**BRAVO AND WOX5 CONTROL ROOT SURVIVAL FROM THE STEM CELL NICHE**

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SUMMARY

Brassinosteroids (BRs) hormones control root growth and development. In the quiescent centre (QC), BRs regulate BRAVO and WOX5 transcription factors, which repress QC division. Here, we combined experimental and mathematical modelling approaches to show that BRAVO and WOX5 interact and regulate each other in the QC. Together they are required to maintain the quiescent state of the QC and to ensure stele stem cell recovery in response to DNA damage.

KEYWORDS

Brassinosteroids, root, cell division, stem cell, quiescent centre, root survival, DNA damage, BRAVO, WOX5, modelling

**INTRODUCTION**

Roots are indispensable organs to preserve plant life and terrestrial ecosystems under normal and adverse environmental conditions. In *Arabidopsis thaliana* (Arabidopsis), the primary root is formed by the activity of the stem cells located at the base of the meristem in the root apex. The root stem cell niche (SCN) is formed by proliferative stem cells that surround the mitotically less active quiescent centre (QC) which prevents their differentiation [1]. The low proliferation rate of the QC provides a way to preserve the genome from replication errors, acting as a reservoir of the root stem cells and dividing to replenish them when they are damaged [2, 3].

In the proximal side of the QC, the stele stem cells (SSC; also called vascular initial cells) generate the stele cells that will give rise to functional xylem and phloem conductive tissues of the plant [4]. Distally located to the QC the columella stem cells (CSC) will give rise to the columella cells [5]. Important factors involved in the regulation of the QC and SCN activity include the auxin regulated PLETHORA genes that are active due to the auxin peak levels in the SCN [6], SCARECROW [7], ETHYLENE RESPONSE FACTOR 115 [8] and GRF-INTERACTING FACTORs [9]. Despite the present understanding of factors regulating stem cell development during root growth, fundamental questions about stem cell signalling mechanisms that control root survival upon environmental or genomic damage are just starting to emerge. Those will be crucial to preserve plant biomass production on earth. Brassinosteroids (BRs) are essential plant steroid hormones for root growth and development and control cell division, elongation and differentiation of root cell types along the longitudinal axis [10].

In the root tip, BRs promote the division of QC cells and differentiation of the distal CSC [5, 11] by controlling the levels of stem cell specific factors such as BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO)and WUSCHEL RELATED HOMEOBOX 5 (WOX5)[5, 12]. *BRAVO* is an R2R3-MYB transcription factor which acts as a specific repressor of BR-mediated QC cell division[12]. The expression of BRAVO is directly regulated by the transcription factor BRI1-EMS-SUPPRESSOR 1 (BES1), which is one of the main effectors of the BR signaling pathway, altogether with its co-repressor TOPLESS (TPL) [12, 13]. WOX5is specifically expressed in the QC [14, 15] and acts by preserving quiescence of the QC and the differentiation of the adjacent CSC [16-18].

Although BRAVO and WOX5 represent the first two plant cell-specific repressors of QC division identified to date, their molecular connection and the possible biological relevance of those in root survival has not yet been established. Here, we show that BRAVO and WOX5 interact at the molecular and genetic level repressing QC division and provide experimental and computational evidence for a joint role of BRAVO and WOX5 in the division of the QC cells in normal conditions. Interestingly, our work also unveils that these two genes are important for overcoming a genotoxic stress, and that their expression regulation to overcome this stress is regulated by a paracrine steroid signal from the damaged cells to the QC and SSC.

**RESULTS AND DISCUSSION**

**BRAVO and WOX5 form a positive feedback loop in the root stem cell niche**

In the root apex, BRAVO and WOX5 control the quiescent status of the stem cells by repressing QC divisions [12, 16]. However, BRAVO and WOX5 levels are oppositely regulated by BRs [5, 12]. To understand the molecular connection between BRAVO and WOX5, we used the pBRAVO:GFP and pWOX5:GFP reporters to analyse the expression pattern and fluorescence levels of both promoters in *bravo* and *wox5* mutant background (Figure 1). In the WT plants, BRAVO expression is specifically located in the QC and the adjacent SSC (Figure 1B, [12]). In contrast, in *wox5* mutants*,* BRAVO expression is reduced in the QC whereas is still normally expressed in the SSC (Figure 1D), suggesting that WOX5 is positively regulating BRAVO expression in the QC. Further supporting this data, 35S:WOX5:GR overexpressor plants show a dramatic increase on BRAVO levels (Figure S1G).

On the other hand, *bravo* mutants displayed a reduction of WOX5 levels (Figure 1G), indicating that BRAVO is necessary to maintain proper WOX5 levels in the QC. Intriguingly, increased BRAVO levels promote a WOX5 overexpression only in the QC cells (Figure S1K). Finally, both *bravo*;pBRAVO:GFP and *wox5*;pWOX5:GFP lines displayed increased levels of GFP compared to the WT controls (Figures 1C, 1H), thus indicating that both transcription factors are negatively regulating themselves. Altogether, these results support a positive feedback loop regulation between BRAVO and WOX5 genes in the QC, and that each of them decreases its own transcription.

To deeper understand the genetic regulation between these two genes, we generated the double mutant *bravo wox5* and evaluated WOX5 expression. Its expression in *bravo wox5* mutantwas increased compared to the WT (Figure S4I) and even more than in the *wox5* single mutant (Figure 1H); suggesting that *bravo* mutation enhances the effect of *wox5* mutation on the WOX5 expression and not vice versa.

An increased expression of WOX5 in *wox5* mutant (Figure 1H) and in *bravo wox5* mutants was observed not only in the QC but extended to the provascular cells (Figure S1I), indicating that WOX5 restricts its own expression in the QC. While WOX5 specific expression is confined by external signals such as ROW1 [14], our results advance that WOX5 expression in the QC cells is controlled also by internal signals like WOX5 itself and BRAVO.

**BRAVO and WOX5 form a complex in the QC cells**

To decipher the molecular connection of BRAVO and WOX5 we next evaluated their possible interaction. It is known that the BR-regulated BES1/TPL complex acts as a transcriptional repressor of BRAVO transcription during QC divisions [12, 13], and that BES1D directly interacts with BRAVO [12] and TPL with WOX5 [18]. Therefore, we also asked if all these components are also interacting with BRAVO and/or WOX5. Yeast-2-hybrid (Y2H) and Förster resonance energy transfer measured by fluorescence lifetime microscopy (FRET-FLIM) assays show that BRAVO can directly interact with BES1, having a stronger interaction with the active form BES1-D [19] (Figure 2), in agreement with [12], and that the EAR domain of BES1 is necessary for BES1/BRAVO interaction (Figure S2A). Our analysis show that BES1 interacts with WOX5 in FRET-FLIM (Figure 2H) and Bimolecular Fluorescence Complementation (BiFC) assays (Figure S2C), at a similar level as BRAVO, and that this interaction is stronger with BES1-D (Figure 2I, 2K). Moreover, both BRAVO and WOX5 also interact with the co-repressor TPL (Figure 2, S2). Finally, we found that BRAVO and WOX5 directly interact in Y2H assays (Figure 2L) as well as in FRET-FLIM (Figure 2B, 2G, 2K). Collectively, our data indicates that BRAVO and WOX5 can biochemically interact between them along with BES1/TPL in the QC cells.

**A mathematical model supports the BRAVO-WOX5 complex formation in the QC cells**

In light of our biochemical data, we constructed a mathematical model to evaluate a minimal set of interactions between BRAVO and WOX5 that can account for the genetic (Figure 1) and biochemical (Figure 2A-L) regulations we observed in the QC. The model assumes that the free BRAVO and WOX5 proteins activate each other´s transcription (Figure 2M-N). These mutual transcriptional activations can drive the positive feedback in promoter activities we show in Figure 1. In addition, the model includes the activation of BRAVO expression by itself [12], assuming it occurs through the free protein. It also takes into account BRAVO-WOX5 biochemical interaction (Figure 2B, 2G, 2L), modelling it as a heterodimer which does not dissociate. The model derivation, discussion of additional assumptions and alternative models are presented as Supplementary Material.

Our results show that these minimal interactions can account for the promoter activities we measured in single mutants, assuming them at the stationary state (see methods). Specifically, the model can explain the increase in the promoter activity in the mutant backgrounds (*bravo* or *wox*5) (i.e. the negative self-regulation each protein has on its own promoter) (Figure 2O-P). In the model, the formation of the BRAVO-WOX5 complex sequesters the free proteins, which are the ones activating each other transcription. Therefore, the formation of the complex inactivates the proteins, yielding an effective negative self-regulation, despite BRAVO directly activates its own transcription (Figure 2M, SI text). Specifically, the computational results predict that the QC in the WT has less free BRAVO and WOX5 protein than in the *wox5* and *bravo* mutants respectively, despite their transcription is higher in the WT.

**BRAVO and WOX5 are required to preserve the QC identity and stem cell maintenance**

We subsequently examined the functional role of BRAVO/WOX5 interaction in stem cell and overall root development. The primary root phenotypes of double mutants *bravo wox5* was analysed with a focus in the SCN, where both transcription factors are localized (Figure 3). The *bravo* and *wox5* single mutants have a higher percentage of QC divisions in comparison to the WT, 80% and 50% respectively (Figure 3A, 3B, 3C, 3E), agreeing with previous data [12, 17]. Strikingly, the *bravo wox5* double mutant shows a 100% of divided QC cells (Figure 3D, 3E), indicating a joint role of BRAVO and WOX5 in maintaining the cellular quiescence at the root SCN. Double mutants were slightly but significantly shorter than the WT in control and BL treatment conditions (Figure S3B), supporting the idea that the impact of BR signalling from the QC can impact overall root growth [3, 5]. In terms of CSC differentiation, we found that *wox5* and *bravo wox5* mutants show the same CSC differentiation phenotype respectively (Figure 3F; [16]), whereas no CSC differentiation defects were observed in *bravo* mutants (Figure 3F; [12]), supporting that BRAVO is not implied in CSC differentiation. Together, our results indicate that BRAVO and WOX5 interact in the root SCN to promote QC divisions during normal root growth.

**Upon DNA damage, BRAVO/WOX5 interaction is essential for stem cell regeneration and root survival**

To address the biological relevance of the 100% of QC divisions in the *bravo wox5* double mutant, we evaluated the stem cell renewal capacity of these plants in response to a genotoxic stress. In the root meristem, the SSC are more sensitive to genotoxic stresses as a consequence of their higher division rate [2]. The QC cells act as a population of non-damaged stem cells that will replenish the damaged ones to guarantee root survival [3, 8, 12]. Plants were treated with bleomycin, a drug that produces double-stranded DNA breaks [2], in order to simulate a genotoxic stress. The 24 hours bleomycin treatment causes SSC death (figures 3G-J,3O) which ends in root exhaustion observable after 72 hours of recovery in the 40% of wild type plants (Figure 3P). Surprisingly, the double mutant *bravo wox5*, which suffers the same damage than the WT after the 24h treatment (Figure 3G, 3J, 3O), has the percentage of exhausted roots increased to 70% (Figure 3N, 3P). Moreover, this high frequency of exhaustion was not observed in the single mutants (Figure 3P).

These results show that when only one of the QC repressors is absent (either BRAVO or WOX5), roots behave as the WT after the treatment (Figure 3K-M, 3P), supporting the existence of a compensation mechanism between BRAVO and WOX5, playing redundant roles, to preserve the capacity of stem cell recovery in response to damage. The increased root exhaustion found in the double mutant *bravo wox5*, where the QC is always divided, can be explained as a consequence of the lack of healthy stem cells in the QC that are able to renew the damaged ones.

**BRAVO and WOX5 function in the SCN**

Altogether, we propose a model for the cell-specific action of BRAVO and WOX5 proteins in the SCN to pilot root survival upon damage.

(I) In the QC, BRAVO and WOX5 form a complex and act preserving its quiescence. Our theoretical and experimental data model suggests a mutual transcriptional activation of BRAVO and WOX5 together with inactivation by BRAVO-WOX5 complex formation. At the same time, this inactivation of the protein by complex sequestration provides a simple framework into which understand the redundant roles that BRAVO and WOX5 have in QC division and root survival upon DNA damage. Assuming that the free proteins are the active ones, the mathematical model predicts their increase in the single mutants, which therefore can compensate the loss of the mutated one. In the double mutants, no compensation can occur for root survival nor repression of QC divisions.

(II) In the SSC, BRAVO and not WOX5 are expressed in normal conditions. However, genotoxic stress triggers the decay of BRAVO levels in the QC and SSC, and the increase of WOX*5* towards the provascular cells (Figure S4). After 72 hours of recovery, BRAVO expression is recovered and WOX5 is restricted to the QC again (Figure S4). These results suggest that, upon genotoxic stress, BRAVO expression decreases in the QC and WOX5 shifts to the damaged vascular cells to renew them. In addition, the regulation of both genes seems to be essential as the lack of both genes resulted in a lower percentage of root survival (Figure 3P). The decrease in BRAVO expression and the migration of WOX5 to the provascular cells is similar than in BL treatment (Figure S1, [5, 12]). Moreover, we unveil that bleomycin treatment increases BR biosynthesis by increasing CPD (CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM) levels (Figure S4A), thus suggesting that this BRAVO and WOX5 regulation is due to a previously unreported activation of BR signaling upon DNA damage. These results are in agreement with the fact that a paracrine steroid signal from the damaged stem cells to the QC in order to divide it [3].

Moreover, upon BL treatment, the expression of WOX5 expands to the SSC, so we asked whether this observation has an impact in the development of the vascular tissues. The analysis of the xylem of *bravo* and *wox5* single and double mutants points to a negative role of WOX5 in xylem differentiation, this repression is occurring when WOX5 expression is shifted to the vascular initial cells (in the knockout mutants after BL treatment) (Figure S5). This observation, together with the fact that *wox5* mutants show mild defects in the differentiated xylem (Figure S5), uncovers the impact of WOX5 from the QC in the proximal cells that might result in the alteration of the xylem fitness. We propose a scenario in which the BR-mediated regulation of WOX5 in the vascular initial cells is necessary to overcome a genotoxic stress that causes stele cell death, and that this regulation has an impact in the normal xylem differentiation. However, the specific WOX5 regulated genes in this cell type and the link between provascular cell replenishment and vascular differentiation remain unknown.

In conclusion, we uncovered that BRAVO and WOX5 form a complex in the QC, and that both proteins repress QC divisions. When a genotoxic stress happens, BR levels are increased in the surroundings of the SCN, promoting a paracrine signal that regulates the expression of both genes in the QC and SSC, and lead to the renewal of the damaged cells (Figure 4). It is not known if the activation of the BR signaling after the stress is through BRASSINOSTEROID INSENSITIVE 1 (BRI1) or the BRASSINOSTEROID INSENSITIVE LIKE 3 (BRL3) receptors. However, after BL treatment, the expression of BRL3 receptor is shifted from the QC to the stele cells [20], in addition it is also known that BRL3 is regulated by SUPRESSOR OF GAMMA RESPONSE 1 (SOG1) under genotoxic stress conditions[21], which could indicate that this BRL3 receptor is also involved in this response.

This study advances in the understanding of how BR signaling, through a paracrine signal and the regulation of cell type specific factors in the stem cell niche, controls important processes in plant development such as QC division, cell replenishment and vascular development.

**AUTHOR CONTRIBUTIONS**

A.I.C-D. designed and supervised the study. N.B., I.B-P. and A.P-R. performed the experiments. J.M., D.F. and M.I. performed the mathematical modelling. J.V-B generated lines used in the study. Y.S. and R.C-B performed the FRET-FLIM assays. S.P and C.M. collaborated in the Y2H and BiFC assays. N.B., I.B-P., A.P-R., M.I. and A.I.C-D wrote the manuscript.

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**STAR METHODS**

**Plant Material and Root Measurement**

All wild-type, mutants and transgenic lines are in the *Arabidopsis thaliana* ecotype Columbia (Col-0) background (Table S1). Seeds were surface sterilized and stratified at 4°C for 48 hours before being plated onto 0.5X Murashige and Skoog (MS) salt mixture without sucrose and 0.8% plant agar, in the absence or presence of Brassinolide (C28H48O6; Wako, Osaka, Japan). Plates were incubated vertically at 20°C and 70% humidity in a 16 hours light/8 hours dark cycle. Primary root length was measured from plates images using ImageJ (<https://imagej.nih.gov/ij/>) and MyROOT [22] softwares.

Beta-Estradiol from Sigma diluted in DMSO was used to induce BRAVO expression. Dexamethasone from Sigma diluted in EtOH was used to induce WOX5 expression. For bleomycin treatment, seedlings were transferred to vertical plates supplemented with 0.6 µg/ml bleomycin (Sigma) 4 days after sowing. For recovery, plants were transferred back to control medium after 1 day of growth in bleomycin-containing medium and analyzed under a confocal microscope after 72h. For zeocin treatment, ….

Basic fuchsin staining protocol for xylem characterization was performed as described in Caño-Delgado et al., 2000. The double mutant *bravo wox5* was generated by crossing the *bravo* and *wox5* single mutants. The double mutant homozygous lines were selected by genotyping. The primers used for *bravo* and *wox5* genotyping are indicated in table S2.

**Confocal Microscopy and Quantification of Fluorescence Signal**

Confocal images were taken with a FV 1000 Olympus confocal microscopy after propidium iodide (PI) staining. PI and GFP were detected with a band-pass 570-670 nm filter and 500-545 nm filter, respectively. The same laser settings were used within the same experiment to quantify the fluorescence signal, using the Integrated Density value measured of each plant. The relative average pixel intensity was obtained by relativizing each series to the wild type GFP expression. The analysis of QC cell division and CSC differentiation was carried out by imaging fixed roots through a modified pseudoSchiff (mPS)-PI staining method[23]. Images were processed with the Olympus FV (Olympus, Tokio, Japan) and ImageJ software (<https://imagej.nih.gov/ij/>).

For bleomycin assays, the percentage of damaged roots was scored after 24h of treatment, identified by the incorporation of PI inside the cells: no damage means that cells did not take up PI; damage indicates that death cells in the vasculature were stained with PI. The percentage of exhausted roots was scored after 24h of treatment plus 72h of recovery, identified by the morphology of the roots.

**Yeast two-hybrid assay**

Yeast two-hybrid assays were performed as the Matchmarker GAL4-based two-hybrid System (Clontech). Constructs were cotransformed into the yeast strain AH109 by the lithium acetate method. The presence of the transgenes was confirmed by growth on SD-LW plates, and protein interaction was assessed by selection on SD-LWH plates. Interactions were observed after 4 days of incubation at 30ºC.

**Transient expression in *Nicotiana benthamiana* for FLIM measurements**

Preparation of transiently expressing *Nicotiana benthamiana* leaves and induction of fusion proteins tagged with either mVenus or mCherry by application of ß-estradiol was carried out as described in [24].

**Acquisition of FLIM data**

Fluorescent lifetime imaging microscopy (FLIM) data acquisition was carried out using a confocal laser scanning microscope (LSM780 inverted microscope, Zeiss) equipped additionally with a time-correlated single-photon counting device with picosecond time resolution (Hydra Harp 400, PicoQuant). mVenus was excited at 485 nm with a pulsed (32 MHz) diode laser at 1.2 µW at the objective (40 x water immersion, C-Apochromat, NA 1.2, Zeiss). The emitted light was collected through the same objective and detected by SPAD detectors (PicoQuant) using a narrow range bandpass filter (534/35, AHF). Images were taken at 12.5 µs pixel time and a resolution of 138 nm/pixel in a 256x256 pixel image. A series of 40 frames was merged into one image and analysed using the Symphotime software package (PicoQuant).

**Analyses and presentation of FLIM data**

The fluorescent lifetime of the collected photons in each merged image was analysed using the Symphotime software (PicoQuant). For this, a ROI covering the whole nucleus was created to reduce background fluorescence. All photons in this ROI were used to build a histogram of the fluorescence decay. A double-exponential fit model was used to approximate the intensity-weighted average fluorescence lifetime  [ns] of all photons of the ROI. The instrument response function was measured with KI-quenched erythrosine and used for reconvolution in the fitting process[25]. The data from replicate measurements was summarized in box plots created in Origin 9.0 (OriginLabs). Boxes represent the 25th to 75th percentile. The square represents the mean value and the line represents the median. Outliers are marked by a x. Statistical significance was tested by one-way ANOVA with a Sidakholm post-hoc test. Different letters indicate statistically significant differences (p < 0.01).

For the creation of FLIM images, photons from individual pixels of a merged image were analysed for fluorescent lifetime using the Symphotime software (PicoQuant). A mono-exponential fit model was used, as the photon number in each pixel was too low for a double-exponential model[26]. The individual pixels are colour-coded according to their fluorescence lifetime.

**Bimolecular fluorescence complementation assay (BiFC)**

The BRAVO and WOX5 coding sequences were inserted by LR-reaction (Invitrogen) into pBiFC binary vectors containing the N- and C- terminal YFP fragments (YFPN43 and YFPC43). Plasmids were transformed into the *Agrobacterium tumefaciens* GV3101 strain and appropriate combinations was infiltrated into *Nicotiana benthamiana* leaves[27]. The p19 protein was used to suppress gene silencing. Infiltrated leaves were imaged two days after infiltration using an Olympus FV1000 laser scanning confocal microscope.

**Quantitative real time PCR**

Total RNA from root tips was extracted with Plant RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen), all according to the manufacturer's instructions. The primers used are indicated in Supplementary Table 2.

PCR products were detected with the SYBR Green I Master (Roche Diagnostics, Mannheim, Germany). LightCycler480 software 1.5.0 release (Roche Diagnostics) was used to calculate relative change in expression levels (3 technical replicates and 2 biological replicates). The expression of UBQ30 was used as an endogenous control.

**Mathematical Model**

We modelled BRAVO and WOX5 protein concentration dynamics through two coupled ordinary differential equations (Figure 2N and SI text). Each equation sets the balance between the production and destruction of each protein species. In the model, mRNA dynamics are assumed to be much faster and are set quasi-stationary. Therefore, the term of production of protein is proportional to the promoter activity (Figure 2 and SI text). To make the comparison between model results and experimental data we assumed that the reporters of promoter activities are linearly proportional to the endogenous promoter activity and correspond to a stationary state. To find the stationary stable solutions of the dynamics we set to zero the two differential equations and solved the remaining system of algebraic equations numerically with custom-made software and using the fsolve routine embedded in Python (Python Software Foundation, [https://www.python.org/](https://www.python.org/" \t "_blank)), which uses a modification of Powell's hybrid method for finding zeros of a system of nonlinear equations.

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