $P < 0.05$; Fig. 3B; Supplemental Table S1), and there was no significant impact on *CLV3* or *STM* transcript levels. This finding, together with *bps1*'s repressive effect on three independently produced *pWUS::GUS* marker lines, indicates that the *bps1* root leads to *WUS* repression.

### CLV-Independent Repression of *WUS* Expression

Finding that the *bps1* root could repress *WUS* expression led us to next consider possible mechanisms. Despite not observing *CLV3* overexpression (Figs. 2 and 3), we considered it still possible that the *bps* signal might work through the CLV3 peptide signaling pathway, which functions to specify the apical boundary of *WUS* expression (Brand et al., 2000; Schoof et al., 2000; Chickarmane et al., 2012). We tested this idea genetically, reasoning that if SAM arrest required CLV, then *bps1 clv* double mutants would be less effective at repressing *WUS*, and so SAM maintenance would be at least partially restored. However, the size and organization of *bps1 clv1-1* and *bps1 clv2-1* SAMs were indistinguishable from those of *bps1* single mutants (Fig. 4A), indicating that neither CLV1 nor CLV2 were required for *bps* signal-mediated SAM arrest.

We also analyzed the *bps1 clv3* double mutant and found that it produced a SAM that was intermediate in size between the *clv3-2* single mutant and the *bps1-2* single mutant (Fig. 4A). This intermediate size could mean that the *bps* signal worked through CLV3 to induce SAM arrest (independently of CLV1 or CLV2). However, another possibility was that the intermediate phenotype resulted from different developmental timing: *CLV3* is required both during embryogenesis and after germination, while production of the *bps* signal starts late in embryogenesis in *bps1* mutants (Schoof et al., 2000; Van Norman et al., 2004, 2011). To distinguish between these possibilities, we compared *pWUS::GUS* expression in *bps1 clv1* and *bps1 clv3* double mutants. As in *bps1* single mutants, *pWUS::GUS* expression was not detectable in the *bps1 clv1* mutants (Fig. 4B). Similarly, *pWUS::GUS* expression in the *bps1 clv3* double mutants ranged from undetectable to very faint. In addition, we examined older *bps1 clv3* seedlings; if shoot arrest by the *bps* signal requires CLV3, then we would expect to observe continued SAM maintenance and expansion in *bps1 clv3* double mutants. However, at 12 d, the *bps1 clv3* seedlings were indistinguishable from *bps1* (Supplemental Fig. S3). Both *bps1* and *bps1 clv3* had only a single pair of small aberrant leaf primordia, in contrast to the four leaves produced by the wild type and the six leaves produced by *clv3*. In addition, the SAM of *bps1 clv3* seedlings failed to be maintained during continued growth, and by 12 d, its size was indistinguishable from that of the *bps1* single mutant. Thus, these data confirmed that the *bps* signal functions independently of CLV signaling and indicated that the *bps* signal can repress *WUS* expression even in the very broad expression domain conditioned by the loss of CLV3.

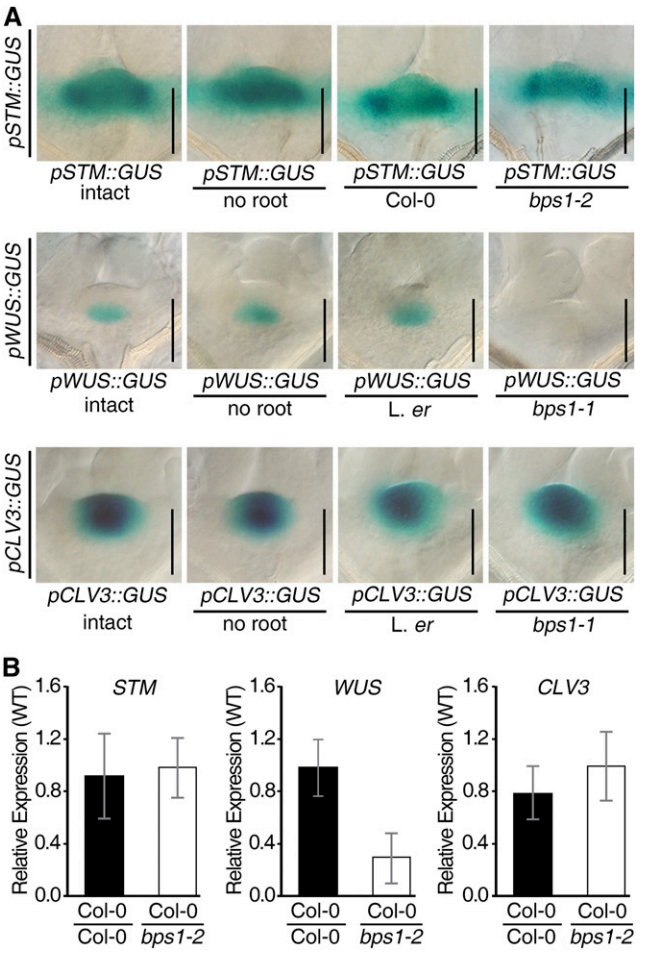


**Figure 3.** The *bps1* root is sufficient to repress *WUS* expression in wild-type scions. A, Analysis of shoot expression of *pSTM::GUS*, *pWUS::GUS*, and *pCLV3::GUS* in 5-d wild-type plants that were intact or in scions that were applied to the agarose grafting apparatus but no root or coupled to wild-type or *bps1* roots. GUS staining was carried out 24 h after transient graft establishment. Bars = 50 $\mu$m. B, Quantitative analysis of *STM*, *WUS*, and *CLV3* expression in 5-d wild-type shoots transiently grafted to either a wild-type (Col-0) or *bps1-2* root. Data are shown as the mean ± SD of three biological and two technical replicates.

### The Root-Derived *bps* Signal Is Sufficient to Reduce Cytokinin Responses

A second potential mechanism by which the *bps* signal might affect *WUS* expression is by interfering with its positive regulation by CK (Kurakawa et al., 2007; Gordon et al., 2009; Chickarmane et al., 2012). We tested whether the *bps* signal affected general CK signaling using the CK response reporter *pARR5::GUS* (D'Agostino et al., 2000). Under normal growth conditions, the wild type showed broad expression across much of the SAM and especially strong expression in the SAM's center (Fig. 5A). By contrast, *bps1* mutants showed only faint expression that was largely found beneath the SAM, and associated with the vascular tissue, suggesting a defect in CK responses in *bps1* mutants. We next analyzed *pARR5::GUS* expression in seedlings 24 h after being transferred to CK-containing medium (N6-benzylaminopurine [BAP]; 0.01–10 $\mu$M). The wild type transferred to 0.01 and 0.10 $\mu$M BAP showed expression that was very similar to the mock-treated control and progressively stronger expression following transfer to 1.0 and 10.0 $\mu$M BAP media (Fig. 5A). The results from *bps1* mutants were similar, except that for all CK concentrations, expression was much lower than that of the wild type. Low expression that was predominantly in the RM was observed for mock-treated, 0.01 $\mu$M, and 0.10 $\mu$M BAP, while expression was progressively stronger in mutants that had been transferred to 1.0 and 10.0 $\mu$M BAP. These observations indicate that *bps1* mutants fail to produce a robust CK response.

To determine whether the reduced CK response was induced by the root-derived *bps* signal, we used transient micrografts; wild-type scions carrying *pARR5::GUS* were coupled to either *bps1* or wild-type roots, and GUS expression analyzed 24 h after coupling (Fig. 5B; Supplemental Fig. S4). The intact and wild-type root

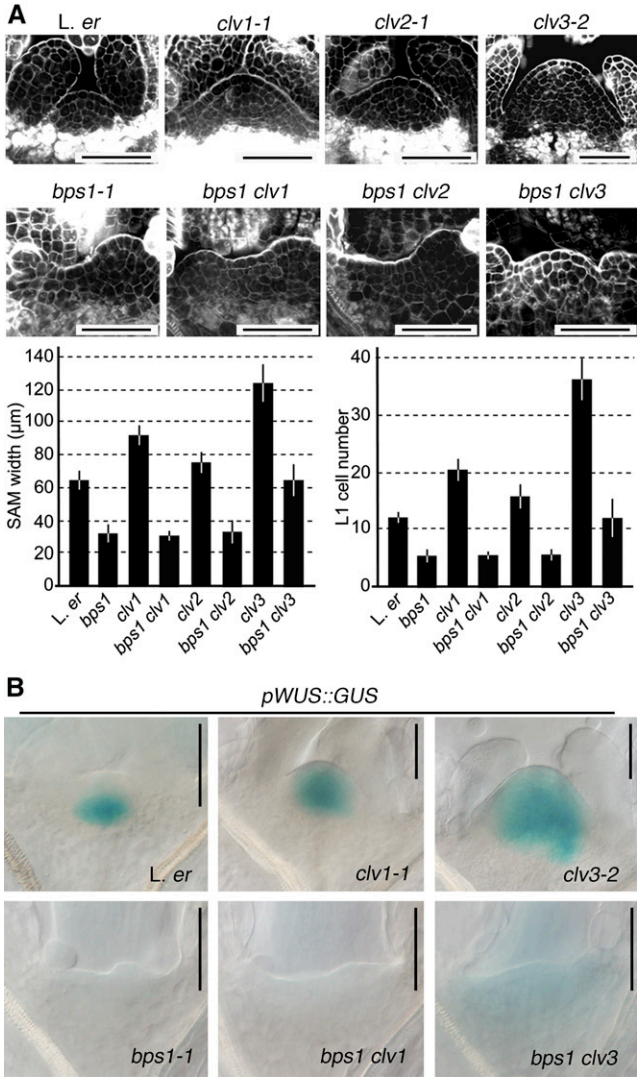


**Figure 4.** CLV3 peptide signaling and the *bps* signal are independent negative regulators of *pWUS::GUS* expression. A, Confocal images depict the SAM of single and double mutants (top) and a quantitative analysis of SAM size at 5 dpi. Graphs depict data from 17 to 20 seedlings for each genotype and are shown as the mean ± SD. B, *pWUS::GUS* expression in single and double mutants at 5 dpi. Bars = 50 μm.

graft controls showed indistinguishable patterns and intensity of GUS staining, indicating that *pARR5::GUS* expression is not sensitive to the experimental treatments involved in micrograft establishment. However, wild-type shoots coupled to *bps1* roots showed strongly reduced *pARR5::GUS* expression, and residual expression was largely restricted to the RM. This pattern matched that of *pARR5::GUS* in *bps1* mutants and indicated that the root-derived *bps* signal was sufficient to reduce *pARR5::GUS* expression.

To get at how the *bps* signal might be interfering with CK responsiveness, we tested whether the *bps* signal repressed expression of cytokinin receptors or response regulators. The Arabidopsis genome encodes three CK receptor genes, *ARABIDOPSIS HISTIDINE KINASE2* (*AHK2*), *AHK3*, and *CRE1/WOL1/AHK4* (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). We used qRT-PCR to assess their relative expression in wild-type shoots transiently micrografted to either wild-type or *bps1* roots (Fig. 5C) and required a fold change of $<-1.5$ or $>1.5$ and $P$ value $< 0.05$ for significance. The expression of *AHK2* and *AHK3* was not significantly affected by grafting to the *bps1* roots; however, wild-type shoots coupled to *bps1* root showed a significant decrease in *AHK4* expression (−1.7-fold change, $P < 0.05$; Supplemental Table S1). Although *WUS* expression has been linked to CK perception through AHK2 and AHK4 (Gordon et al., 2009), the impact of the *bps* signal on *WUS* expression (Fig. 3B) was much stronger than its effect on *AHK4* expression.

We also used qRT-PCR to assess the impact of the *bps1* root on expression of the *ARABIDOPSIS HIS PHOSPHOTRANSFER* (*AHP*) gene family, of which six genes are encoded by the Arabidopsis genome (Suzuki et al., 2000; Fig. 5C). Only *AHP6* showed a significant response to the *bps1* root (−1.6-fold change, $P < 0.05$; Supplemental Table S1). AHP6 is an unusual member

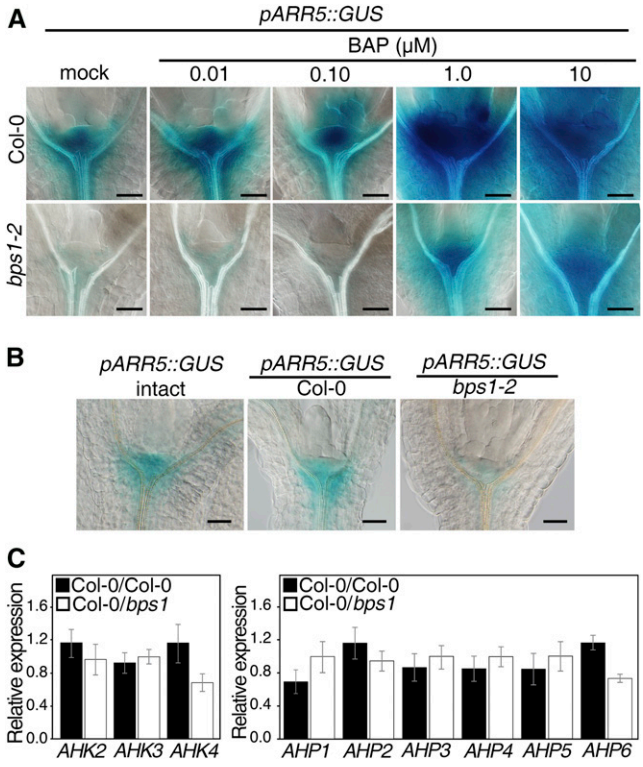


**Figure 5.** The *bps* signal is sufficient to reduce cytokinin responses. A, Shoot expression of the CK response marker *pARR5::GUS* in 5-d wild-type (Col-0) and *bps1-2*, mock-treated or transferred to 0.01 to 10 μM CK (BAP) for 24 h. Bars = 50 μm. B, Transient graft assay of *pARR5::GUS* in 5-d wild-type plants that were intact or scions that were coupled to wild-type or *bps1* roots. GUS staining was carried out 24 h after transient graft establishment. Bars = 50 μm. C, Quantitative analysis of *AHK* and *AHP* expression in 5-d wild-type shoots transiently grafted to either a wild-type (Col-0) or *bps1-2* root. Data are shown as the mean ± SD of three biological and two technical replicates.

of this gene family, as it inhibits rather than promotes phosphotransfer, thus inhibiting cytokinin signaling (Mähönen et al., 2006). In the root, AHP6 plays important roles in regulating differentiation of protoxylem, while in the inflorescence meristem (IM), *AHP6* expression at the flanks of the IM appears to negatively regulate IM size (Mähönen et al., 2006; Bartrina et al., 2011). However, if APH6 function in the SAM is similar to its functions in the IM, we would expect its reduced expression to lead to enhanced SAM size, the opposite of the *bps1* phenotype. Thus, reduced *AHP6* is unlikely to explain the diminished *bps1* SAM size or the loss of *WUS* expression in *bps1* mutants.

### CK Treatment Recovers *WUS* Expression in *bps1*, But Only in the RM

Because CK provided to *bps1* mutants led to partially restored *pARR5::GUS* expression, we tested whether *pWUS::GUS* expression in *bps1* also responded to exogenous CK. As with the stronger and broader *WUS* expression reported for CK-treated wild-type inflorescences (Lindsay et al., 2006; Gordon et al., 2009; Chickarmane et al., 2012), wild-type seedlings transferred to media supplemented with CK (BAP) also showed broader *pWUS::GUS* expression, though only at concentrations of 1 $\mu$M + (Fig. 6A), in agreement with our earlier results using *pARR5::GUS* (Fig. 5A). Similarly, *bps1* mutants transferred to 0, 0.01, and 0.10 $\mu$M BAP showed no *pWUS::GUS* expression, but expression was partially recovered in seedlings transferred to 1.0 and 10 $\mu$M BAP (24 h). This expression, though, was not in its normal domain; instead, it was limited to the RM and did not include the OC (Fig. 6, B and C). These data suggest that these high CK treatments allow *WUS* expression to occur in two separable domains, an upper OC domain and a lower RM domain, and highlight the importance of the *bps* signal in specifically inhibiting expression in the OC domain.

### *WUS* Repression by the *bps* Signal: Altered CK Signaling or SAM Differentiation?

Our analysis of SAM arrest in *bps1* mutants showed that the *bps* signal repressed *WUS* expression and reduced CK responsiveness, but whether the reduction in *WUS* expression was a direct result of altered CK, was unclear. An alternative explanation of these linked responses was that the *bps* signal induces general SAM differentiation. To distinguish between these possibilities, we assessed SAM differentiation status using several approaches.

First, we tested whether the CK-induced *WUS* expression in *bps1* mutants (in the RM) conferred stem cell maintenance. In wild-type seedlings, CK treatment resulted in a SAM that was wider than the mock-treated control and that had small meristematic cells extending deeper into the RM (Fig. 7A). The SAM from CK-treated

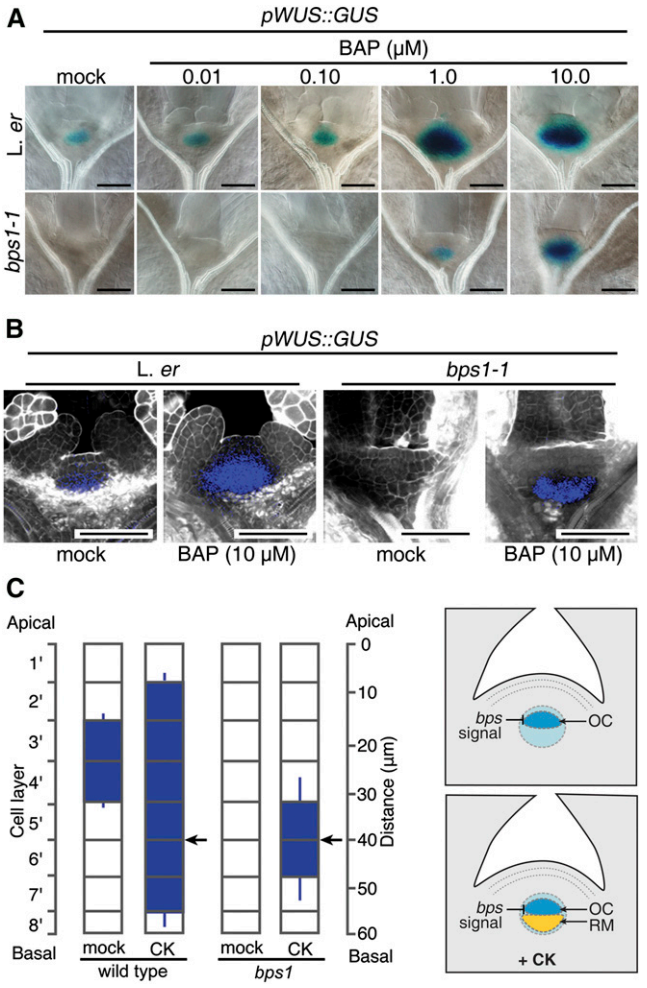


**Figure 6.** CK treatment restores *WUS* expression in the *bps1* rib meristem. A, Shoot expression of the *pWUS::GUS* in 5-d wild-type (L*er*) and *bps1-1*, mock-treated or transferred to 0.01 to 10 $\mu$M CK (BAP) for 24 h. Bars = 50 $\mu$m. B, Confocal images of *pWUS::GUS* expression (4 dpi) in mock-treated or in seedlings transferred to 10 $\mu$M BAP for 24 h. C, Quantitative analysis of CK-treated *pWUS::GUS* expression domain positions; scale on the left indicates cell layers, and scale on the right indicates distance from the apex ($n = 20$). Arrows point to strongest GUS staining (error bar, SD). Cartoon to the right depicts the two domain model for *WUS* expression.

*bps1-1* mutants was also deeper and had additional layers of small meristem-like cells in the RM. However, the SAM apex remained narrow and flat. These observations revealed that the RM domain of the *bps1* SAM remained responsive to *WUS* expression and was consistent with the pattern of *pARR5::GUS* induction (Fig. 5). Nevertheless, it was unclear why the SAM L1 or L2 cells failed to respond to this restored *WUS* expression, but possibilities include their distance from the *bps1 WUS* expression domain (the RM), because the *bps* signal had altered the responsiveness of these cells, or because these cells had differentiated.

As another strategy, we used a genetic approach and assessed the response of *bps1 clv* double mutants to

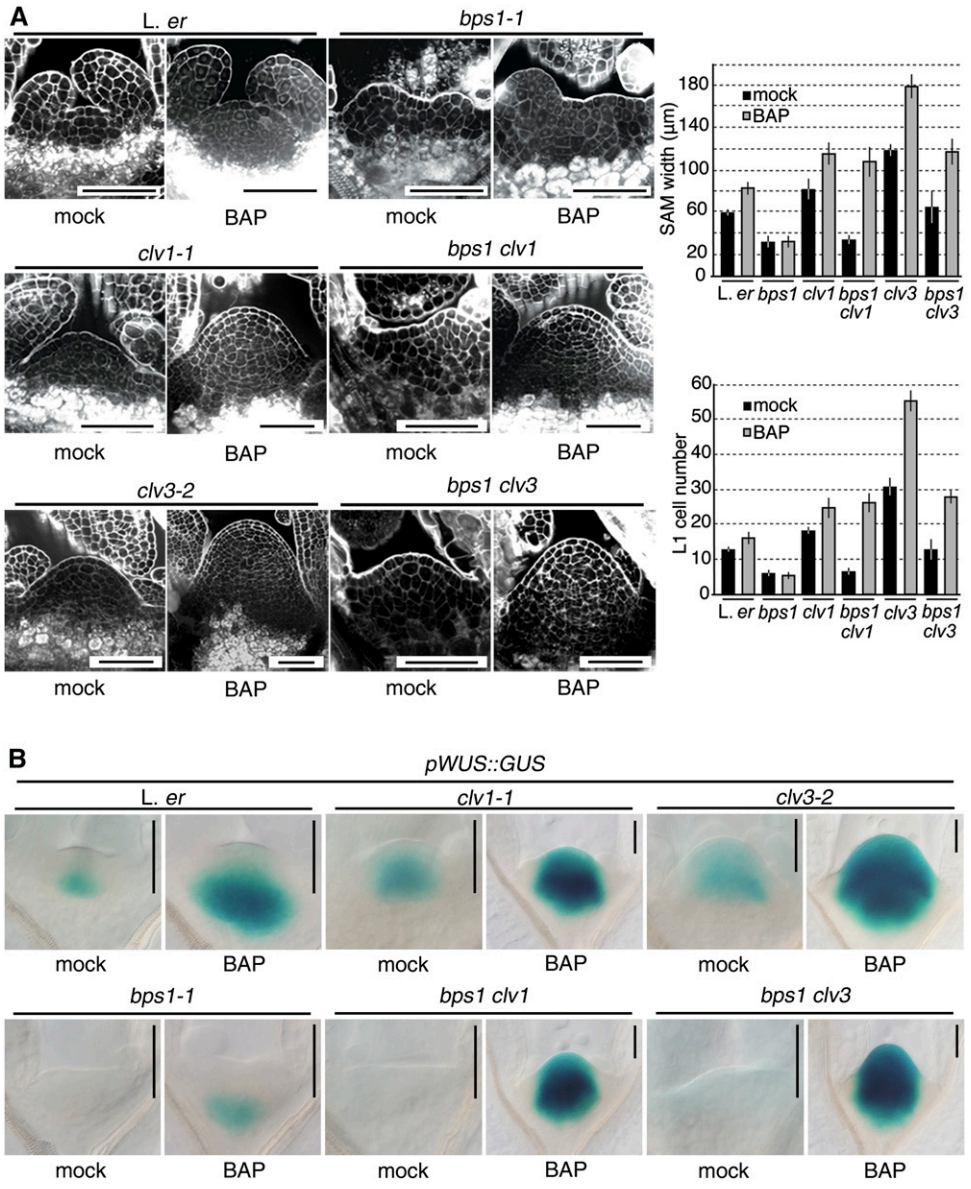


**Figure 7.** In *clv* mutant backgrounds, CK overrides the repression of *WUS* expression provided by the *bps* signal. A, Confocal images of the SAM of 5 dpi seedlings, mock-treated and grown on medium containing CK (0.1 $\mu$M BAP). Seed germination and plant growth were on GM without or with 0.1 $\mu$M BAP until 5 dpi. To the right, histograms depict SAM sizes of mock-treated and CK-treated seedlings. Error bars indicate SD ($n$ = 10, at 5 dpi). B, *pWUS::GUS* expression in single and double mutants at 5 dpi from the experiment condition in A. Bars = 50 $\mu$m.

exogenous CK. As reported by others, *clv1* and *clv3* mutants produce a very large SAM in response to exogenous CK (Lindsay et al., 2006; Gordon et al., 2009; Zhao et al., 2010). Although *bps1* mutants do not respond to CK by SAM apex expansion and do not require CLV signaling for repression of *WUS* expression, we found that both *bps1 clv1-1* and *bps1 clv3-2* double mutants grown on CK-supplemented medium produced large SAMs (Fig. 7A); this revealed that cells of the L1 and L2 were able to proliferate in *bps1* backgrounds. Furthermore, this proliferation appeared to be driven by *WUS*, as *pWUS::GUS* expression extended to the L1 of CK-supplemented *bps1 clv* double mutants (Fig. 7B). Notably, these seedlings (12 d) still showed other features of the *bps1* phenotype, including short abnormal roots and small cotyledons, which indicated that the *bps* signal continued to be produced (Supplemental Fig. S5). Because this experiment involved germinating the *bps1 clv* mutants on CK-containing medium, and we were interested in whether the *bps1* SAM had differentiated, we repeated the *bps1 clv* CK induction experiment but by transferring germinated *bps1 clv1* double mutants to CK-supplemented growth medium (GM). Because *bps1* and *clv1 bps1* are phenotypically indistinguishable when grown on GM, we expected that if the *bps1* SAM was partially differentiated, then the same would be true for *bps1 clv1* double mutants. Seedlings transferred to CK-supplemented medium were analyzed for *pWUS::GUS* expression 24 h later (Fig. 8). In *bps1 clv1* double mutants, transfer induced *pWUS::GUS* in a pattern that was very similar to that of the wild type; expression was detected in the OC and also deeper into the RM (Fig. 8). Remarkably, these *bps1 clv1* double mutants also had a slightly dome-shaped SAM, suggesting that in the absence

of CLV, restored *WUS* expression also restored normal SAM maintenance. These observations revealed that the CZ cells in *bps1 clv1* double mutants remained able to respond to *WUS*, even when it is expressed several days after germination. Moreover, this restored *WUS* response required enhancing CK responsiveness, which was conditioned through the loss of CLV signaling.

We also used the *pCLV3>>WUS-GR* system (Yadav et al., 2010) to induce *WUS* expression in the upper layers of the *bps1* SAM. This construct produces an inactive form of WUS (WUS-GR) in the SAM's central zone, and addition of dexamethasone (DEX) allows WUS-GR to be activated. In wild-type seedlings, this leads to strong SAM expansion (Fig. 9, A and B). Transgenic *bps1* mutants supplied with DEX also produced an enlarged and dome-shaped SAM that included normal-sized cells in the upper SAM. Although ectopic *WUS* expression has been shown to lead to SAM formation, even in differentiated tissues (Gallois et al., 2002, 2004; Xu et al., 2005), these results support the CK-mediated rescue of SAM maintenance in *bps1 clv1* double mutants.

## DISCUSSION

Long-distance signaling in plants is necessary for both normal development and to coordinate physiological responses. Signaling between roots and shoots is especially important for coordinating stress perception (e.g. water availability and nutrient deficiencies) with growth. A possible new pathway functioning in root-to-shoot signaling was implicated by analyses of the Arabidopsis *bps1* mutant. This mutant overproduces a small metabolite (which we call the *bps* signal) in its roots that moves to the shoot, reversibly arrests shoot growth, and is sufficient to arrest shoot growth in wild-type plants (Van Norman et al., 2004). The intriguing hormone-like activity of the *bps* signal prompted us to characterize how it affected shoot growth. The findings presented here revealed that the *bps* signal leads to repression of *WUS* expression, perhaps by repression of CK responses (Supplemental Fig. S6).

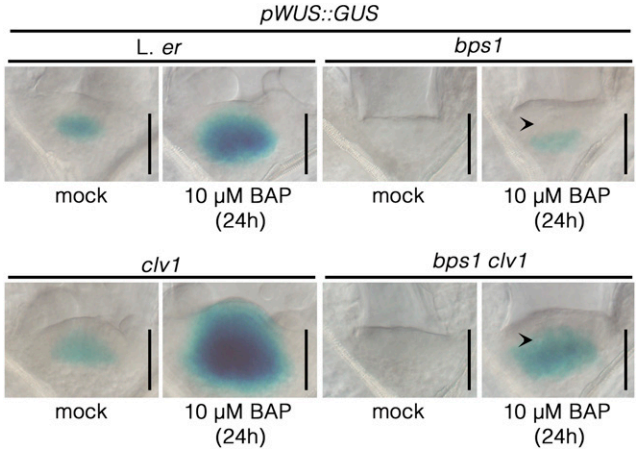


**Figure 8.** CK restores *pWUS::GUS* expression and rescues SAM maintenance in 4-d seedlings. Three-day seedlings that germinated on GM were transferred to medium containing 10 $\mu$M BAP and GUS stained 24 h later. Arrowhead in BAP-treated *bps1* and *bps1 clv1* indicates the OC position. Bars = 50 $\mu$m.

The first hint that the *bps* signal might interfere with SAM maintenance came from comparing the SAM morphology of 3 d *bps1* and wild type; although the embryonic SAMs were indistinguishable, the 3 d *bps1* SAM was small and flat in contrast to the domed SAM of the 3 d wild type. This timing was consistent with previous analysis of *bps* signal responses (Van Norman et al., 2011), suggesting that the loss of BPS1 allows synthesis of the *bps* signal during germination. The change in SAM size further suggested that following recruitment of the first pair of leaf primordia (Medford et al., 1992), the SAM failed to restore a normal number of cells.

We linked the *bps1* SAM maintenance defect to a loss of *WUS* expression. *pWUS::GUS* transgenes were not expressed in *bps1* mutants. Moreover, transient micrograft experiments revealed that the *bps1* root was sufficient to repress *WUS* expression in wild-type shoots. Experiments coupling the *bps1* root to a wild-type shoot indicated that the *bps* signal can exit the root, diffuse across a small agarose block, enter the wild-type scion through the hypocotyl, and repress *WUS* expression, all in 24 h. Thus, the *bps* signal appears to be highly potent and suggested an attractive model for the long-distance regulation of plant growth: root production of the *bps* signal might modulate *WUS* expression to coordinate shoot growth with conditions in the rhizosphere.

Normally the size of the SAM is primarily governed by a WUS/CLV3 feedback loop (Brand et al., 2000; Schoof et al., 2000), and so we considered that *WUS* repression might arise if the *bps* signal induced higher or ectopic *CLV* activity. We showed that neither the CLV1 nor the CLV2 receptors were required for *bps* signal-induced arrest; however, it is a formal possibility that the other CLV3 receptors, *CORYNE* and *RECEPTOR-LIKE KINASE2*, do function in this pathway. We consider this unlikely, though, as we still observed down-regulation of *pWUS::GUS* in the modestly enlarged 5 d SAM of *bps1 clv3* double mutants, and the 12 d *bps1 clv3* double mutants showed shoot growth defects very similar to those of *bps1* single mutants. Although we cannot rule out that the *bps* signal itself might be perceived by the other CLV3 receptors, leading to *WUS* repression, a simpler interpretation is that the *bps* signal influenced *WUS* expression through a CLV-independent mechanism.

Instead, our data suggest that the *bps* signal interferes with activation of *WUS* expression by disrupting CK signaling. The general CK reporter *pARR5::GUS* (D'Agostino et al., 2000) showed low expression in *bps1* mutants, and in transient micrografts, the *bps1* root was sufficient to induce a similar low expression in wild-type scions. An attractive mechanism for *bps* signal repression of CK responsiveness is down-regulation of

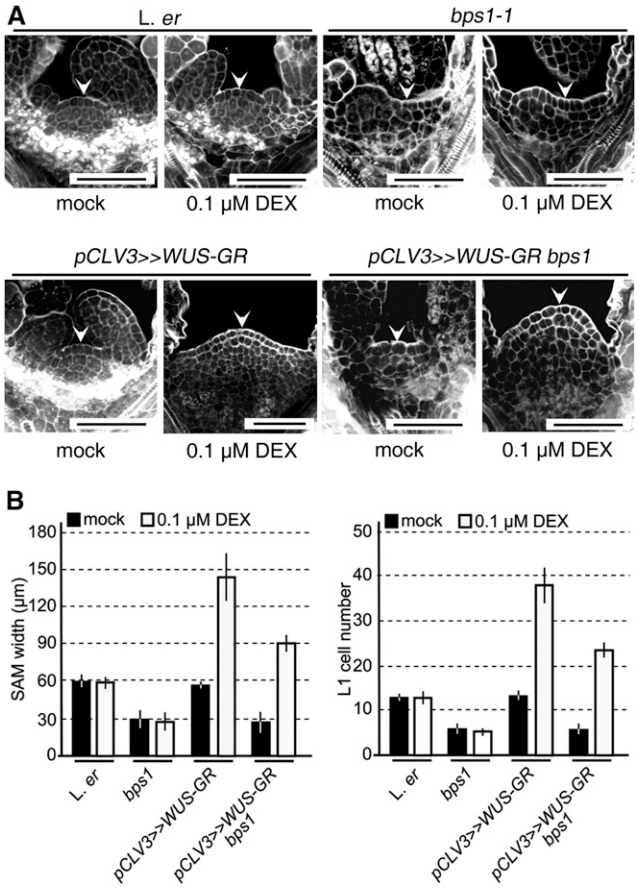


**Figure 9.** Ectopic *WUS* expression in the CZ is sufficient to restore stem cell maintenance in *bps1*. *WUS* expression was under control of the *CLV3* promoter, and activity was regulated by protein fusion with GR. A, Confocal images of SAM of mock-treated and DEX-treated seedlings, including nontransgenic controls, at 5 dpi. Bars = 50 $\mu$m. B, Quantitative analysis of the SAM from the experiment depicted in A. Error bars indicate SD ($n$ = 20 for each genotype).

CK receptors; indeed, we found *AHK4*, a receptor previously linked to reduced *WUS* expression (Gordon et al., 2009), to be down-regulated in wild-type tissue transiently grafted to the *bps1* root. However, within these same wild-type scions, the *bps1* root led to a much stronger repression of *WUS* expression (*AHK4* transcripts down 1.7-fold and *WUS* transcripts down 5-fold). Our expression analysis does not exclude the possibility that *AHK4* is highly down-regulated in just the SAM nor that the *bps* signal might also *AHK* protein activity; however, the differential impact of the *bps* signal on *WUS* and *AHK4* transcripts is difficult to reconcile with a simple receptor repression model.

Elements of CK signaling remained intact in *bps1* mutants, as providing CK to *bps1* mutants partially restored *WUS* expression, albeit limited to the internal RM. This restricted *WUS* expression domain was supported by the restored stem cell maintenance that was restricted to the RM when *bps1* mutants were provided an exogenous supply of CK. The level of CK required to activate *WUS*, however, was quite high and thus not a physiologically normal level, though it was the same as that required to expand the *WUS* expression domain in the wild type. Nevertheless, the data do indicate that it is possible to separate *WUS* expression into two domains. How the *bps* signal restricts CK responsiveness from the upper tier of the SAM is currently unclear. One possibility is that the *bps* signal could function in a manner analogous to CLV3 and provide stronger CK buffering (Lindsay et al., 2006; Gordon et al., 2009; Chickarmane et al., 2012). Alternatively, the *bps* signal might, in addition to reducing *AHK4* expression, disrupt production of CK in the SAM epidermis (Kurakawa et al., 2007; Kuroha et al., 2009), enhance CK degradation (Bartrina et al., 2011), or affect mechanical signaling from the epidermis (Savaldi-Goldstein et al., 2007; Hamant et al., 2008; Bozorg et al., 2014; Gruel et al., 2016).

The *bps1* mutant provided a unique opportunity to examine the activity of WUS when its expression was limited to the RM domain. This localized activity induced a strictly localized response; small meristem-like cells were restored to the RM, but the SAM apex showed no restored maintenance and instead remained flat, with no increase in SAM width or L1 cell number. The localized response to restored *WUS* might not be surprising, as it was consistent with the pattern of *pWUS::GUS* expression; however, an important feature of WUS is its movement, which allows it to confer stem cell identity to the surrounding cells (Daum et al., 2014). Because studies of WUS movement have focused on OC-specific expression, and we find *WUS* expression lower in the RM, it is possible that WUS mobility might depend on where it is expressed. In *bps1*, the failure of RM-expressed WUS to rescue the stem cell identity in the tunica (L1 and L2) might be caused by a loss of cell-to-cell movement, e.g. if the *bps* signal altered plasmodesmata aperture. Indeed, the induced closure of the plasmodesmata in the vicinity of the OC using *pWUS::CalS3m* also caused SAM termination, albeit more modest than the termination induced by the *bps* signal (Daum et al., 2014). It is also possible that plasmodesmata connecting cells of the RM and OC are normally closed, regardless of the *bps* signal. Supporting this idea, a tracer study mapping symplastic domains in Arabidopsis inflorescences showed that at that developmental stage, the RM was symplastically uncoupled from the upper three cell layers (Gisel et al., 1999).

Given the hormone-like activity of the *bps* signal, an attractive model is that the *bps* signal can be deployed to alter the balance of WUS activity in the upper and lower regions of the SAM's OC. WUS activity in the RM leads to stem elongation, while expression in the adjacent OC promotes the activity of the overlying stem cells, which are available to contribute to organogenesis. Separable regulation of these two domains might have important roles in controlling plant architecture, or it could be deployed to arrest organogenesis while still maintaining a residual stem cell population. Indeed, studies of rhizosphere stress implicate long-distance growth

coordination by unknown mobile signaling compounds (Blackman and Davies, 1985; Saab and Sharp, 1989; Gowing et al., 1990; Mulholland et al., 1996; Stuurman et al., 2002). Root signaling associated with drought causes production of both fewer and smaller leaves, which is strikingly similar to the *bps1* mutant. If the *bps* signal is the drought-induced compound, then we would predict its activity in the shoot to result from specific targets that cause reversible effects. We previously showed that growth arrest was reversible when we removed the source of the *bps* signal (the root; Van Norman et al., 2004). Moreover, although *wus* mutants have severe defects in SAM maintenance, they also frequently produce a new SAM (Laux et al., 1996). Whether the *bps* signal is the compound produced by a drought-treated root, however, awaits a deeper understanding of the full set of responses to the *bps* signal and biochemical identification of this enigmatic molecule.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Plants used in this study were Arabidopsis (*Arabidopsis thaliana*), and specifically the Columbia-0 (Col-0) and Landsberg *erecta* (L*er*) accessions. The *bps1-1* (L*er*) and *bps1-2* (Col-0) alleles have been described previously (Van Norman et al., 2004). For double mutant analyses, the *bps1-1* allele was crossed to *clv1-1* (L*er*), *clv2-1* (L*er*), and *clv3-2* (L*er*) (Clark et al., 1993; Laux et al., 1996; Kayes and Clark, 1998). Molecular markers included *pSTM::GUS* (Col-0), *pWUS::GUS* (L*er*), *pCLV3::GUS* (L*er*), and *pARR5::GUS* (Col-0) (D'Agostino et al., 2000; Kirch et al., 2003; Bäurle and Laux, 2005; Williams et al., 2005). Homozygous markers in heterozygous *bps1* and wild-type background were identified and then the expression was tested in next generation. Seeds were plated on plant GM (0.5× MS salts [Caisson Labs], 1% Suc, 0.5 g/L MES [Fisher Scientific], and 0.8% agar [MP Biomedicals]), incubated for 2 or 3 d in the dark at 4°C, and transferred to the growth chamber at 22°C under continuous light (80–140 $\mu$E).

### Transient Micrografts

Transient micrografts, a modification of the Arabidopsis hypocotyl graft, were described previously (Turnbull et al., 2002; Adhikari et al., 2013). Briefly, the rootstock-scion junction was stabilized inside a silicone sleeve (0.012-in. internal diameter; Helix Mark Co.) filled with 0.8% agarose (Fisher Scientific, Molecular Biology Grade). Grafts used 4 dpi seedlings, which were incubated on sterile GM (2% agar) for 24 h prior to analysis.

### Microscopy

SAM anatomy was analyzed using a Zeiss 510 Meta laser scanning confocal microscope. Embryos (isolated from mature seeds) and seedlings were fixed in 50% methanol and 10% acetic acid, and then cell walls were stained using a modified pseudo-Schiff propidium iodide method (Truernit et al., 2008). Confocal microscopy to analyze the GUS staining patterns was carried out using the same modified pseudo-Schiff propidium iodide staining procedure, with the confocal microscope set to reflection mode. Quantitative analysis used 71 to 90 mature seeds, 16 to 24 individuals for each seedling stage, and 17 to 20 for each genotype of double mutants. SAM serial sections were generated using sectioned wax-embedded tissue (8 $\mu$m thick and stained with toluidine blue) as previously described (Long and Barton, 1998).

### GUS Analysis

GUS staining followed previously described methods (Sieburth and Meyerowitz, 1997). Staining incubation time was optimized for each marker using 2 mM 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide (X-gluc). After GUS staining, tissue was incubated in 70% ethanol for 2 d and cleared with saturated chloral hydrate for 6 to 24 h. Observation of the GUS staining pattern was carried out using an Olympus BX-50 microscope and images collected using a digital camera system (Olympus PD71).

### Quantitative Analysis of Transient Micrografts

Each transient micrograft experiment included three controls: intact (scion genotype), root cut (placed into the agarose-containing sleeve but with no rootstock), and coupled to a wild-type rootstock. Each experiment used between 16 and 48 micrografted plants and repeated with similar results in at least three independent experiments. Staining intensity was classified as strong if the staining was easily observed, classified as weak if the blue staining product was difficult to observe and/or diffuse, and classified as absent if no trace of the blue staining product was detectable.

### Quantitative Real-Time RT-PCR

Total RNA was isolated from 5 dpi wild-type (Col-0) scions 24 h after transient micrografting to *bps1-2* rootstocks using the Qiagen Plant RNeasy Kit. The quantity, quality, and purity of the total RNA was estimated using a NanoDrop 2000 (Thermo Scientific) and a Bioanalyzer 2100 (Agilent). RNA was converted to cDNA using the Reverse Transcription System (Promega) and analyzed using the Maxima SYBR Green qPCR Master Mix (Fermentas) and the Mastercycler realplex EP (Eppendorf). *ACTIN2* was used as an internal control to normalize gene expression. The qRT-PCR was carried out using three biological and two technical replicates. Gene-specific primers used for qRT-PCR are listed in Supplemental Table S2.

### Cytokinin Treatment

The cytokinin BAP (Acros Organics) was used for *WUS* induction experiments. BAP was provided to the seedlings in two ways. For transient induction, seedlings carrying *pARR5::GUS* and *pWUS::GUS* (3 or 4 dpi) were transferred to GM supplemented with BAP and GUS staining 24 h later. Optimum BAP concentrations were established by testing 0.01 to 10 $\mu$M, and 10 $\mu$M BAP was selected for the remaining experiments; this is a 100× lower concentration than those used for induction in floral and inflorescence meristems (Lindsay et al., 2006; Gordon et al., 2009; Chickarmane et al., 2012). CK induction experiment were also carried out by germinating seedlings on GM supplemented with 0.1 $\mu$M BAP, as higher concentrations inhibited germination. For testing of SAM differentiation, 4 dpi seedlings were transferred to GM with 10 $\mu$M BAP and analyzed 24 h later.

### DEX Induction

*WUS* expression was induced in *bps1-1* plants by introducing *pCLV3::LhG4 6XOP::WUS-GR* (*pCLV3>>WUS-GR*) (Yadav et al., 2010), and homozygous *pCLV3>>WUS-GR* lines that segregated *bps1-1* were established. The minimal effective dose of DEX (Sigma-Aldrich) for *pCLV3>>WUS-GR* was determined by germination on GM supplemented with 0.01 to 10 $\mu$M DEX; 0.1 $\mu$M DEX was selected because it was the lowest DEX concentration that led to SAM expansion in the wild type. Quantitative analyses were carried out with 20 individuals for each genotype.

### Accession Numbers

Genes from this article can be found in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: *BPS1* (At1g01550), *STM* (At1g62360), *CLV1* (At1g75820), *CLV2* (At1g65380), *CLV3* (At2g27250), *WUS* (At2g17950), *ARR5* (At3g48100), *AHK2* (At5g37550), *AHK3* (At1g27320), *AHK4* (At2g01830), *AHP1* (At3g21510), *AHP2* (At3g29350), *AHP3* (At5g39340), *AHP4* (At3g16360), *AHP5* (At1g03430), and *AHP6* (At1g80100).

### Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** The *bps* signal disrupts shoot apical meristem maintenance.

**Supplemental Figure S2.** Transient micrograft establishment and analysis.

**Supplemental Figure S3.** Seedling and SAM phenotypes of 12 d *bps1 clv3* seedlings reveal SAM arrest in *bps1 clv3* double mutants.

**Supplemental Figure S4.** Quantitative analysis of *pARR5::GUS* by transient grafting assay.

**Supplemental Figure S5.** CK-treated 12 d *bps1 clv3* seedlings.

**Supplemental Figure S6.** Model for mechanism by which the root-derived *bps* signal arrests SAM maintenance.

**Supplemental Table S1.** Expression in wild-type shoots transiently grafted to Col-0 and *bps1-2* roots.

**Supplemental Table S2.** qRT-PCR primers.

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