Regulation of Plant Stem Cell Quiescence by a Brassinosteroid Signaling Module

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SUMMARY

The quiescent center (QC) maintains the activity of the surrounding stem cells within the root stem cell niche, yet specific molecular players sustaining the low rate of QC cell division remain poorly understood. Here, we identified a R2R3-MYB transcription factor, BRAVO (BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER), acting as a cell-specific repressor of QC divisions in the primary root of Arabidopsis. Ectopic BRAVO expression restricts overall root growth and ceases root regeneration upon damage of the stem cells, demonstrating the role of BRAVO in counteracting Brassinosteroid (BR)-mediated cell division in the QC cells. Interestingly, BR-regulated transcription factor BES1 (BRI1-EMS SUPPRESSOR 1) directly represses and physically interacts with BRAVO in vivo, creating a switch that modulates QC divisions at the root stem cell niche. Together, our results define a mechanism for BR-mediated regulation of stem cell quiescence in plants.

INTRODUCTION

Cellular quiescence is a temporary and reversible cell cycle arrest characterized by programmed events that avoid proliferation. However, in eukaryotes, little is known about the molecular determinants for the quiescent state (Cheung and Rando, 2013). Self-renewal of quiescent cells acts as a replenishment source, e.g., in the hematopoietic case it ensures long-term maintenance of multipotent stem cells throughout the organismal lifespan (Wilson and Trumpp, 2006). In plants, the root stem cell niche is composed of different sets of stem cells that give rise to specific root cell lineages, which are surrounding a group of cells with low proliferation rate termed quiescent center (QC) (Scheres, 2007; Figure 1A). The QC cells maintain the stemness of neighboring cells, which function as a major signaling hub maintaining the proliferation/differentiation rates (Cheung and Rando, 2013; Scheres, 2007), where retinoblastoma (RBR) plays an autonomous control in the regulation of QC division (Wachsmann et al., 2011). The proper balance between quiescence and proliferation ensures organismal longevity and prevents both genetic damage and stem cell exhaustion (Cheung and Rando, 2013).

Plant steroid hormones, Brassinosteroids (BRs), are essential regulators of plant architecture, growth and development. BR perception through the plasma membrane-localized BRASSINOSTEROID INSENSITIVE 1 (BRI; Li and Chory, 1997), a leucine-rich-repeat receptor-like-kinase (LRR-RKL) protein promoting the translocation of BRI1 EMS SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1; Wang et al., 2002; Yin et al., 2002) to the nucleus where they regulate gene expression (Sun et al., 2010; Yu et al., 2011). Despite the high degree of knowledge regarding BR-signaling components, how the regulatory events downstream of BES1 and BZR1 are translated into specific developmental outputs remains poorly understood.

The local action of BRs in stomata patterning and establishment of organ boundary (Bell et al., 2012; Gendron et al., 2012; Gudesblat et al., 2012; Kim et al., 2012) argues for cell-specific BR pathways in different organs. However, the majority of the BR signaling components are ubiquitously expressed in the plant, yet cell-specific components have not been identified. In the primary root, BRs are essential regulators of growth and development (Fàbregas et al., 2013; González-García et al., 2011; Hacham et al., 2011; Müssig et al., 2003). BRs promote the division of QC cells at the root stem cell niche, suggesting that counteracting BR signaling is a mechanism to preserve the low rates of cell division in the QC (González-García et al., 2011; Heyman et al., 2013); however, cell-specific repressors of the BR pathway remain to be identified.

In this study, we used a cell-based transcriptomic approach to identify cell-specific regulators of the BR-mediated signaling in...
the root stem cell niche. We report the identification of a R2R3-MYB transcription factor, BRAVO (BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER), acting as cell-specific repressor of BR-mediated divisions in the stem cell niche of the Arabidopsis root. BRAVO mutant plants show strong dividing QCs, whereas BRAVO overexpression under an inducible promoter represses root growth, leading to root growth exhaustion upon genotoxic stress. BES1 directly represses and physically interacts with BRAVO in vivo, enabling strong BRAVO expression in QC cells and null or low BES1 signaling, which together ensure quiescence. In addition, the BR-activated BES1 signaling drives an ultrasensitive response toward the repression of BRAVO, thereby promoting QC divisions at the root stem cell niche. Our study reveals that the BRAVO/BES1 signaling module defines a mechanism for BR-mediated regulation of stem cell quiescence in plants.

RESULTS

BRAVO Defines a BR-Regulated Transcription Factor Specific to Root Stem Cells

To identify cell-specific BR-signaling components, the primary roots of the stele marker WOODEN LEG (Mähoën et al., 2000; pWOL:GFP) were treated with 10 nM Brassinolide (BL, the most active BR compound) at 0.5, 1, 2, and 4 hr (Figure S1 available online). The stele cells were isolated with fluorescent-activated cell sorting (FACS) of green fluorescent protein (GFP) marked cells and subjected to microarray analysis (Birnbaum et al., 2005), revealing a total of 309 significantly differentially regulated genes (fold change > 1.5; p < 0.01; see Experimental Procedures; Table S1). Time-course analysis showed a peak of 120 deregulated genes after 2 hr BL treatment (Figure S1), whereas gene ontology (GO) enrichment analysis (López-Bigas et al., 2008) disclosed cell cycle, histone modification, gravitropism, and phloem/xylem histogenesis among the most enriched categories (Figure S1). To further refine our search, we used the CORONA/ATHB15 (Zhiponova et al., 2013; pAthb15;YFP) marker that labels a few provascular meristematic cells (Figure S1) to perform FACS and microarray analysis after 2 hr of BL treatment (724 genes, fold change > 1.5; p < 0.01; Table S1). The differentially expressed genes were then compared with both BES1 and BZR1 direct targets (Sun et al., 2010; Yu et al., 2011) to identify cell-specific regulators. Venn-diagram comparison of these genes with a set of vascular initial/quiescent center (QC) enriched genes (Brady et al., 2007; Nawy et al., 2005) identified a single gene that matched all criteria (Figure 1B). The gene corresponds to an R2R3-MYB transcription factor, MYB56 (At5g17800), hereafter renamed BRAVO (BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER).

In agreement with the microarray data, BRAVO expression (pBRAVO:GFP) appeared to be specific to vascular initial and QC cells of the root apical meristem (Figure 1C). BRAVO transcription was specifically downregulated by BRs in a dose- and time-dependent manner (Figures 1D and 1E; Figure S2). Exogenous treatments with different plant hormones such as abscisic acid, gibberelins, and ethylene previously related to root stem-cell maintenance failed to significantly modify BRAVO expression in short-term applications (Figure S2). Similarly, BRAVO protein (pBRAVO:BRAVO-GFP) was localized at the nuclei of vascular initials and QC cells and disappeared rapidly upon short-term BL treatment (Figures 1F and 1G). The BR-activated bes1-D mutant that accumulates the active (dephosphorylated) form of BES1 (Yin et al., 2002) exhibited a dramatic reduction in BRAVO levels (Figures 1C and 1H; Figure S2). Collectively, these results show that the BRAVO locus defines a cell-specific component of the BR signaling pathway at the root stem cell niche of Arabidopsis.

BRAVO Is a Negative Regulator of QC Divisions

Previous analyses established that BR-activated BRI1 and BES1 signaling promotes the division of QC cells in the root stem cell
Figure 2. Phenotypic Analysis of bravo Mutants in the Primary Root

(A) Schematic representation of BRAVO gene with the T-DNA insertions in the second exon of the gene.

(B) Relative BRAVO levels in bravo-1 and bravo-2 mutant alleles.

(C) Six-day-old seedlings mPS-PI stained of bravo mutants. White arrows indicate QC position and black arrows the position of QC-divided cells.

(D) Quantification of columella stem cell layers.

(E) Root length of Col-0, bravo-1, and bravo-2.

(F) Six-day-old roots counterstained with PI, white arrow indicates the end of meristematic cells.

(G) Quantification of meristem length of Col-0, bravo-1, and bravo-2.

(H) Transverse root sections of 6-day-old col-0, bravo-1, and bravo-2 seedlings stained with toluidine blue. ***p < 0.005. Error bars ± SEM.
niche (González-García et al., 2011), yet the mechanism for such regulation is not known. To determine whether the BR-signaling component BRAVO controls QC divisions, we analyzed loss-of-function bravo mutants in the primary root apex (Figure 2). Six-day-old seedlings were analyzed for two independent knockout T-DNA insertion lines (Figures 2A and 2B). Unless for the increased QC divisions (Figure 2C), the bravo mutants did not show apparent phenotypes observed in root growth and development (Figures 2D–2I). Microscopic analysis revealed an ~3-fold increase in the frequency of QC divisions in bravo mutants as compared to wild-type (WT; 70% versus 15%; N > 100 for each genotype), and this was restored to the WT levels in pBRAVO::BRAVO-YFP; bravo plants (Figures 3A–3C, 3O). Moreover, the additionally divided cells of bravo mutants express the QC and endodermis identity marker SCARECROW (SCR; Sabatini et al., 2003; Figures 3F and 3G). A progressive fade-out of SCR expression over time in the newly rootward QC cell indicates an asymmetric QC division (Figures 2F and 2G, Figure S3), in agreement with previously published work (Wachsmann et al., 2011). Furthermore, QC markers WOX5 (Sarkar et al., 2007) and AGL42 (Nawy et al., 2005) were also present in the divided QC cells of bravo mutants (Figures 3H–3K, Figure S3), yet their expression appeared to be below WT levels.

BRAVO loss-of-function mutant phenotype resembles that of plants with excess of BR signaling, such as the gain-of-function best-1-D (70%) and plants exogenously treated with BL (0.04 nM; 70%; Figures 3D, 3E, and 3O). In agreement, the additional QC divisions observed in plants with excess of BRs appeared concomitantly with BRAVO downregulation (Figures 1E and 1H). In contrast to the best-1-D, the bravo mutants did not exhibit defects in neither distal nor proximal stem cell differentiation (Figures 2C, 2D, 3A, 3B, and 3D), indicating that BRAVO specifically functions as a local repressor of QC self-renewal in the primary root.

Figure 3. BRAVO Controls QC Divisions

(A–E, L–N) Microscopy images of mPS-PI stained 6-day-old roots with indicated genotypes, Brassinolide (BL, 0.004 nM).

(F–K) Six-day-old seedlings counterstained with PI; (F) pSCR:GFP; (G) bravo/pSCR:GFP; (H) pWOX5:GFP; (I) bravo/pWOX5:GFP; (J) AGL42-GFP and (K) bravo/AGL42-GFP.

(O) Quantification of the QC divisions in 6-day-old roots expressed in percentage, (n > 50 seedlings for each genotype, see Table S1).

See also Figure S3.

Next, we investigated whether QC divisions are downstream of BES1 and BZR1 in the BR pathway. Analysis of RNAi BES1 roots indicated that the BR-mediated QC divisions are downstream of BES1 (Figure 4). First, we observed that BR-mediated QC divisions are downstream of BRII1 (Figures 4A–4D, and 4G–4J). However, the br1-16-D mutants showed mild QC defects and BZR1 expression could not be detected in the QC cells (Figures 4E, 4F, 4O, 4P, 4T–4V). We observed that only BES1 and not BZR1 became activated in the QC upon BL treatment (Figures 4O–4V), supporting a predominant role of BES1 in BR-mediated QC divisions. To unveil the regulation of QC divisions by both BES1 and BRAVO, we generated bri1-16/bravo and bravo/bes1-D double mutants. The absence of QC divisions in bri1-16/bravo double mutants, like in bri1 mutants, pointed to the requirement of BR signaling in order to promote QC divisions (Figures 3N, 3O, and 4). In addition, the stronger QC division phenotype in bravo/bes1-D mutant compared to the single mutants (Figures 3L and 3O) indicates that BES1 and BRAVO do not regulate QC divisions in a linear pathway. In the same direction, local expression of BES1 in the QC cells in pWOX5::BES1-D-GFP displayed a stronger phenotype than bravo mutants (Figure 3M). Together, these data suggest that QC divisions are both activated by BES1 and repressed by BRAVO to preserve quiescence in the root meristem.

Biological Significance of the BRAVO Pathway in Root Development

In the stem cell niche, the activation of stress-associated BR-signaling triggers increased QC division and premature stem cell differentiation that results in aberrant root growth (González-García et al., 2011; Heyman et al., 2013). Our data indicate that BRAVO acts as a highly regionalized repressor, counteracting BR-mediated divisions in the QC. To further investigate its role as a repressor of cell division, we expressed BRAVO outside its native expression domain by generating inducible BRAVO lines. Ectopic induction of BRAVO led to a 50% reduction in root length of 6-day-old seedlings and a reduction of the meristematic cell number (Figures 5A–5D). Furthermore, this induction led to a significant deregulation of widely expressed cell cycle regulators such as CYCB1;2, CYCD3;3, CYD2;2, RBR, KRP1,
Figure 4. BR-Mediated QC Divisions Are Downstream of BES1

(A–F) Six-day-old seedlings counterstained with PI.

(A and B) bri1-116; pWOX5:GFP.

(C and D) bri1-116; pWOX5:GFP treated with BL continuously.

(E) pSCR:GFP expression.

(F) bzr1-D; pSCR:GFP.

(G–O) Six-day-old seedlings mPS-PI stained of the indicated genotypes.

(P) Quantitative analysis of QC divisions.

(Q–V) Six-day-old seedlings counterstained with PI.

(Q) pBES1:BES1-GFP.

(R and S) pBES1:BES1-GFP after continuous BL treatment.

(T) pBZR:BZR-CFP.

(U) pBZR:BZR-CFP expression in the root epidermis. Note that BES1 is expressed in the QC cells, whereas there is no detectable expression of BZR.
KRP2, and WEE1 (Figure 5E) concomitantly with BRAVO induction, in agreement with microarray data of BR-responsive genes (Figure S2). Thus, BRAVO can repress cell divisions by interfering with the normal cell cycle.

The continuous renewal of stem cells ensures proper root growth and development (Scheres, 2007). In light of our results, we hypothesized that BRAVO functions in conferring to the QC the capacity to overcome external stresses, i.e., DNA stress. Thus, using a radiolabeled drug, which promotes stem cell death by chemical induction of DNA damage (Cruz-Ramírez et al., 2013; Fulcher and Sablowski, 2009; Heyman et al., 2013), we investigated the role of BRAVO in controlling stem cell regeneration. Upon bleomycin treatment, WT plants expressing pBRAVO:GFP undergo a downregulation of BRAVO concomitantly with QC division. This indicates that BRAVO regulates the preceding QC division necessary to guarantee replenishment of the stem cell compartment and to promote root growth (Figures 5F and 5G). Moreover, BRs promoted DNA damage-mediated death of the QC cells and both bes1D and bravo mutants exhibited a reduced root growth recovery upon bleomycin treatment (Figure S4). In contrast, bleomycin treatment of plants that ectopically express BRAVO blocked root growth, and end up with organ exhaustion (Figures 5G and 5H). Hence, BR-mediated regulation of BRAVO functions to restrict quiescence and ensures the maintenance of regeneration potential of stem cells upon damage. Together, these analyses uncover a role for the BR-mediated BRAVO pathway in root development.

**BRAVO and BES1 Module Creates a Switch in the QC Cells**

Because both BRAVO and BES1 are found in QC cells and drive antagonistic effects on QC divisions in a nonlinear pathway, we evaluated whether they regulate each other to ensure a univocal response. First, we tested whether BES1 downregulation of BRAVO is transcriptional. Chromatin immunoprecipitation (ChIP) data showed that the dephosphorylated, active form of BES1 binds to the E-boxes of the BRAVO promoter (Figure 6A; Figure S1). This transcriptional repression was confirmed by transactivation assays in *Nicotiana benthamiana* and was released in the presence of BRAVO (Figures 1H and 6B). In addition, both ChIP and transactivation analysis revealed that...
BRAVO binds to and promotes its own expression (Figures 6B and 6C), demonstrating that BRAVO can be transcriptionally regulated by both BRAVO and BES1.

The heterodimerization of BES1 with other MYB transcription factors has been reported (Yu et al., 2011). We next tested BRAVO/BES1 heterodimerization with fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) and with coimmunoprecipitation experiments in planta. Our results showed that BRAVO interacts with the dephosphorylated, active form of BES1 in the nucleus (Figures 6D and 6E; Figure S4). To assess the biological activity of BRAVO/BES1 interaction, bes1-D; pBRAVO::BRAVO-GFP double mutants were generated. Increased levels of BRAVO suppressed the QC division phenotype of bes1-D (Figures 6F–6H), similar to the exogenous BL treatment of pBRAVO::BRAVO-GFP (Figures 6F–6H). In agreement, transactivation assays with BES1/BRAVO dimer suppress repression/activation activities of BES1 and BRAVO (Figure 6B), respectively. Altogether, these results show that BRAVO/BES1 proteins heterodimerize in the QC cells.

Finally, to understand how the cross-regulations between BES1 and BRAVO integrate the BR signaling that controls QC divisions, we built a mathematical model that takes into account the BRAVO-BES dimerization and BRAVO transcriptional control by BRAVO and BES1 (Figure 7A, see Mathematical Model within Experimental Procedures). Computational simulations predicted that these interactions drive robustly opposite amounts of BRAVO and active BES1 with a switch-like response to BR signaling (Figures 7B and S5). Specifically, a high amount of free active BES1 (i.e., [HIGH, LOW] state) at low BR signaling (i.e., low BES1 dephosphorylation rates). As BR signaling increases, a switch (i.e., sharp transition) to an opposed state with strong free active BES1 and no free BRAVO (i.e., [LOW, HIGH] state) occurs. Taking into account the roles of BES1 and BRAVO in QC divisions unveiled by the mutant analyses, the (HIGH, LOW) state drives quiescence, whereas QC divisions are induced in the (LOW, HIGH) state. Together, these results provide a fine mechanism for BR-controlled QC divisions.

Parameter space exploration of the mathematical model indicated that the physical interaction between BES1 and BRAVO is crucial to drive opposed states of BRAVO and BES1 and a sharp transition (Figures 7C and S5). This is in agreement with the known ultrasensitive responses driven by molecular titration through heterodimer formation (Buchler and Louis, 2008; Cross and Buchler, 2009). In addition, our computational results show that the functional role of the BRAVO-BES1 heterodimer in BRAVO transcription is not relevant for these features to hold (Figure 7D). Moreover, BES1 transcriptional repression of BRAVO and BRAVO auto-activation facilitate that this sharp transition becomes bistable (Figure S5).

Importantly, the states of BRAVO and dephosphorylated BES1 expression predicted by the model are in agreement with the extent of BRAVO and dephosphorylated BES1 in the QC of WT plants and in plants treated with BL (Figures 1C–1E and 4). We next evaluated whether an abrupt switch in BRAVO expression with BR signaling occurs in vivo, as predicted by the model. To this end, we quantified pBRAVO::GFP fluorescence in individual QC cells after continuous BL treatments.
uncovering that BRAVO displays an ultrasensitive response to BRs (Figure 7E).

**DISCUSSION**

BRs play key roles in cell division associated to developmental programs such as root meristem, the formation of organ boundaries, and stomata patterning, yet BR components operating at a cellular scale have not been disclosed. Our data unveil how BR signaling operates with cellular resolution, and defines BRAVO as a molecular repressor counteracting steroid-mediated divisions in the stem cell niche. This mechanism ensures the low rates of cell proliferation in the QC, whereas the behavior of the BRAVO/BES1 signaling module can confer the QC cells with the plasticity to adapt to environmental changing conditions. Collectively, our results support that BRAVO is a master regulator of cellular quiescence in plants.

The identification of BRAVO as the single gene appearing in a Venn diagram in a search for stem cell-specific BR-signaling components using FACS coupled to transcriptomics hinted at the potential significance of this locus. Despite that, BRAVO gene belongs to a large multigene family, MYB transcription factors (Dubos et al., 2010), the bravo knockout mutants exhibit cell-specific defects at the quiescent center cells of the root stem cell niche.

The regulation of quiescence in the stem cell niche, where the quiescent cells are surrounded by rapidly dividing stem cells, has been an outstanding question in developmental biology (Hsu and Fuchs, 2012; Morrison and Spradling, 2008). In the plant root, the quiescent cells provide short-range signals to maintain stem-cell-specific defects at the quiescent center cells of the root stem cell niche.

The Venn diagram in a search for stem cell-specific BR-signaling elements bound to the promoter. Parameter values as in (B) with (left) $K_{C1} = K_M$ and (top) $\varepsilon_1 = 0.1$, (middle) $\varepsilon_1 = 1$, (bottom) $\varepsilon_1 = 3.9$; (right) $K_{C2} = K_B$; and (top) $C_{2C} = C_0 = 0.068$, (middle) $C_{2C} = 1$, (bottom) $C_{2C} = 2$.

(E) $pBRAVO:GFP$ expression in QC cells as a function of BL concentration. The dots indicate the mean fluorescence per QC cell averaged over $n$ ($18 < n < 100$) QC cells of 6-day-old seedlings continuously treated at each indicated concentration of BL. Data from two experiments. SEM is indicated. Fluorescence (in arbitrary units) has been normalized to the CTL average fluorescence. The curved line represents the function $y = 1 - 0.91x/(xh+0.0027h)$ with $h = 2.8$ and $x$ is the BL concentration, denoting the ultrasensitive response ($h > 1$) of $pBRAVO:GFP$ expression to BL.

See also Figure S5.
Quiescence Regulation by BRAVO

regulators that maintain homeostasis and the long-term function of stem cell niches. In this context, the identification of a negative regulator, BRAVO, that inhibits the steroid hormonal pathway of the root stem niche will serve as a paradigm that will be of relevance for other stem cell niches, beyond the root.

We propose that BRAVO negatively regulates QC divisions by acting as a safe-lock to retain QC cells in a mitotically inactive status. The lack of BRs signaling in the nondividing QC cells is supported by (1) the specific BRAVO expression, and (2) the lack of BRs-promoted ERF115 expression (Heyman et al., 2013), which suggests that the activation of the BR-signaling pathway is detrimental for proper QC function. This notion is further supported by radiolabeled drug treatment of BR-signaling mutants (Figure S4). Importantly, BRAVO dynamics upon DNA damage suggest its involvement in promoting quiescence, ensuring proper root growth regeneration. Oppositely, BRAVO downregulation would release the BR-dependent ERF115 expression (Heyman et al., 2013). QC cells are a reservoir of both auxin transport and biosynthesis (Overvoorde et al., 2010). Despite any specific quantification of BRs in planta has been carried out, it is attractive to speculate that plant levels of BRs in the QC will result in lower proliferation activities. Understanding the hierarchy of those among other regulators will further refine our understanding of quiescence.

Cell response to stimuli is fundamental for proper plant adaptation to environmental cues, and these reversible responses account for its renowned plasticity (Siegel-Gaskins et al., 2011). QC cells divide upon stimulation by hormones (González-García et al., 2011; Ortega-Martínez et al., 2007; Zhang et al., 2010, 2013), stem cell damage (Heyman et al., 2013), or cell cycle interference (Cruz-Ramírez et al., 2013) enabling replenishment of the stem cells upon damage. To preserve the QC function as a reservoir of stem cells, this transition between divided and nondivided QC cells should be reversible. Our mathematical modeling taking into account BES1/BRAVO mutual interaction, BRAVO autoactivation, and BRAVO transcriptional inhibition by BES1 results in a robust response to stimuli that is switch-like and is accompanied by opposed expressions of BRAVO and BES1. When considering that BRAVO and BES1 can both antagonistically regulate QC divisions, this switch-like response becomes relevant to ensure univocal responses on whether the QC needs to divide. Although alternative mechanisms driving switch-like responses cannot be discarded, we found that with BR hormone stimulation, BRAVO switches its expression in vivo sharply, thereby enabling the change from quiescence to the induction of QC divisions. Notably, the three elements involved in BRAVO and BES1 cross-regulation—(1) dimerization of two proteins, (2) transcriptional autoactivation of one of them, and (3) transcriptional repression by the other protein drive bistable cellular responses as shown with in silico evolutions of genetic circuits (François and Hakim, 2004). In agreement, our modeling results indicated that the sharp transition of BRAVO expression with BR signaling can robustly involve bistability of BRAVO expression states. The mechanism provided by the BES1/BRAVO signaling module gives a fine example for a selective control of cellular quiescence in eukaryotes.
Fluorescence Microscopy
Phenotypic analysis of roots of mPS-PI stained plants and GFP fluorescence were depicted as described (González-Garcia et al., 2011). The CFP and YFP fluorescence in *N. benthamiana* and Arabidopsis epidermal cells was analyzed with a confocal laser scanning microscope (TCS SP2-SE; Leica) using a 63x water immersion objective lens (numerical aperture 1.20; PL APO). CFP fluorescence was excited with the 458 nm ray line of the argon laser and recorded in one of the confocal channels in the 455–520 nm emission range. YFP fluorescence was excited with the 514 nm ray line of the argon laser and detected in the range between 520 and 575 nm. Images were acquired in the sequential mode (20 Z plams per stack of images; 0.5 μm per Z plain) using Leica LCS software (version 2.61).

Fluorescence Lifetime Microscopy and Data Analysis
Fluorescence lifetime of the donor was experimentally measured in the presence and absence of the acceptor. FRET efficiency (E) was calculated by comparing the lifetime of the donor in the presence (τ<sub>0</sub>W) or absence (τ<sub>0</sub>W) of the acceptor: E = 1 – (τ<sub>0</sub>W)/τ<sub>0</sub>W. Statistical comparisons between control (donor) and assay (donor + acceptor) lifetime values were performed with Student’s t test. FRET–FLIM measurements were performed using a FLIM system coupled to a streak camera. The light source (λ = 439 nm) was a pulsed diode laser working at 2 MHz (Hamamatsu Photonics, Japan). All images were acquired with a 60x oil immersion lens (Plan APO 1.4 numerical aperture, IR) mounted on an inverted microscope (Eclipse TE2000E, Nikon, Japan) coupled to the FLIM system. The fluorescence emission was directed back out into the detection unit through a band pass filter. The FLIM unit was composed of a streak camera (Streakscope C4334, Hamamatsu Photonics, Japan) coupled to a fast and high sensitivity CCD camera (model C8800-53C, Hamamatsu). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with tri-exponential function using a nonlinear least-squares estimation procedure.

Fluorimetric QUS Assays
For GUS reporter assays, the indicated constructs were transiently expressed in *N. benthamiana* leaves using Agrobacterium. Leaf discs were collected 36 hr after agroinoculation, frozen in liquid nitrogen and stored at −80°C until processing. After protein extraction, 1 μg of total protein was used in replicate to measure enzymatic activities of individual samples. GUS activity was measured using the substrate 4-methylumbelliferyl-D-glucuronide as described (Froidure et al., 2010).
inhibition and activation, respectively, driven when both dephosphorylated BES1 and BRAVO are bound to the BRAVO promoter, whereas $e_3 = 1$ indicates that production is not modified from the basal one.

Using mass action kinetics, we can translate these reactions into the following six ordinary differential equations:

\[
\begin{align*}
\dot{D}_1 &= k_{1a} \cdot D_{1M} - k_{2a} \cdot MB_1; \\
\dot{D}_2 &= k_{3a} \cdot D_{2B} - k_{4a} \cdot BD_2; \\
\dot{M} &= a \cdot (D_2 + e_1 \cdot D_1 + e_2 \cdot D_1 + e_3 \cdot D_2) + k_{1M} \cdot D_{1M} + k_{1D} \cdot D_{1D} + k_{1'c} \cdot D_{1D'} - k_{2M} \cdot MB_1 - k_{2D} \cdot D_{2D} - a_2 D_{1D} + a_2 D_{1D'} \\
B_2 &= k_{3b} \cdot B + k_{2b} \cdot D_{2B} - k_{4b} \cdot BD_2 - k_{5b} \cdot MB_2 - a_2 B_{1D} + a_2 B_{1D'} \\
B &= b_1 \cdot k_{b1} \cdot B_1 - b_2 \cdot B + a_2 B_{1D} - a_2 B_{1D'} \\
C &= k_{c1} \cdot MB_2 - k_{c2} \cdot C - d_1 C \\
\end{align*}
\]

(Equation 2)

Parameters $e_1 = 3.9$, $e_2 = 0.088$, $e_3 = 1.353$, $k_{1M}/k_{2M} = 72.66 \text{ nM}^{-1}$, $k_B = k_{b1}/k_{b2} = 82.06 \text{ nM}^{-1}$, and $k_{2D} = k_{2D}/k_{2D'} = 164.76 \text{ nM}^{-1}$ were estimated from the experimental data of Figure 5B following the procedure described in the Supplemental Experimental Procedures. For all remaining nontitlable parameters, we chose their values within biologically reasonable ranges, setting: $D_{1D'} = D_{2D'} = 0.6 \text{ nM}$, $\beta = 3 \text{ nM}^{-1} \cdot h^{-1}$, $d_{MB} = d_{BD} = 0.02 \text{ h}^{-1}$, $d_{MC} = 0.002 \text{ h}^{-1}$, $k_B = 0.01 \text{ h}^{-1}$, and $k_{cD} = 0.002 \text{ h}^{-1}$. Because the stationary solutions depend also on $k_{b1}$ and $k_{2D}$, we set them as $k_{b1} = 329.52 \text{ h}^{-1} \cdot \text{nM}^{-1}$ and $k_{2D} = 2 \text{ h}^{-1}$ with $k_B = k_{2b}/k_{2D} = 164.76 \text{ nM}^{-1}$.

**Mathematical Model with Active Heterodimer**

To explore the possibility that the dephosphorylated BES1-BRAVO heterodimer could also transcriptionally regulate BRAVO, we considered two distinct scenarios, generating two different submodels. In the first one, the heterodimer binds to the same site as BRAVO promoter as BRAVO itself with a binding constant $k_{C1} = k_{C1} \cdot k_{C2}$, and drives BRAVO transcription at a rate $e_{C2}$ times the basal one. When both BES1 and the heterodimer are bound to the promoter, the transcription is modified by a factor $e_B$, as when BRAVO is bound. These considerations give four new additional reactions to those in Equation 1:

\[
\begin{align*}
D_1 + C &\rightarrow C_1 \\
D_1 C + D_2 &\rightarrow D_1 C + D_2 + M \\
D_1 C + D_2 B &\rightarrow D_1 C + D_2 B + M \\
D_1 C + D_2 B &\rightarrow D_1 C + D_2 B + M
\end{align*}
\]

(Equation 3)

The resulting dynamics from Equations 1 and 3 are obtained following the procedure used to obtain Equation 2. The stationary states of these dynamics were evaluated as described below for different parameter values of $k_{C1}$, $k_{C2}$, and $e_{C2}$.

In the second scenario, the heterodimer binds to the binding site of BES1 with a binding constant $k_{C2} = k_{C2} \cdot k_{C2}$, and activates or represses BRAVO transcription by a factor $e_{C2}$. When both BRAVO and the heterodimer are bound to the promoter, the transcription is modified by a factor $e_{B}$, as when BRAVO is bound. The corresponding reactions, which need to be added to those in Equation 1, are

\[
\begin{align*}
D_1 + C &\rightarrow C_1 \\
D_1 + D_2 &\rightarrow D_1 + D_2 + M \\
D_1 + D_2 B &\rightarrow D_1 + D_2 B + M \\
D_1 + D_2 B &\rightarrow D_1 + D_2 B + M
\end{align*}
\]

(Equation 4)

The resulting dynamics from Equations 1 and 4 are obtained following the procedure used to obtain Equation 2. The stationary states of these dynamics were evaluated as described below for different parameter values of $k_{C2}$, $k_{C2}$, and $e_{C2}$.

**Mathematical Analysis of the Model**

We obtained all the steady states of the above dynamics by setting the derivatives to 0 using Mathematica Software (Wolfram Research, Mathematica, Version 9.0). Solid lines with symbols in bifurcation diagrams denote the stable steady states found. Dashed lines in bifurcation diagrams are a guide to the eye for transitions. In parameter space explorations, solutions are computed at the center of the plotted points, and colored overlays are guides to the eye. Stability of the solutions was checked through numerical integration of the dynamics using custom-made software.
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