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Brassinosteroid-mediated stem cell divisions in response to DNA damage in Arabidopsis thaliana

Ainoa Planas-Riverola

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CENTER FOR RESEARCH IN AGRICULTURAL GENOMICS (CRAG)

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Author:
Ainoa Planas-Riverola

Supervisor:
Dr. Ana I. Caño-Delgado

January 14th, 2022

UAB Universitat Autònoma de Barcelona

CRAG CENTRE FOR RESEARCH IN AGRICULTURAL GENOMICS

UNIVERSITAT AUTÒNOMA DE BARCELONA
Genetics and microbiology department
Genetics PhD program

CENTER FOR RESEARCH IN AGRICULTURAL GENOMICS (CRAG)
Molecular Genetics department

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Dissertation submitted in partial fulfillment of the requirements for obtaining the degree of Doctor (PhD) by Universitat Autònoma de Barcelona (Barcelona, Spain)

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CENTRE FOR RESEARCH IN AGRICULTURAL GENOMICS

“We are only as strong as we are united,
as weak as we are divided.”
J. K. Rowling

Per a tu,

ABSTRACT

The present PhD thesis dissertation reports novel mechanisms for stem cell divisions mediated by brassinosteroids (BRs) upon DNA damage conditions in plants.

BRs are steroid hormones involved in multiple process of plant growth and development, and the adaptation to the environment. One of the processes that are known to be regulated by BRs is the stem cell divisions. Plants stem cells are in niches, which are mainly found in the root and shoot apexes. At the core of the root stem cell niche it is placed the quiescent center (QC), a group of cells with very low mitotic activity that maintain the undifferentiated status of surrounding stem cells and act as a cell reservoir. QC cells only trigger their divisions when need to replenish the stem cells, for example, after a DNA damage. BR signaling is in charge of triggering these QC divisions, but the exact mechanisms of how this process is regulated is still unknown. Here, we use an interdisciplinary approach, using *Arabidopsis thaliana* as a model system, including molecular genetics, physiology and bioinformatics to decipher the role of BR receptors upon DNA damage regulating the QC divisions.

The results presented in this thesis dissertation uncover novel roles for the BR-receptor kinase BRL3 (BRI1-like 3) protein in DNA damage and DNA repair machinery in plants. This is important in order to control DNA repair mechanisms and cell cycle progression

in the root meristem, which requires a balance of these processes to ensure plant adaptation to adverse conditions. We found that, at the root apex, BRL3 downstream signaling events modulate an enzyme that is specifically expressed in the QC cells, the RNR2A (RIBONUCLEOTIDE REDUCTASE 2A), in charge of maintaining dNTPs (deoxynucleotide triphosphates) supply during DNA synthesis. Overall, we found that RNR2A is crucial for a proper QC division in response to DNA damage conditions. Moreover, we also discovered that BRL3 pathway is also involved in the triggering of ROS (reactive oxygen species), probably by using RBOHD (RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D) machinery, an enzyme in charge of producing ROS. ROS act as signal molecules, involved in myriad of developmental and adaptive responses, also including QC divisions.

Overall, our studies untap novel roles for the cell-specific steroid receptor kinase BRL3, that has remained unknown for twenty years since their original discovery, while open new avenues for the study of BRL3 signaling pathway in plants.

TABLE OF CONTENTS

Abbreviations

amiR Artificial micro RNA

ATM ATAXIA-TELANGIECTASIA MUTATED

ATR ATM/RAD3-RELATED

BAK1 BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1

BES1 BRI1-EMS-SUPRESSOR 1

BIN2 BRASSINOSTEROID-INSENSITIVE 2

BL Brassinolide

BR(s) Brassinosteroid(s)

BRAVO BRASSINOSTEROID AT VASCULAR AND ORGANIZING CENTER

BRI1 BRASSINOSTEROID INSENSITIVE 1

BRL1 BRI1-Like 1

BRL3 BRI1-Like 3

BZR1 BRASSINAZOLE RESISTANT 1

CPD CONSTITUTIVE PHOTOMORPHOGENIC DWARF

CSC Columella stem cells

DDR DNA damage response

DNA Deoxyribonucleic acid

DSB Double strand break

DWF4 DWARF 4

DZ Differentiation zone

dNDPs Deoxyribonucleosides diphosphates

dNTPs Deoxynucleotide triphosphate

(e)H_2O_2 (Extracellular) hydrogen peroxide

EdU 5-ethynyl-2’-deoxyuridine

ERF115 ETHYLENE RESPONSE FACTOR 115

EZ Elongation zone

FACS Fluorescence activated cell sorting

FLS2 FLAGELLIN-SENSING 2

GFP Green fluorescent protein

HU Hydroxyurea

H_2DCFDA 2’,7’-dichlorodihydrofluorescein

LRR-RLK Leucine-rich repeat receptor-like kinase

QC Quiescent center

MAPK Mitogen-activated protein kinase

MZ Meristematic zone

NDPs Ribonucleotide diphosphates

nls Nuclear signal localization

NO Nitric oxide

O_2^- Superoxide anions

PCD Programmed cell death

PI Propidium iodide

RBOH Respiratory burst oxidase homolog

RNA Ribonucleic acid

RNR Ribonucleotide reductase

ROS Reactive oxygen species

SCN Stem cell niche

SOG1 SUPRESSOR OF GAMMA RADIATION 1

SSB Single strand break

TPL TOPLESS

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labelling

WOX5 WUSCHEL-RELATED HOMEOBOX 5

WT Wild-type

YFP Yellow florescence protein

Chapter 1

General Introduction

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Planas-Riverola A., Markaide E., Caño-Delgado AI. (2020) *Trends in Plant Science.*

General Introduction

1.1 Brassinosteroid ligand perception and signal transduction

Brassinosteroids (BRs) are phytohormones that were originally discovered in *Brassica napus* pollen, based on their ability to promote growth (Mitchell et al., 1970). BR molecules are composed of a steroid nucleus and resemble to animal steroids, not only because of the chemical structure, but also in the functions that they regulate (Thummel and Chory, 2002). However, their signal transduction differs from the one of animals. While perception of animal steroids occurs in the nucleus (Aranda and Pascual, 2001), in plants the steroid perception takes place in the cytoplasmatic membrane.

BR signaling mainly drives cellular growth (Belkhadir and Jaillais, 2015; Zhao and Li, 2012). Accordingly, mutations in genes encoding the main components of the BR synthesis and signaling pathways result in severe dwarfism, impaired organ growth and development, and limited plant fertility and yield (Li and Chory, 1997; Singh and Savaldi-Goldstein, 2015). Nevertheless, BRs are also regulating adaptation to biotic (De Bruyne et al., 2014) and abiotic (Lozano-Durán and Zipfel, 2015; Nolan et al., 2017) stresses.

Since their discovery, the main components of the canonical BR signaling pathway have been identified through multiple genetic and biochemical screens, mainly performed in the model plant *Arabidopsis thaliana* (arabidopsis) (Vert et al., 2005; Zhu et al., 2013). BR hormones are perceived extracellularly by members of the BRI1 (BRASSINOSTEROID INSENSITIVE 1) leucine-rich repeat receptor-like kinase (LRR-RLK) family (Li and Chory, 1997; Wang et al., 2001). The BR hormones bind directly to a 93-amino-acid region located within the extracellular domain of membrane-bound BRI1 (Hothorn et al., 2011; Kinoshita et al., 2005; Sun et al., 2013). Direct binding of the hormone triggers the formation of a BRI1-BAK1 (BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1, also known as SERK3 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3)) heterodimer, which in turn initiates an intracellular phosphorylation relay cascade (Li and Nam, 2002; Russinova et al., 2004).

The cascade (Figure 1.1 A) activates BSU1 (BRI1-SUPRESSOR 1) phosphatase (Kim et al., 2009; Kim et al., 2011) to inhibit BIN2 (BRASSINOSTEROID-INSENSITIVE 2). This culminates in promoting the activity and stability of the plant-specific transcription factors BZR1 (BRASSINAZOLE RESISTANT 1) (Wang et al., 2002) and BES1 (BRI1-EMS-SUPPRESSOR 1) (Yin et al., 2002a), which directly control the transcription of thousands of BR-responsive genes and hence regulate a plethora of developmental events in the plant (He et al., 2002; Sun et al., 2010). When BRs are absent, the GSK3-like kinase BIN2 phosphorylates BZR1/BES1

proteins and inactivates them, promoting their binding to 14-3-3 proteins and leading to their cytoplasmic retention and degradation (Gampala et al., 2007; Li and Nam, 2002; Peng et al., 2008). This inhibits their ability to bind DNA, thereby causing pathway inactivation.

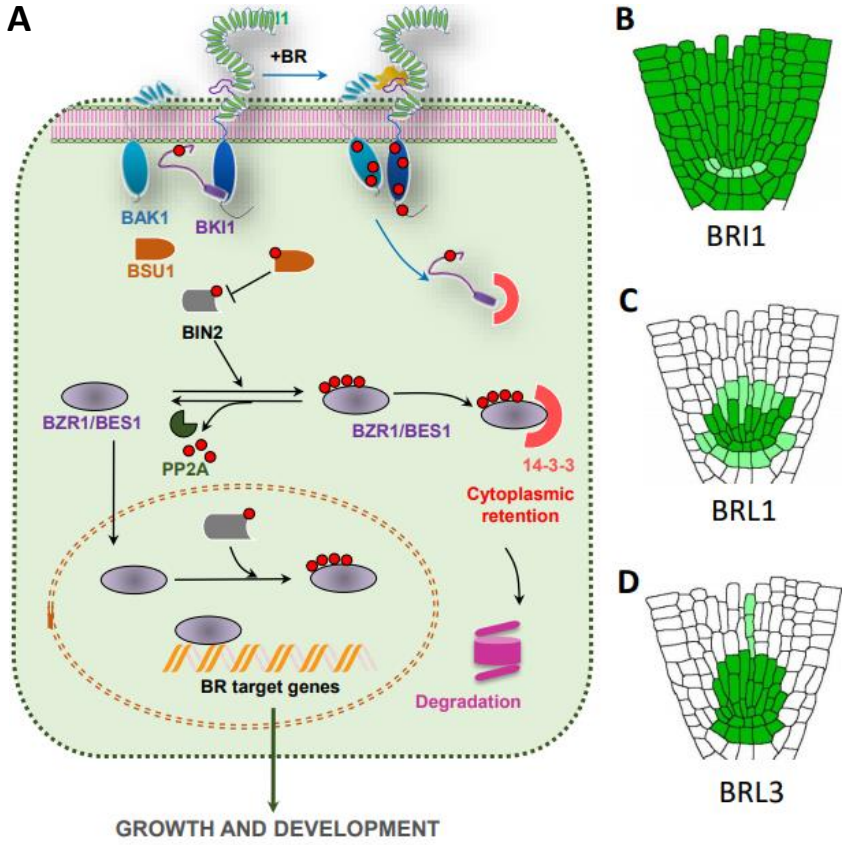


Figure 1.1 An overview of the BR signaling pathway. (A) Schematic representation of the BRI1 signaling pathway. In the absence of BR, BIN2 phosphorylates BZR1 and BES1 proteins, inactivating them by promoting their binding to 14-3-3 proteins, leading to their cytoplasmic retention and degradation. When BRI1 perceives BR molecules, it heterodimerizes with BAK1, initiating an intracellular phosphorylation relay cascade that activates BSU1, which inhibits BIN2 and ends with the dephosphorylation and consequent activation of BZR1 and BES1. **(B-D)** Schematics of the root tissue-specific expression of BR receptors. BRI1 is expressed throughout the root (B), whereas BRL1 (C) and BRL3 (D) exhibit a more discrete expression pattern, being active mainly in the root SCN area. Dark green represents high expression of the protein, whereas light green represents lower expression.

Based on the presence of the extracellular BR-binding domain, there are three membrane-localized BRI1-like homologs named BRL1, BRL2 and BRL3 (BRI1-LIKE 1, 2 and 3). Whereas BRL1 and BRL3 are functional BR receptors that, like BRI1, can bind to brassinolide (BL), a bioactive form of BR with high affinity, BRL2 appears not to be a functional BR receptor (Caño-Delgado et al., 2004; Lozano-Elena and Caño-Delgado, 2019). Furthermore, whereas BRI1 is expressed nearly ubiquitously in the root (Friedrichsen and Chory, 2001) (Figure 1.1 B), the BRLs are found only in some specific tissues (Figure 1.1 C,D). For example, BRL1 and BRL3 are localized in vascular stem cells, where they govern cell-specific BR-response pathways (Caño-Delgado et al., 2004; Fàbregas et al., 2013; Salazar-Henao et al., 2016). Under native conditions, both BRL1 and BRL3 can heterodimerize with the BAK1 co-receptor, but not with BRI1, and form a complex (Fàbregas et al., 2013). These studies suggest that BRI1 and the BRLs are able to form distinct receptor complexes in different cell types, thereby performing unique signaling roles, but the specific downstream components of the BRL1 and BRL3 pathways remain unknown.

1.2 The primary root as a model for deciphering cell-specific brassinosteroid signaling

Owing to its simple and radial organization of cell types, the primary root of arabidopsis provides an excellent model for dissecting signaling mechanisms with cell-specific resolution (Dolan et al.,

1993; Scheres et al., 1994). Indeed, a number of studies of the primary root have shown that BRs control specific cellular processes in distinct root cell types (Figure 1.2). The arabidopsis primary root displays a radial pattern of cell files, with the stem cell niche (SCN) located in the inner core of the root apex (Figure 1.2). The four outer concentric layers are the epidermis, cortex, endodermis and pericycle, which surround the inner vascular tissues (Figure 1.2). Along the longitudinal axis, three zones can be identified, the meristematic zone (MZ), the elongation zone (EZ) and the differentiation zone (DZ). The stem cells originating from the MZ will differentiate and divide in this region. Cells will stop division when entering the EZ and will start to elongate. Finally, when entering to DZ, cells will stop elongating as they will be mature enough to be differentiated (Figure 1.2).

BRs play an important role in overall root development (Figure 1.2). Lack or excess of these phytohormones are both detrimental to primary root growth and development, so, BR levels are crucial for a proper regulation of these processes. On the one hand, mutants lacking BR compounds or BRI1 receptor exhibit short roots, indicating that BRI1 signaling is required for root growth (Chaiwanon and Wang, 2015; González-García et al., 2011; Hacham et al., 2011; Müssig et al., 2003). On the other hand, short roots are also observed in *bes1-D* (gain-of-function) mutants, or in plants treated with high concentrations of BRs (González-García et al., 2011; Müssig et al., 2002). The short roots of mutants with impaired BR biosynthesis can be rescued by treatment with low concentrations

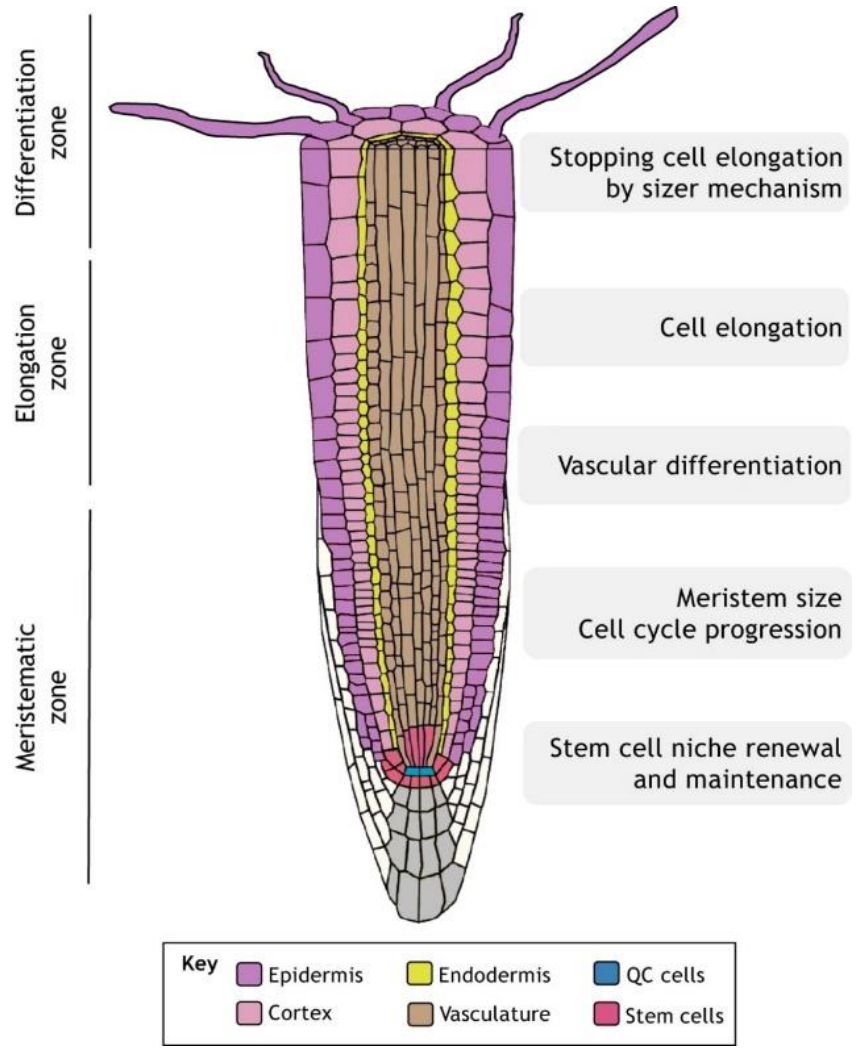


Figure 1.2 BR functions in the primary root. BRs are involved in a variety of cell-specific processes that occur within the different zones of the root. These include processes such as cell cycle division, cell elongation and cell differentiation.

of BR (Chaiwanon and Wang, 2015). Moreover, supporting the notion that BRs can promote root growth, it has been shown that wild-type (WT) roots treated with low concentrations of BRs increase their length (González-García et al., 2011; Müssig et al., 2003), although this enlargement is small and not always detectable

(Chaiwanon and Wang, 2015). Altogether, these results suggest that, rather than controlling root growth in a linear fashion, the correct balance of BR levels appears to be crucial.

Root growth depends on cell proliferation at the MZ and on cell elongation prior to differentiation at the EZ, and BRs impinge on both processes. BRs modulate meristematic proliferation (González-García et al., 2011; Hacham et al., 2011) and have been proposed as key regulators in the optimal control of cell cycle progression (González-García et al., 2011). BRs have been also proposed to be crucial for optimal cell expansion (Chaiwanon and Wang, 2015; Clouse and Sasse, 1998). Recent mathematical and computational modeling has further demonstrated that root growth features depend on the mechanism by which cell elongation terminates, e.g. whether cells stop elongating according to their spatial position along the root, according to a time interval, and/or according to their cell size (Pavelescu et al., 2018). Quantification of cell length in single roots, together with mathematical and computational modeling, suggests that the dominant mechanism for cell elongation termination is a size-based mechanism whereby root cells stop expanding when they reach a determined length, and that BRI1 facilitates this mechanism (Pavelescu et al., 2018). In addition, this suggests that BR signaling at least partially controls these three separate functions: cell division, cell elongation rate and termination of cell elongation (Pavelescu et al., 2018). Indeed, plants treated with high concentrations of BR increase expansion at the meristem and reduce the number of

meristematic cells, but do not exhibit an increase in meristem cell length (Chaiwanon and Wang, 2015).

The control of root growth by BR signaling is also spatially segregated throughout the root. BR signaling is not found homogeneously throughout the root, with BZR1 being more strongly activated at the transition from the MZ to the EZ and in the EZ itself (Chaiwanon and Wang, 2015). Moreover, BR signaling induces target genes in the epidermis (the outer layer) but mostly represses genes in the stele (the inner layer) (Vragović et al., 2015), highlighting that BR signaling can elicit tissue-specific responses. Based on these results, it has been proposed that BR signaling can function in a non-cell-autonomous manner, signaling from the epidermis to inner cells (Hacham et al., 2011; Vragović et al., 2015).

1.3 The role of brassinosteroid signaling in stem cell self-renewal and differentiation

The root SCN comprises a small group of stem cells located at the base of the meristem in the root apex. These cells are essential for sustaining root growth, as they continuously provide the precursors of more-specialized cells, and to replace tissues that have been damaged (Dolan et al., 1993; Sabatini et al., 2003; van den Berg et al., 1997). The core of the niche contains a group of cells with very low mitotic activity that are collectively known as the quiescent center (QC). The QC maintains the undifferentiated state of the

surrounding stem cells (Sarkar et al., 2007; van den Berg et al., 1997) whilst maintaining its own stemness, but it can also act as a reservoir of cells that can replenish damaged stem cells (Heyman et al., 2013; Vilarrasa-Blasi et al., 2014).

BRs play a key role in maintaining the identity and quiescence of QC cells (González-García et al., 2011), and thereby affect the maintenance of the root SCN. BR signaling acts within the root SCN by modulating BRAVO (BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER) (González-García et al., 2011). This transcription factor, also named MYB56, belongs to the R2R3-MYB family and is expressed specifically in vascular initials and QC cells (Vilarrasa-Blasi et al., 2014). Phenotypic analyses have shown that BRAVO represses QC cell divisions (Figure 1.3 A), as *bravo* mutants show a significant increase in QC division frequency. When BR signaling is activated (Figure 1.3 B), the BR downstream effector BES1 becomes activated and downregulates the levels of BRAVO transcript. It also heterodimerizes with BRAVO protein itself, strongly inhibiting its action and promoting the division of QC cells (Figure 1.3B, Vilarrasa-Blasi et al., 2014). This constitutes a regulatory circuit that controls QC division via interactions at both the transcriptional and protein levels. Another transcription factor that acts as a co-repressor of BRAVO is TPL (TOPLESS), which can bind to the BRAVO promoter as well as interact with BES1 via its ERF-associated amphiphilic repression (EAR) motif (Figure 1.3 B, Espinosa-Ruiz et al., 2017).

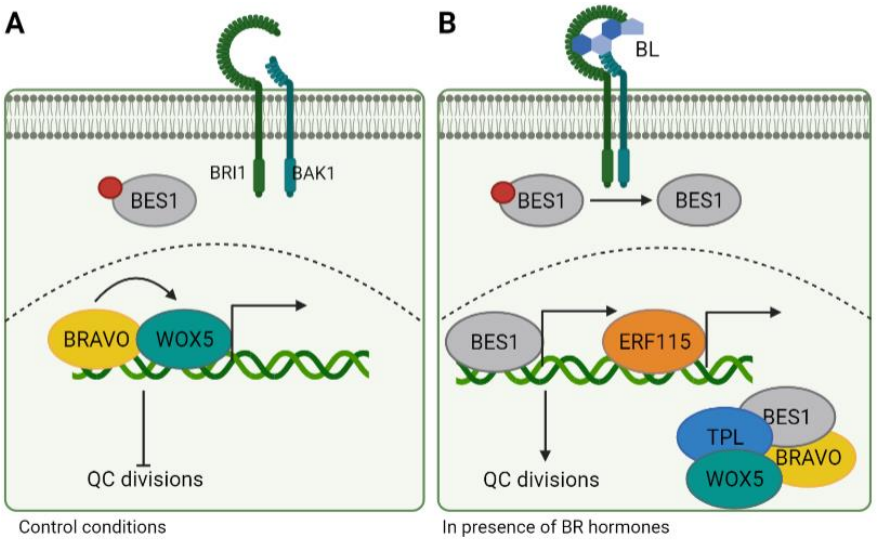


Figure 1.3 Functional role of BRs in stem cell regeneration. (A) In control conditions, BR signaling in the QC is not active. This maintains BES1 in a phosphorylated and inactive state, permitting BRAVO and WOX5 to interact and regulate their expressions to repress QC divisions. **(B)** In the presence of BR hormones, the cascade of phosphorylations and dephosphorylations started by the heterodimerization of BRI1 with BAK1 leads to the dephosphorylation and activation of BES1. The inactivation of BRAVO through the formation of a complex with TPL and BES1, as well as being transcriptionally downregulated, and the induction of ERF115 expression, together promote the division of QC cells to replenish the stem cell population when needed.

BRs can also induce the expression of ERF115 (ETHYLENE RESPONSE FACTOR 115), a transcription factor that belongs to the ethylene response factor family and plays an important role in root growth and development. Specifically, ERF115 acts as a limiting factor for QC divisions as it regulates the expression of PSK5 (PHYTOSULFOKINES 5), a peptide hormone that enhances the frequency of QC divisions (Heyman et al., 2013; Heyman et al., 2016). Collectively, BR signaling represses BRAVO activity and, at the same time, activates ERF115 to promote QC divisions when needed (Figure 1.3 B). However, it is still unknown where the signals that activate the BR pathway originate from, i.e. if they come from

external tissues or if this process is carried out in a cell-autonomous way.

BRs also promote the differentiation of columella stem cells (CSCs), which are located distally to the QC. This occurs in a dose-dependent manner (González-García et al., 2011; Lee et al., 2015) via the regulation of the transcription factor WOX5 (WUSCHEL-RELATED HOMEOBOX 5). WOX5 is a homolog of WUSCHEL, a transcription factor that maintains the identity of stem cells in the shoot (Mayer et al., 1998). In the root, WOX5 is required to maintain the identity of stem cells (Sarkar et al., 2007), and its transcript expression is restricted to the QC controlled by CLE40 (CLAVATA3/ESR-RELATED 40). CLE40 peptide is perceived through the receptor-like kinase ACR4 (ARABIDOPSIS CRINKLY 4) and its recognition leads to the exclusion of WOX5 expression outside of the QC, limiting its expression to the QC cells (Ding and Friml, 2010; Zhang et al., 2015).

In the QC WOX5 represses CYCD (cyclin-dependent kinase/cyclin D) activity to establish quiescence. Moreover, WOX5 protein also moves from the QC to the CSC, where it directly represses the transcription factor CYCLIN DOF FACTOR 4 (CDF4), avoiding differentiation (Forzani et al., 2014; Pi et al., 2015). Importantly, the expression of WOX5 is regulated by BRs; WOX5 expression decreases in *bri1-116* mutants (lacking an active version of BRI1 receptor) and in plants treated with brassinazole (BRZ_{220}, a potent inhibitor of BR biosynthesis (Asami et al., 2000)). In contrast, WOX5

expression increases in plants treated with BL and in *bes1-D* or BRI1 overexpressor mutants (González-García et al., 2011). Recent studies uncovered that BRAVO and WOX5 form a transcription factor complex in the QC that modulates their gene expression to preserve the quiescence of the QC cells, as well as maintain the overall root growth and architecture (Figure 1.3A, Betegón-Putze et al., 2021). Furthermore, by using mathematical modelling it was also established that BRAVO uses the WOX5/BRAVO complex to promote WOX5 activity in the stem cells (Figure 1.3A, Betegón-Putze et al., 2021). Indeed, WOX5 is also interacting with BES1 and TPL (Figure 1.3B, Betegón-Putze et al., 2021), indicating that BRAVO and WOX5 directly interact forming a heterodimeric complex, and that each can bind active BES1 and TPL, suggesting these proteins are able to compete for their mutual binding. All these results, unveil the importance of transcriptional regulatory circuits in plant stem cell development.

1.4 The importance of DNA damage repair mechanisms in the stem cell population

Stem cell division is a process tightly regulated, as the balance between self-renewal and differentiation is crucial for its maintenance (Dolan et al., 1993; Nawy et al., 2005). An asymmetric cell division regulates one daughter cell remaining as a stem cell meanwhile the other can differentiate. The proper balance among them is maintained by both intrinsic programs and environmental

regulatory signals (Li and Xie, 2005). Moreover, plant stem cells are the precursors of tissues during the whole life of the plant, and, as plants cannot escape stresses that could lead to oxidative stress or DNA damage, the protection against those is especially important within the SCN.

In arabidopsis plants, the DNA damage response (DDR) is mainly driven by ATM (ATAXIA-TELANGIECTASIA MUTATED), which primarily responds to double strand breaks (DSBs), and ATR (ATM/RAD3-RELATED), which primarily responds to single strand breaks (SSBs) (Figure 1.4, Maréchal and Zou, 2013). Both, ATM and ATR, are protein kinases and their downstream effects include cell cycle arrest, transcriptional activation of genes involved in DNA metabolism, repair and changes in chromosome structure (Hu et al., 2016). In animals, the ATR and ATM pathways of DDR signaling converge to activate the p53 tumor suppressor, a transcription factor that controls both DNA repair and cell cycle arrest (Yoshiyama et al., 2013a). Plant genomes lack a p53 homolog, but its functional equivalent was isolated through a genetic screen and was named SOG1 (SUPRESSOR OF GAMMA RADIATION 1) (Preuss and Britt, 2003). SOG1 is a transcription factor of the NAC (NAM, ATAF1/2, and CUC2) family and is the central regulator of the plant DDR, as it governs multiple responses involved in DNA damage mechanisms (Figure 1.4, Ogita et al., 2018; Yoshiyama et al., 2009).

SOG1 is rapidly phosphorylated in response to DNA damage being dependent on ATM and ATR activity (Figure 1.4, Sjogren et al.,

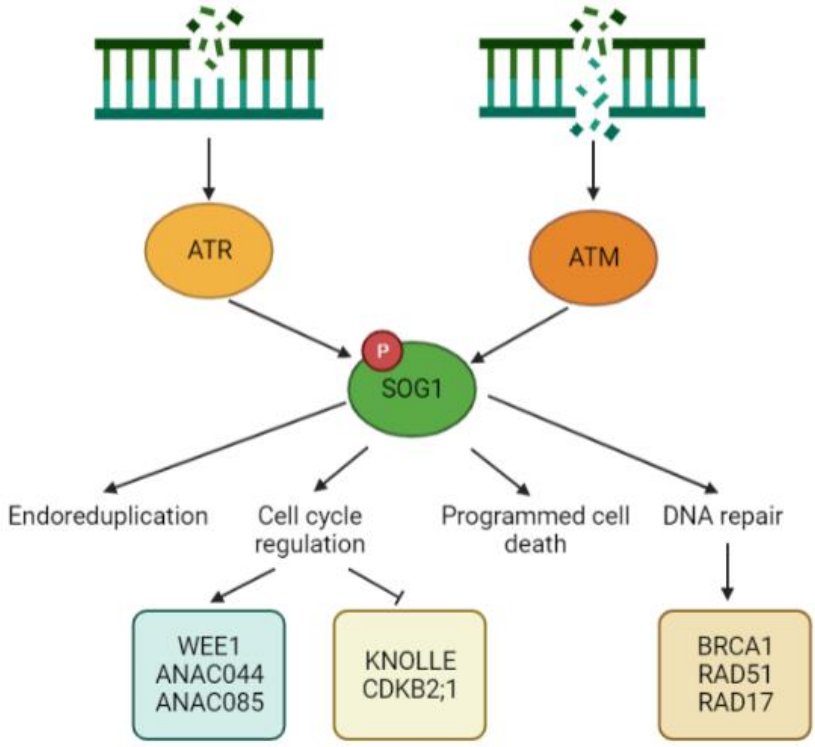


Figure 1.4 Overview of DNA damage response pathways in plants. ATM is activated upon DSBs and ATR upon SSBs. Both kinases rapidly phosphorylate SOG1, the master key regulator of the DDR. SOG1 is involved in all the processes that undergo after a DNA damage. SOG1 is needed to activate endoreduplication and PCD, as well as to activate the DNA repair machinery through the direct activation of different proteins such as BRCA1, RAD51 and RAD17. SOG1 is also key to regulate the cell cycle upon a DNA damage, it activates different genes such as WEE1, ANAC044, ANAC085 and CYCB1 as well as downregulates KNOLLE and CDKB2;1 in order to promote the cell cycle arrest.

2015; Yoshiyama et al., 2013b). Its activity directly regulates the expression of many DNA repair genes, such as BRCA1 (BREAST CANCER SUSCEPTIBILITY 1), RAD51 and 17 (RADIATION SENSITIVE 51 and 17) (Figure 1.4, Ogita et al., 2018). SOG1 also regulates endoreduplication, which consist in several rounds of DNA replication without mitosis, leading to an increase in nuclear DNA content. Endoreduplication prevents DNA-damaged cells from

proliferating but also from dying, being a crucial mechanism for the survival upon DNA damage (Adachi et al., 2011). In addition, SOG1 also inhibits the cell cycle progression, by directly regulating the expression of different genes. SOG1 is directly upregulating negative regulators of the cell cycle transition, such as WEE1, as well as the transcription factors ANAC044 and ANAC085 (Figure 1.4, Ogita et al., 2018; Takahashi et al., 2019). SOG1 also downregulates genes that promote the cell cycle progression such as KNOLLE and CDKB2;1 (Figure 1.4, Adachi et al., 2011). Lastly, SOG1 can trigger programmed cell death (PCD), as in *sog1* mutants PCD is not activated upon DNA damage (Figure 1.4, Adachi et al., 2011; Furukawa et al., 2010).

Genotoxic agents always cause PCD in a meristem cell-type dependent manner at the root apex. In particular, stem cells and vascular initial cells appear to be more susceptible than QC cells (Fulcher and Sablowski, 2009). The PCD susceptibility in this region over other mechanisms to cope with the DNA damage could be beneficial for plants, since fast-growing and dividing regions, as the meristems, could not afford spending time and resources on accurate DDR. Moreover, a proper mechanism regulating the DNA repair and the cell cycle progression in the QC cells must be crucial to avoid propagation of genomic errors to the rest of the cells that will form all the tissues. However, despite the acquired knowledge about DDR and stem cell division regulation it is still unknown which is the interactions between hormone pathways that controls SCN maintenance, such as BR, and the responses to repair the DNA or to

trigger the PCD in a SOG1 or ATM/ATR dependent or independent manner. As an example, ERF115 induction, which is mediated by BR signaling (Heyman et al., 2013), was also shown to depend partially on SOG1 activity (Johnson et al., 2018), but the connection between these two pathways is still unrelated. Future studies aiming to understand this relation could be key to comprehend the mechanisms by which stem cells are being protected against DNA damage or other hazardous elements such as oxidative stresses.

1.5 Brassinosteroid signaling in adaptation to stress

The ability of a plant to tolerate stress, such as changes in water availability, temperature or soil salinity, depends on its ability to switch between growth activation and repression in unfavorable conditions (Bechtold and Field, 2018; Feng et al., 2016). A key pathway that controls responses to environmental stresses is the abscisic acid (ABA) signaling pathway (Yoshida et al., 2014; Zhu et al., 2017). However, compelling evidence indicates that BRs also play a prominent role in controlling the balance between normal growth and resistance against environmental assaults, acting either via crosstalk with the ABA pathway or independently. Several mechanisms have been proposed to explain how BR signaling mediates adaptation to stress. These include: (*i*) fine-tuning stress-responsive transcript machineries (Ye et al., 2017); (*ii*) activating antioxidant machineries (Kim et al., 2012; Lima and Lobato, 2017; Tunc-Ozdemir and Jones, 2017; Zou et al., 2018); and (*iii*) promoting

the production of osmoprotectants (Fàbregas et al., 2018). The interplay between BR signaling and redox signaling appears to be crucial for plant development under stress. For decades, reactive oxygen species (ROS) were considered as harmful byproducts generated by metabolism in aerobic organisms. as they can react with macromolecules including lipids, proteins, and nucleic acids, resulting in oxidative modification and cell damage which can lead to cell death (Sies, 2014). However, recent evidence demonstrates that low levels of ROS have key regulatory roles in cell fate signaling, including plant growth and developmental processes, as well as in plant biotic and abiotic stress responses (Mittler, 2017; Waszczak et al., 2018). In plants, ROS can be generated in nearly every subcellular compartment, and numerous enzymatic reactions have evolved to actively generate ROS. Plant NADPH oxidases (NOX) (also termed respiratory burst oxidase homologs; RBOHs) are a major component of the production of extracellular ROS, specifically of extracellular hydrogen peroxide ((e)H2O2) (Kimura et al., 2017); therefore, its activity is strictly controlled to avoid damaging consequences of unrestricted H_2O_2 production.

Arabidopsis present ten different RBOH proteins, named from A to J. Of those, RBOHD is the best-characterized one, being reported to be involved in both biotic and abiotic stress responses. Receptor-like cytoplasmic kinases, such as BIK1 (BOTRYTIS-INDUCED KINASE 1), are responsible, among other several protein kinases, for phosphorylating the N-terminus region of RBOHD (Kaya et al., 2019) (Figure 1.5). BIK1 is activated as an immunity response

triggered by FLS2 (FLAGELLIN-SENSING 2), an LRR-RK that recognizes the bacterial peptide flagellin (flg22) (Chinchilla et al., 2006; Li et al., 2014). When FLS2 is activated by flg22, it forms a complex with BAK1, which leads to a succession of trans-phosphorylation events between several intracellular kinases, including BIK1. Furthermore, the FLS2–BAK1 union also activates

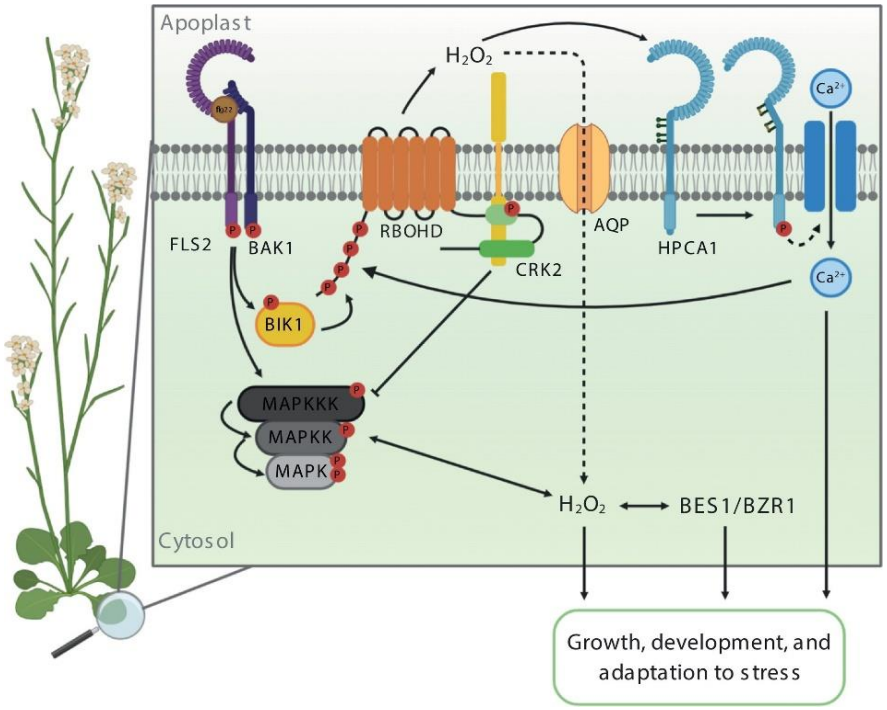


Figure 1.5 Current model for reactive oxygen species production, and signaling pathways. Extracellular ROS, mainly H_2O_2, is produced by RBOHs. Specifically, RBOHD can be activated through phosphorylation of its N-terminus or C-terminus domains by interaction with different molecules and receptors BIK1 and Ca^{2+} have been shown to interact with the N terminus and CRK2 with the C terminus. BIK1 activation is dependent on the activity of the complex FLS2–BAK1, which can sense pathogen peptides, such as flg22. Moreover, FLS2–BAK1 activation also leads to the activation of the MAPK cascade directly controlling ROS activity. Extracellular H2O2 can also enter the cells via aquaporins (AQP) and can then interact with different transcription factors in the cytosol, such as BES1 and BZR1. These interactions lead to developmental changes and adaptation to stresses, which, through different mechanisms, can also be triggered by Ca^{2+} influx.

other downstream signaling, including the mitogen-activated protein kinase (MAPK) cascades, which also control H_2O_2 production and are regulated by its levels (Nühse et al., 2000) (Figure 1.5). Interestingly, another recent study also uncovered that, upon bacterial pathogen infection, CYSTEINE-RICH receptor-like protein kinase (CRK2), which exists in a preformed complex with RBOHD, phosphorylates its C-terminal region and regulates its H_2O_2-production activity in vivo (Kimura et al., 2020) (Figure 1.5). While previous studies only reported phosphorylation of the N-terminal region as a unique way of RBOHD regulation, these new results uncover a novel mechanism of regulation and highlight a key role of CRK2 in the control of the apoplastic H_2O_2 burst in response to biotic stress (Figure 1.5, Kimura et al., 2020).

It is known that BR induces the antioxidant system during abiotic stress tolerance (Jiang et al., 2012; Zhou et al., 2014). BR has also been reported to utilize H_2O_2 and nitric oxide (NO) mediated mechanisms to provide stress tolerance (Cui et al., 2012; Xia et al., 2009). For example, during oxidative stress, BR increases ABA production through NO-mediated machinery (Zhang et al., 2011a). BR-mediated transient H_2O_2 production via NADPH oxidase also triggers ABA biosynthesis, which, along with enhanced H_2O_2 production, acts as a positive-feedback mechanism for prolonged heat and oxidative stress tolerance (Zhou et al., 2014). The over-accumulation of superoxide anions (O_2^-) in the BR biosynthesis-defective mutant *det2-9* highlights yet another node of crosstalk between the BR and ROS pathways that is implicated in controlling

root growth and development (Lv et al., 2018). Interestingly, this BR-mediated control of O_2^- accumulation was found to occur through the peroxidase pathway rather than the NADPH oxidase pathway (Lv et al., 2018). H_2O_2-mediated oxidative modifications enhance the transcriptional activity of BZR1 and promote its interaction with ARF6 (AUXIN RESPONSE FACTOR 6) and PIF4 (PHYTOCHROME INTERACTING FACTOR 4) (Tian et al., 2018). In contrast, the TRXh5 (thioredoxin h5) interacts with BZR1 and catalyzes its reduction (Tian et al., 2018). Exogenous BR application also increases H_2O_2 production in the root SCN, contributing to BR-induced QC division and cell elongation (Tian et al., 2018). However, despite all the gathered knowledge the question of how BR are leading to these changes in ROS accumulation is still unanswered.

1.6 Concluding remarks

BRs are key for maintaining proper plant growth, both under normal conditions and in response to environmental stress, and ample evidence now supports the idea that modifying the BR response pathway can be a powerful strategy for designing better-adapted crops. However, our understanding of the main functions of BR signaling during stress is only generic, and the investigation of precise spatiotemporal- and context-specific regulatory mechanisms has only just begun (Kang et al., 2017; Lozano-Durán and Zipfel, 2015; Lozano-Elena et al., 2018; Vragović et al., 2015). Further

studies are clearly required to obtain a more mechanistic understanding of the global and local actions of the BR pathway.

A deeper understanding on the mechanisms that are controlling the stem cell divisions, SNC maintenance, DDR and coping mechanisms for DNA damage and oxidative stress could be key not to only understand better how those special cells are being regulated but also to improve the plant growth and adaptation as stem cells are continuously acting as precursors for all the tissues during the whole life cycle of the plant.

Objectives

The general objective of the present PhD thesis was to investigate how brassinosteroids regulate the stem cell niche maintenance and how they regulate the stem cells divisions upon stress conditions in the plant model *Arabidopsis thaliana*.

To this end, the following specific objectives have been accomplished:

1. Dissect the molecular connection between brassinosteroid signaling pathway and DNA damage response.

2. Study the specific roles of BRL3 receptor during the response to DNA damage.

3. Identify novel stem-cell specific components that controls QC divisions in a brassinosteroid-dependent manner.

4. Unravel one of the mechanisms by which brassinosteroids are able to enhance hydrogen peroxide production in response to abiotic stress.

Chapter 2

Results

Brassinosteroid signaling modulates DNA damage response in the root meristem

Part of this chapter published as:

Paracrine brassinosteroid signaling at the stem cell niche controls cellular regeneration

Lozano-Elena, F.*, Planas-Riverola A.*, Vilarrasa-Blasi, J., Schwab, R. and Caño-Delgado AI. (2018) *Journal of Cell Science*, 131:2

Brassinosteroid signaling modulates DNA damage response in the root meristem

2.1 Introduction

Hormonal stimulation has an important role in regulating the SCN maintenance (González-García et al., 2011; Heyman et al., 2013; Zhang et al., 2010). As an example, BRs promote the cell division in the QC and the differentiation of the CSC, having an impact in the global SCN maintenance (Fàbregas et al., 2013; González-García et al., 2011; Vilarrasa-Blasi et al., 2014). More specifically, they control different transcription factors, like ERF115, which activated by BRs promotes QC divisions and stem cell regeneration after DNA damage (Heyman et al., 2013; Heyman et al., 2016) or BRAVO and WOX5, which act as repressors of QC divisions (Betegón-Putze et al., 2021; Vilarrasa-Blasi et al., 2014).

Maintenance of genome integrity is essential in all living organisms, and especially in the plant SCN. The DDR signaling pathway has extensively studied in mammals, due to its importance in cancer research, but has also been studied into details in plants during the last 15–20 years (Nisa et al., 2019). Plants have different mechanisms to cope with DNA damage, as (1) stopping the cell cycle to allow DNA repair, (2) promote the endoreduplication and (3) activate the PCD when errors cannot be repaired to avoid hazardous mutations

(Hu et al., 2016). When the plants suffer DNA damage the stem cells are the ones especially undergoing to PCD to avoid propagating genome errors (Borges et al., 2008; Fulcher and Sablowski, 2009), and after that the division of the QC cells is key to replenish the stem cell population and ensure the survival of the plant.

Despite the importance of BR signaling controlling QC divisions and the resemblance of the outputs obtained by exogenous BR application or DNA damage inducing treatments, such as QC divisions and the regulation of many transcription factors (such as BRAVO, ERF115 and WOX5) (Heyman et al., 2013; Johnson et al., 2018; Vilarrasa-Blasi et al., 2014), it is still unknown which is the link between these two processes. Moreover, although it is known that all BR receptors are involved in the modulation of QC cell division and differentiation of surrounding stem cells under normal conditions (Fàbregas et al., 2013), the specific contribution of each receptor to this process remains unknown, as well as whereas BR-regulated QC function is maintained in a cell-autonomous way or if it requires external signaling.

To further investigate the BR-mediated regulation of quiescence at the local level and its impact on stem cell regeneration after DNA damage in this chapter we used a tissue-specific approach in order to determine the ability of QC cells to integrate exogenous steroid signals. We specifically overexpressed two BR signaling components specifically in the QC cells, the BRI1 membrane receptor and the activated BES1 transcription factor. Moreover, we also investigated

how BR signaling was being activated and transduced when DNA damage occurs and what were the consequences of this activation. For that we analyzed the regulation of BR receptors, BR biosynthesis genes and BES1/BZR1 transcription factors under DNA damage treatment, and which was the biological relevance of these regulations during DDR.

Altogether, the results in this chapter demonstrate that: (*i*) active BES1 is a key factor for cell-autonomous QC divisions; (*ii*) the BR hormone itself is the limiting factor for BR-induced QC divisions; (*iii*) upon DNA damage BR signaling pathway is activated via BRL3 by the direct regulation of SOG1; (*iv*) the signal activated via BRL3 upon DDR is specifically transduced by BZR1 and (*v*) BRL3 signaling during DDR is inhibiting DNA repair mechanisms while activating the cell cycle progression, including the promotion of QC divisions.

2.2 The local BR hormone level limits the QC division

To elucidate whether the BR-induced division signals of the QC were transduced in a cell-autonomous manner through the canonical BR signaling cascade, on the one hand we used the gain-of-function BES1 mutant, *bes1-D*, which is known to be constitutively active (Yin et al., 2002a). On the one hand, we used the pWOX5:bes1-D-YFP (Vilarrasa-Blasi et al., 2014) lines in Col-0 WT and the knock-out BRI1 mutant *bri1-116* backgrounds (Figure 2.1 A-D). On the

other hand, we transformed the pWOX5:BRI1-YFP construct into the same backgrounds to evaluated the local contribution of the BRI1 receptor to QC division (Figure 2.1 E,F). As the WOX5 promoter drives a higher expression compared with the endogenous BRI1 promoter, BRI1 expression upon WOX5 promoter ended in a local overexpression of the receptor in the QC (Figure 2.2).

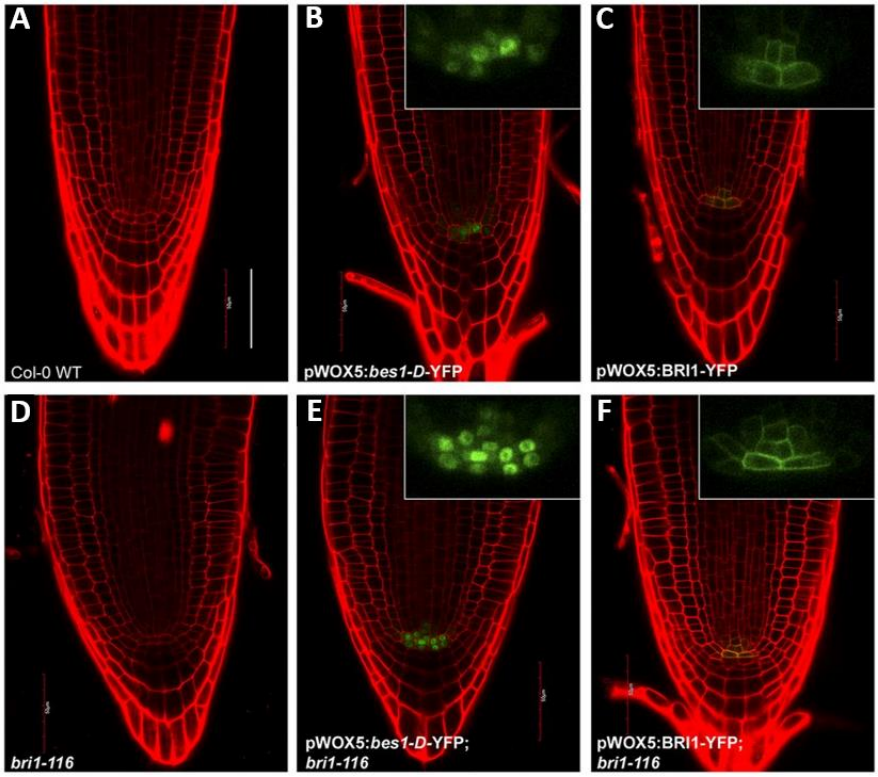


Figure 2.1 QC-specific expression of BR pathway components. (A–F) Confocal images of 6-day-old WT and mutant Arabidopsis roots in control conditions. Insets show the YFP channels at higher magnification. Red for PI, green for YFP. Scale bar: 50 µm.

Confocal microscopy of 6-day-old roots revealed an increase in the number of QC divisions in both the WT and the *bri1-116* mutant upon expressing *bes1-D* under the WOX5 promotor (Figure 2.3 A,D,F,M). This indicates that active BES1 locally promotes division

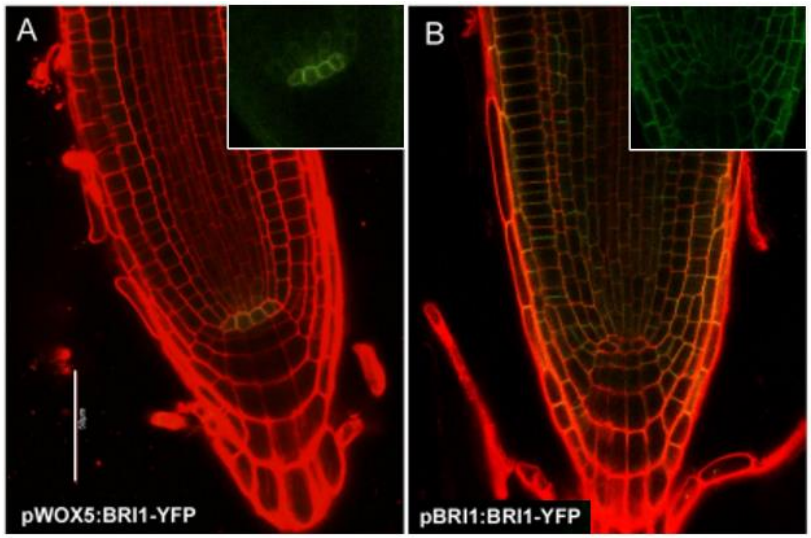


Figure 2.2 WOX5-controlled BRI1 expression causes an overexpression in the QC. (A-B) Confocal images of 6-day-old Arabidopsis roots grown under control conditions. pWOX5:BRI1-YFP (A) and pBRI1:BRI1-GFP (B) (Geldner et al., 2007). Insets show the YFP-tagged BRI1protein. Red for PI, green for YFP. Scale bar: 50 µm.

at the QC in a cell-autonomous manner. However, the QC division rates in the *bri1-116* background were lower than those in the WT background (Figure 2.3 M), suggesting that BR signaling from surrounding tissues also participates in activation of QC divisions. In addition, treatment of WT plants harboring the pWOX5:*bes1-D*-YFP construct with BL did not result in a significant increase in cell division rates (Figure 2.3 D,J,M). This is probably due to a saturated BRs signal contributed also by basal receptor-transduced signaling. Conversely, upon BL treatment, a significant increase in cell division rate was observed for the *bri1-116* plants that contained pWOX5:*bes1-D*-YFP (Figure 2.3 F,L,M). This suggests that the signal is not saturated in these plants, and that the BRL receptors are also contributing factors regulating this process.

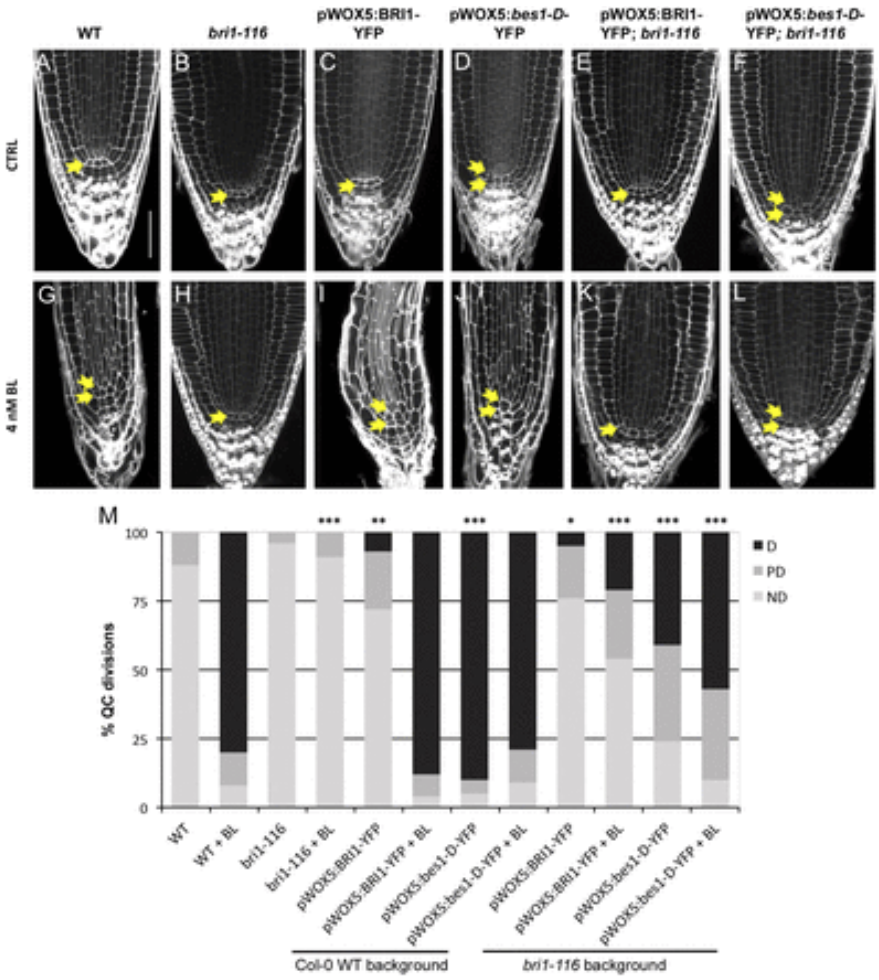


Figure 2.3 BES1 promotes QC division in a cell-autonomous manner. (A–L) Confocal images of fixed 6-day-old WT and mutant Arabidopsis roots in control conditions (A-F) and supplemented with 4 nM BL (G-L). Arrows indicate the number of QC cell layers identified. **(M)** Quantification of QC division rate. ND: non-divided; PD: partially divided; D: divided. Asterisks indicate statistically significant differences due to genotype, comparing against WT either in control or 4 nM BL conditions. Differences in QC division frequencies were assessed with a two-sided Fisher's test (*p<0.05, **p<0.01, ***p<0.005). Data are generated from three independent replicates (n > 21). White for PI. Scale bar: 50 µm.

When BRI1 is locally overexpressed using the WOX5 promoter, a small increase in QC division rate was observed in both the WT and the *bri1-116* backgrounds (Figure 2.3 C,E,M). This increase,

however, was substantially smaller than that observed upon expression of *bes1-D* using the same promoter (Figure 2.3 D,F,M). Upon application of exogenous BL, a dramatic increase in the QC division rate was observed for those plants expressing pWOX5:BRI1-YFP in the WT background but not in the *bri1-116* background (Figure 2.3 C,E,I,K,M). This implies that BRI1 signaling in the QC alone is not sufficient to promote QC divisions, but rather additional external signaling is required.

The fact that overexpression of BRI1 in the QC did not result in a large increase in QC division until exogenous BL was applied, indicates that the BR hormone itself is the limiting factor of QC division. These results suggest two possible scenarios: (*i*) there is an insufficient level of BRs in the root SCN to promote QC division, or (*ii*) BRI1-like receptors (i.e. BRL1 and BRL3) act as competitors for BR ligand binding.

To address the second scenario, we crossed the pWOX5:BRI1-YFP plants with double and triple mutants lacking two (*brl1brl3*) or all receptors (*bri1-116brl1brl3*), respectively, and assessed the occurrence of spontaneous QC divisions or an increased sensitivity to BL. Application of BL to the *brl1brl3* double mutant backgrounds yielded similar effects to those in the WT background, showing that the loss of these genes does not affect QC division rates even when applying lower concentrations of BL (0.04 nM) (Figure 2.4). With respect to the triple mutant, results similar to those found in the *bri1-116* background were found (Figure 2.4). Altogether, these results

indicate that the BRL1/3 receptors do not compete with the BRI1 receptor for hormone binding. Interestingly, a lack of BRL receptors attenuates the slight increase in QC division that is observed upon overexpressing BRI1 in the QC (Figure 2.3 M; Figure 2.4 K). In

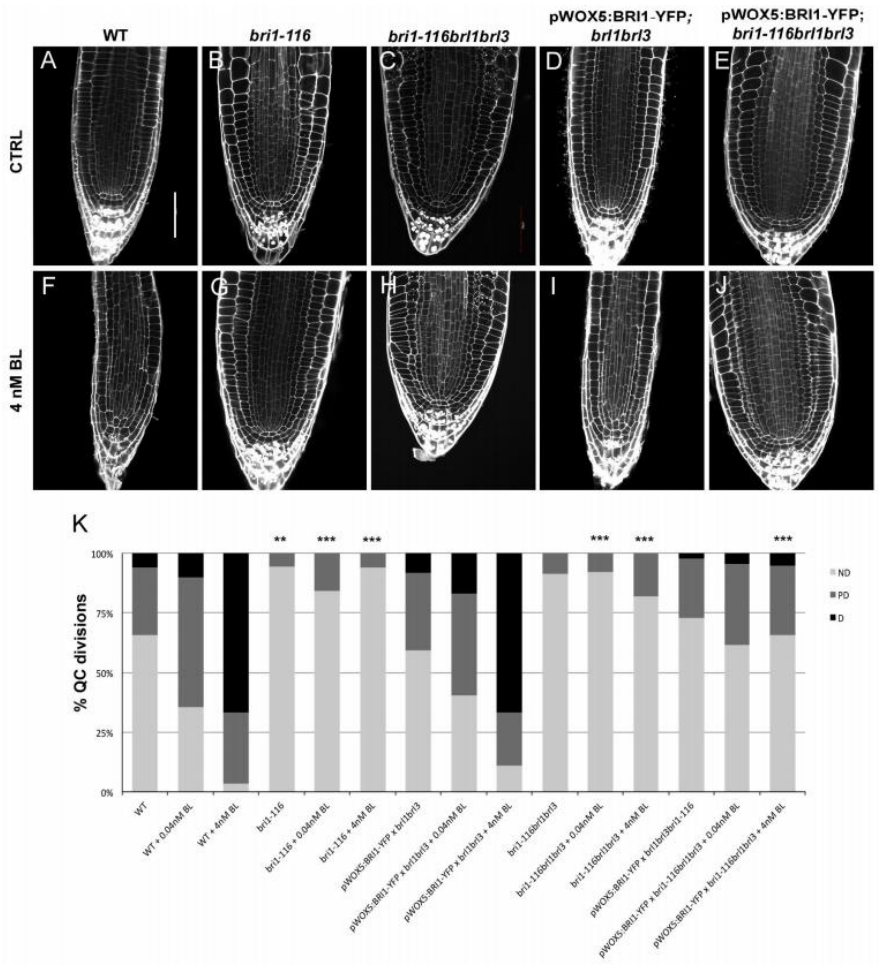


Figure 2.4 The BRL1 and BRL3 receptors do not compete with BRI1 for steroid-ligand binding in the QC microenvironment. (A-J) Phenotype of 6-day-old roots grown under control conditions (A-E) and treated with BL (F-J). **(K)** Quantification of QC division. Statistical differences in division rates were evaluated through a two-sided Fisher's test. Asterisks mean statistically significant differences respect to WT. Differences in QC division frequencies were assessed with a two-sided Fisher's test (**$p<0.01$, ***$p<0.005$)**.** Data are generated from three independent replicates ($n > 34$). ND = QC non-divided, PD = QC partially divided, D = QC totally divided. White for PI. Scale bar: 50 µm.

agreement with previously reported data (Fàbregas et al., 2013), this supports a marginal role for the BRL1 and BRL3 receptors in promoting BR-mediated QC divisions in normal conditions. These results, together with the previous ones, exclude the possibility that BRL receptors compete with BRI1 for ligand binding. Thus, we conclude that the BR hormone concentration must be the limiting factor for promoting QC division.

2.3 Paracrine BR signaling triggers QC division

Since the QC has been proposed to act as a stem cell reservoir and is known to divide in the face of environmental stresses, we decided to evaluate whether the BR receptors are essential for carrying out such stress-induced division. For this purpose, we decided to use bleomycin, a chemotherapeutic drug that has been described to preferentially harm root vascular stem cells and induce QC division (Fulcher and Sablowski, 2009; Vilarrasa-Blasi et al., 2014). As such, this system triggers QC division independently of BR treatment. We compared the local knockout lines (i.e. pWOX5:BRI1-amiR, which express an artificial micro RNA (amiR) specifically against BRI1 expressed only in the QC) against both the null *bri1-116* mutant and WT roots. While the pWOX5:BRI1-amiR lines were damaged at the same rate as the WT plants (Figure 2.5 A,B,C,I;), the *bri1-116* mutant remained free of any visible damage (Figure 2.5 D,I). As previously described, this is probably due to its slow cell cycle progression

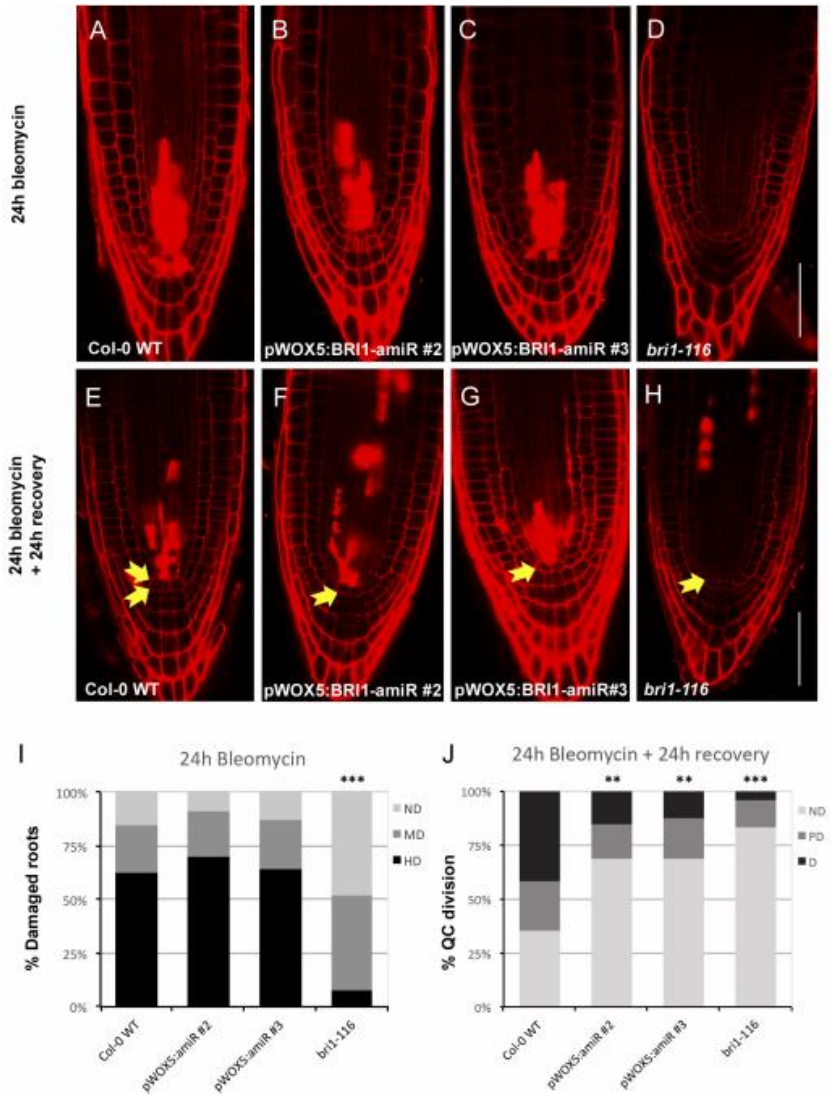


Figure 2.5 BR receptors in the stem cell niche modulate QC divisions upon DNA damage. (A–D) Confocal images of 5-day-old seedlings treated with bleomycin for 24 h. **(E–H)** Confocal images of 5-day-old seedlings subjected to 24 h of bleomycin treatment and a subsequent 24 h of recovery. **(I)** The proportion of roots showing cell death in the root apex after 24 h of bleomycin treatment. HD, hard damage; MD, mild damage; ND, no damage. Differences in the proportion of damaged roots were assessed with a two-sided Fisher's test (***$p<0.005$). Data are generated from three independent replicates ($n > 25$). **(J)** Quantification of QC divisions after 24 h of bleomycin treatment and 24 additional hours of recovery. ND, QC non-divided; PD, QC partially divided; D, QC totally divided. Differences in the QC division frequencies were assessed with a two-sided Fisher's test (**$p<0.01$, ***$p<0.005$). Data are generated from three independent replicates ($n > 25$). Red for PI. Scale bar: 50 µm.

(González-García et al., 2011; Vilarrasa-Blasi et al., 2014). Interestingly, in contrast to what was observed for the WT roots, the QC of the pWOX5:BRI1-amiR lines remained undivided following 24 h of bleomycin treatment plus 24 h of recovery (Figure 2.5 E,F,G,J). In the case of *bri11-116*, the QC also remained undivided, but as previously mentioned, the roots were not damaged by bleomycin (Figure 2.5 H,I). Given that the pWOX5:BRI1-amiR lines and WT show similar levels of provascular cell death after 24 h of bleomycin treatment (Figure 2.5 A,B,C,I), our results argue that the absence of QC divisions in bleomycin-treated pWOX5:BRI1-amiR lines is not due to an inherent resistance against DNA damage.

Although it has been demonstrated that downregulation of BRAVO is implicated in this type of QC division upon DNA damage (Vilarrasa-Blasi et al., 2014), the exact nature of signal progression from the damaged cell to the QC is still unclear. Even if we cannot discern between BRI1 and the BRLs perceiving this signal, results obtained by treating the pWOX5:BRI1-amiR lines with bleomycin have revealed that these signals are perceived by BR receptors acting in the SCN, so the signal should be of a steroid nature and act in a paracrine manner. Since our previous results suggested that DNA damaging agents may promote the accumulation of BRs in the SCN and that this increase of the BR concentration would be the fact that will promote the QC divisions during the DDR, we decided to evaluate the levels of the BRs synthesis genes CONSTITUTIVE PHOTOMORPHOGENIC DWARF (CPD), whose expression domain is restricted to procambial cells in root meristems

(Vukašinović et al., 2021), and DWARF 4 (DWF4), considered the rate-limiting factor to have active BRs (Vukašinović et al., 2021), upon DNA damage.

To better understand a possible regulation of these two genes, we used stable transcriptional markers with a nuclear localization tag (nls), pCPD:nlsGFP and pDWF4:nlsGFP (Vukašinović et al., 2021), and checked their expression in the confocal microscope. In the case of CPD it appears upregulated upon 24 h of bleomycin (Figure 2.6 A,B,D), and it goes back to the normal levels after 24 h recovery (Figure 2.6 A,C,D). In the case of DWF4, its localization in the QC cells was increased after 24 h bleomycin treatment (Figure 2.6

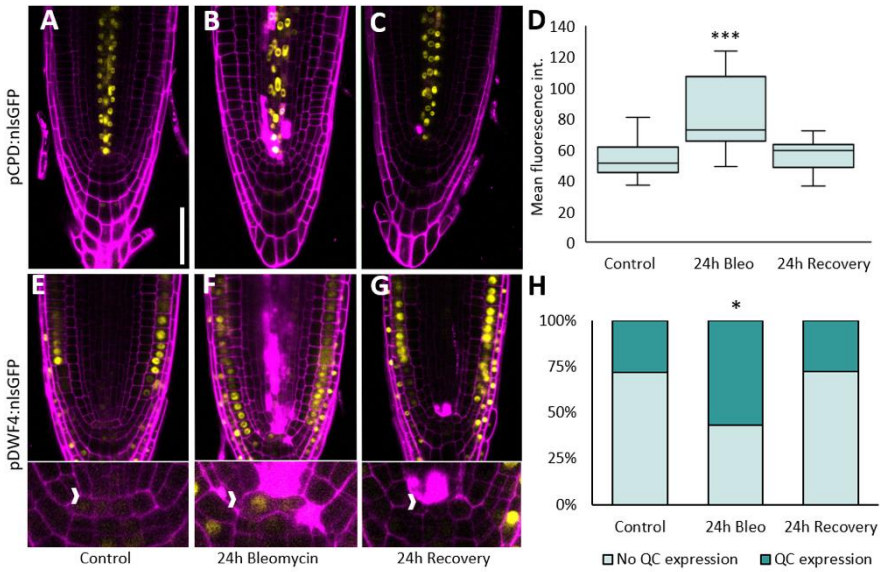


Figure 2.6 BR biosynthesis is activated upon genotoxic stress. (A-C) Confocal images of 6-day-old Arabidopsis roots showing CPD transcriptional expression. **(D)**. Averages from three independent biological replicates (n >35). Differences were assessed with a two-tailed t-test (***p<0.005) **(E-G)** Confocal images of 6-day-old Arabidopsis roots showing DWF4 transcriptional expression. Insets show the SCN at higher magnification. **(H)** Averages from three independent biological replicates (n > 28). Differences were assessed with a two-tailed t-test (*p<0.05). Magenta for PI, yellow for GFP. Scale bar: 50 µm.

E,F,H). Also, we observed DWF4 localization recovered its normal expression in the QC after 24 h recovery (Figure 2.6 E,G,H). These results indicate that the biosynthesis of BR is promoted during the DDR, leading to an increased available hormone in the SCN to be sensed by nearby BR receptors to activate the BR signaling cascade.

2.4 DNA damage activates brassinosteroid signaling through BRL3 receptor pathway

BR signaling and DDR have similar effects in regulating downstream components on signaling pathways, as inhibiting BRAVO and enhancing WOX5 transcription factors (Heyman et al., 2013; Johnson et al., 2018; Vilarrasa-Blasi et al., 2014). As BRL3 receptor expression is also regulated by BL treatment (Salazar-Henao et al., 2016) we wondered if it could be also regulated by DNA damage. A major contribution of BRI1 has been attributed to DDR, as *bri1* mutant does not trigger the PCD, neither the QC divisions, after DNA damage (Lozano-Elena et al., 2018; Vilarrasa-Blasi et al., 2014). However, *bri1* mutant has severe pleiotropic phenotypes even in normal conditions (Clouse et al., 1996; Noguchi et al., 1999). Whereas *brl1* and *brl3* mutants show no major phenotypes in normal growth conditions (Caño-Delgado et al., 2004; Fàbregas et al., 2013).

By analyzing the transcriptional reporter of BRL3 fused with GUS, using different promoter lengths (1796 bp and 755 bp) upon 24 h

zeocin treatment, BRL3 was found upregulated in the root meristem (Figure 2.7 A-D). Interestingly, when using the longer promoter construct, normally BRL3 is expressed specifically in the QC and in the vasculature (Figure 2.7 A, Salazar-Henao et al., 2016). Upon the DNA damage treatment, caused with zeocin, a drug with similar effects as bleomycin, a strong induction its native domain, but also in the epidermis can be observed (Figure 2.7 A,B). Moreover, in the case of the shorter promoter construct of 755 bp it is normally expressed only in the vasculature, as the expression in the QC is lost (Figure 2.7 C, Salazar-Henao et al., 2016). Upon DNA damage

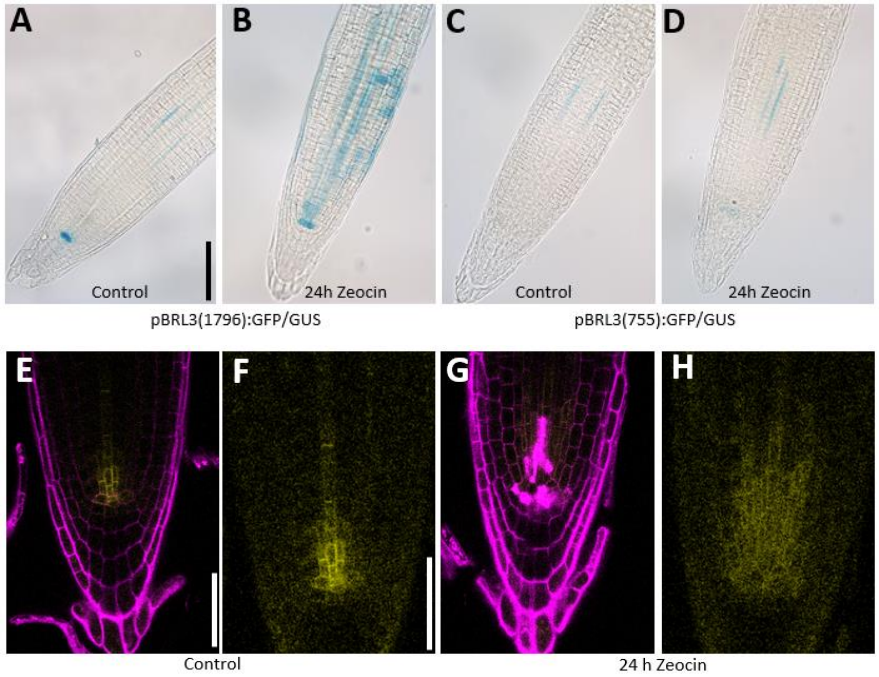


Figure 2.7 BRL3 receptor is upregulated upon DNA damage. (**A-D)** GUS staining of 6-day old seedlings expressing different length BRL3 promoters (1796 bp (A,B) and 755 bp (C,D)) fused with GUS in control conditions (A,C) and upon 24 h of zeocin (B,D). **(E-H)** 6-day old seedlings confocal imaging of pBRL3:BRL3-YFP reporter line in control conditions (E,F) and upon 24 h of zeocin (G,H). Blue for GUS, magenta for PI, yellow for GFP. Scale bar: 100 µm. (A-D) 50 µm (E-H).

conditions, BRL3 transcript appears induced in the vasculature, while seems to partially recover its expression in the QC cells (Figure 2.7 C,D). We also confirmed those transcriptional changes were correlated with differential expression of the BRL3 protein, as native expression lines (pBRL3:BRL3-YFP) displayed an increased fluorescence signal towards the meristem after 24 h bleomycin treatment (Figure 2.7 E-H).

As BRL3 levels were enhanced upon DNA damage, we also wondered if BRI1 and BRL1 could have a similar regulation. For that we analyzed the native expressions of BRI1 and BRL1 by measuring the fluorescence of pBRI1:BRI1-GFP and pBRL1:BRL1-YFP lines using confocal microscopy. In the case of BRI1 no changes were found upon 24 h of zeocin treatment (Figure 2.8 A-C), indicating that BRI1 does not suffer any change expression change during DDR. In the case of BRL1, a decrease in the protein levels was found after 24 h of zeocin (Figure 2.8 D-F). Moreover, by using RNAseq of WT root tips treated during 2 h with zeocin, we also sought to investigate an early regulation of BRI1-like receptors in response to DNA-damage at the transcriptional level. Upon 2 h zeocin, BRL3 was being upregulated (Table 3.1), whereas no transcriptional regulation could be seen neither in the case of BRI1, nor of BRL1 (Table 3.1). These results indicate that the BRI1 receptor is not having any differential expression during DDR, whereas the upregulated levels of BRL3 point to an important role of BRL3 signaling during the DNA damage. Noteworthy, the observed downregulation of BRL1 levels observed at the root apex upon 24 h of zeocin treatment may be

mediated by the direct degradation of the receptor, as its transcription is not changing.

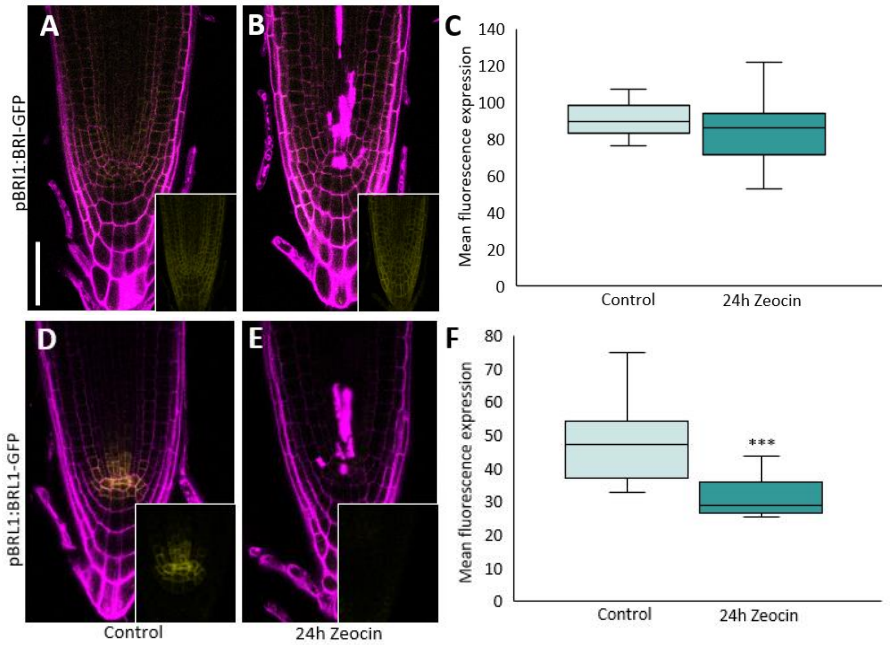


Figure 2.8 BRI1 and BRL1 protein expression upon DNA damage. (**A-B)** 6-day old seedlings confocal imaging of pBRI1:BRI1-GFP reporter line in control conditions (A) and upon 24 h of zeocin (B). **(C)** Mean fluorescence intensity for BRI1 protein. **(D-E)** 6-day-old seedlings confocal imaging of pBRL1:BRL1-GFP reporter line in control conditions (A) and upon 24 h of zeocin (B). **(F)** Mean fluorescence intensity for BRL1 protein. Data are generated from three independent replicates (n > 23). Differences were assessed with a two-tailed t-test (***p<0.005, *p<0.05). Magenta for PI, yellow for GFP. Scale bar: 50 µm.

Table 2.1 BR receptors levels upon 2 h of zeocin in 6-day-old root tips.

Gene	Fold Change CTRL vs. zeocin	Significance
BRI1 (AT4G39400)	0.194404803	8.051459818
BRL1 (AT1G55610)	-0.165429404	0.46703506
BRL3 (AT3G13380)	1.411026718	1.70E-09

Recent results reported that SOG1 can directly bind to BRL3 promoter, activating its expression during the DDR (Ogita et al., 2018). In order to confirm this, we analyzed the BRL3 promoter sequence and found one SOG1-binding consensus motifs in it (CTT(N)7AAG), placed in the first 500 bp of the promoter. ChIP-PCR of pSOG1:SOG1-Myc lines in control conditions and upon DNA damage confirmed that SOG1 was able to directly bind to BRL3 promoter during DDR (Figure 2.9 A). Moreover, the upregulation of the BRL3 transcription during DDR was impaired in the *sog1* mutant (Figure 2.9 B), in agreement with previous reports (Ogita et al., 2018). Altogether these results evidence that BRL3 activity is upregulated in the root meristem, directly promoted by the action of SOG1 directly binding to BRL3 promoter.

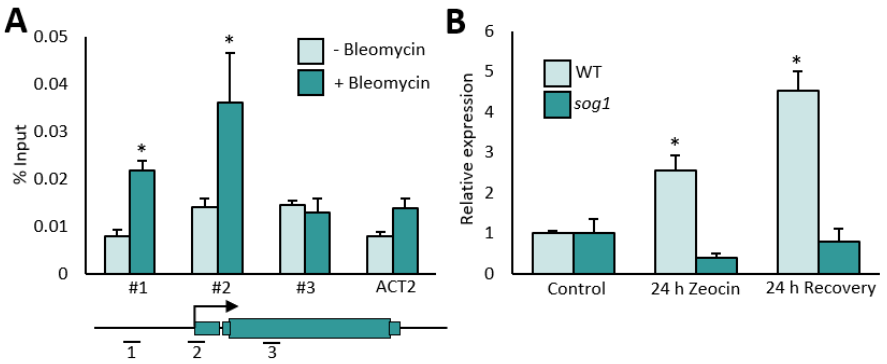


Figure 2.9 BRL3 upregulation upon DNA damage is SOG1-mediated. A) Schematic representation of BRL3 promoter, 1 and 2 are indicating the region selected for ChiP-PCR, being the 2 a putative binding sequence of SOG1 (ATCGTG), 3 indicates an intergenic region used as a negative control. ChiP-PCR of pSOG1:SOG1-Myc lines in control conditions and upon bleomycin treatment showing binding to two sites of BRL3 promoter and using ACT2 as a negative control. Differences were assessed with a two-tailed t-test (*p<0.05). **(B)** qRT-PCR showing the relative expression of BRL3 transcript in control conditions, upon 24 h zeocin and after 24 h recovery in WT background and *sog1* mutant background. Differences were assessed with a two-tailed t-test (*p<0.05). Data are generated from three independent replicates.

In light of our findings, we hypothesized that if the observed increase of BR biosynthesis and the upregulation of BRL3 transcription was actually activating this cell-specific signaling pathway, the downstream BR-regulated transcription factors BES1 and BZR1, should be activated by being dephosphorylated and translocated to the nucleus. To test that, we used stable protein reporter lines of both transcription factors, pBES1:BES1-GFP and pBZR1:BZR1-YFP, we checked the subcellular localization of the two proteins upon DNA damage treatments to analyze shuttling from the cytoplasm to the nuclei. In the case of BZR1, the protein was translocated to the nucleus upon 24 h of zeocin (Figure 2.10 A,B). As our previous results suggest that this activation is mediated by BRL3 signaling, we also analyzed the subcellular localization of BZR1 in the *brl3* mutant. BZR1 shuttling to nuclei upon DNA damage is disrupted in the *brl3* background, suggesting that activation of BZR1 upon DNA damage

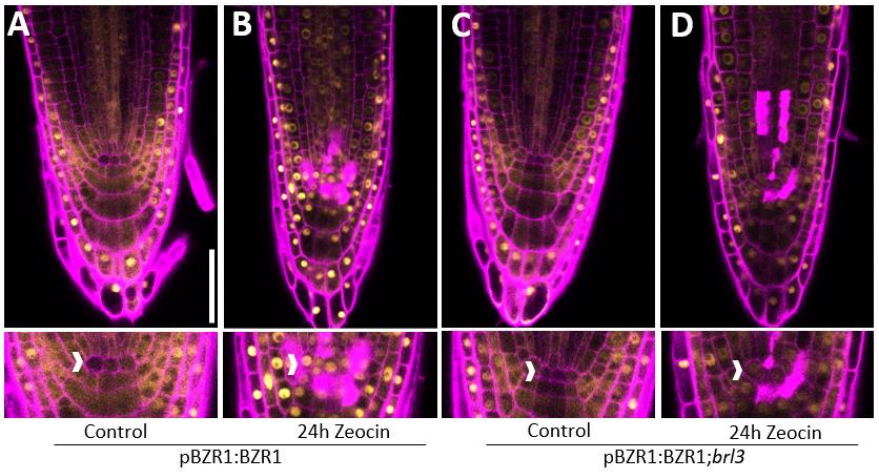


Figure 2.10 DNA damage activates BZR1 in a BRL3 dependent manner. A-D) 6-day-old seedlings showing the expression and localization of BZR1 in control conditions (A,C) and upon 24 h zeocin (B,D) in WT (A,B) and *brl3* backgrounds (C,D). Insets show the SCN at higher magnification and white arrows indicates QC position. Magenta for PI, yellow for YFP. Scale bar: 50 µm.

is mediated specifically by BRL3 (Figure 2.10 A-D). In the case of BES1, we did not observe any translocation upon 24 h of zeocin treatment (Figure 2.11 A,B), while it was happening upon 24 h of BL (Figure 2.11 A,C). These results indicate that BES1 is not involved in the BR signaling involved in the DDR. All together, these results reveal that upon DNA damage in the root apex the BR pathway is activated, specifically by the BRL3 receptor and its downstream effector BZR1.

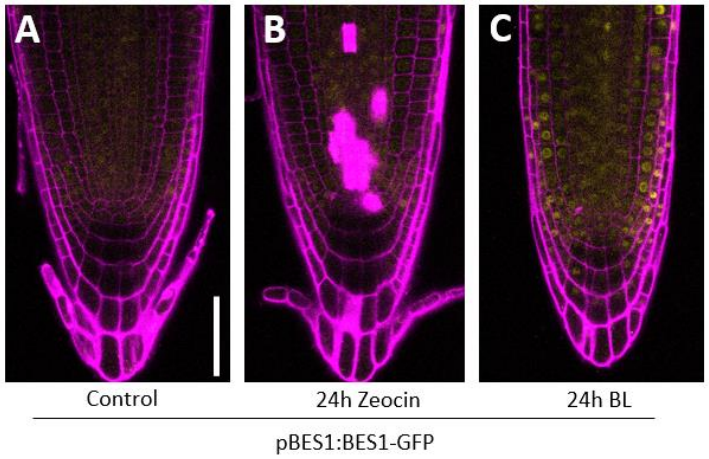


Figure 2.11 BES1 appeared to be not involved in the DDR. A-C) 6-day-old seedlings showing the expression and localization of BES1 in control conditions (A), upon 24 h zeocin (B) and upon 24 h BL (C). Magenta for PI, yellow for YFP. Scale bar: 50 µm.

2.5 BRL3 inhibits DNA repair while activating cell cycle progression during DDR

In order to understand BRL3-dependent processes during DDR we performed a double transcriptomic approach: On the one hand we treated WT and *brl3-2* mutant with 2 h of zeocin and collected root

tips to perform a transcriptomic profiling in order to find the genes regulated by BRL3 during the DDR. On the other hand, roots expressing pBRL3:nlsGFP were treated with 10 nM of BL for 2 h, and cells expressing GFP were selected by fluorescence activated cell sorting (FACS) to find BR-regulated genes in the BRL3 expression domain, as BR signaling and DNA damage treatments are giving very similar responses.

First, we compared the differences between WT and *brl3-2* mutant in control conditions and upon 2 h zeocin. In control conditions 195 genes deregulated were found (p-value (FDR) < 0.05 and FC > |2|, see methods). Gene ontology (GO) enrichment analysis of these genes indicated that in control conditions BRL3 is controlling cell wall remodeling and epidermal differentiation (Figure 2.12 A). When comparing *brl3* mutant upon 2 h zeocin vs. WT upon 2 h zeocin we found 58 genes deregulated (p-value (FDR) < 0.05 and FC > |2|, see methods). GO enrichment analysis of these genes indicated that upon DNA damage BRL3 is important to promote the response to the extracellular stimulus, as they are downregulated in the mutant (Figure 2.12 B). To actually identify the genes that are controlled by BRL3 in response to the zeocin treatment genes were plotted in a bidimensional space, where responses to zeocin in WT and *brl3-2* mutant are in X and Y axes respectively (Figure 2.13). Genes falling near the diagonal are those not affected by the interaction, whereas the further from the diagonal, the strongest is the influence of the interaction. 43 genes significantly regulated in a different way in

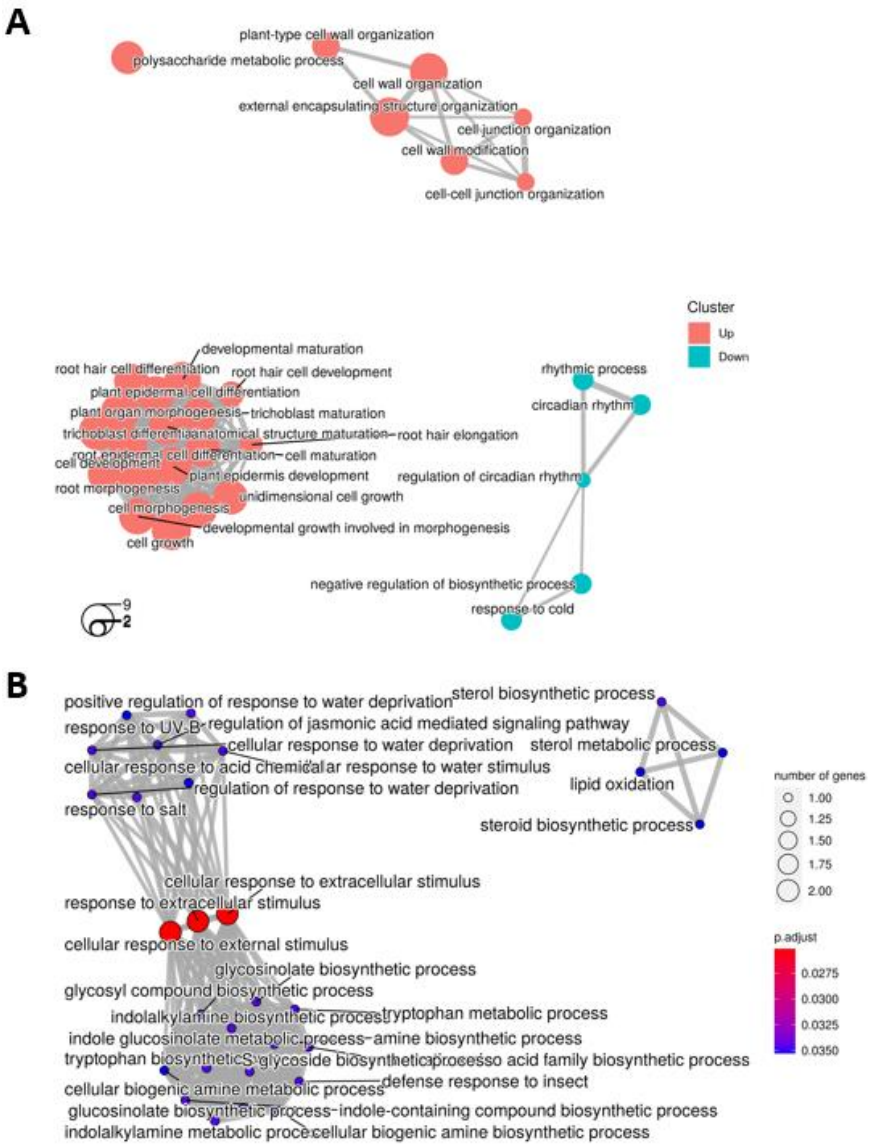


Figure 2.12 GO enrichment analysis of deregulated genes in *brl3* mutant. A) Network representation of GO categories enriched among DEG genes in *brl3* vs. WT at control conditions. Red upregulated genes in the mutant and blue downregulated genes in the mutant**. B)** Network representation of GO categories enriched among genes under-responding to bleomycin respect the WT (Putatively activated by BRL3 under stress). Colors represent the values for scaled data.

Figure 2.13 Genes with a differential response to zeocin between WT and *brl3*. 45 genes were found with a differential response to zeocin treatment in *brl3* and WT backgrounds (lineal model accounting for interaction).

brl3-2 in response to DNA damage were found (Interaction Genotype*Treatment, see methods), being most of them upregulated in the *brl3-2* mutant (36 out of 45). GO analysis showed that the most affected categories in the over-responding genes were the ones related to DNA response and cell cycle process (Figure 2.14). Whereas the most representatives' terms in the under-responding genes were more related to signaling pathways and catabolic processes (Figure 2.14). By analyzing the 7 deregulated genes annotated in the "Cellular response to DNA damage stimulus" (GO:0006974) genes that are involved in repairing DSBs by the homologous recombination process were mainly found, such as SYN2 (SISTER CHROMATID COHESION 1 PROTEIN

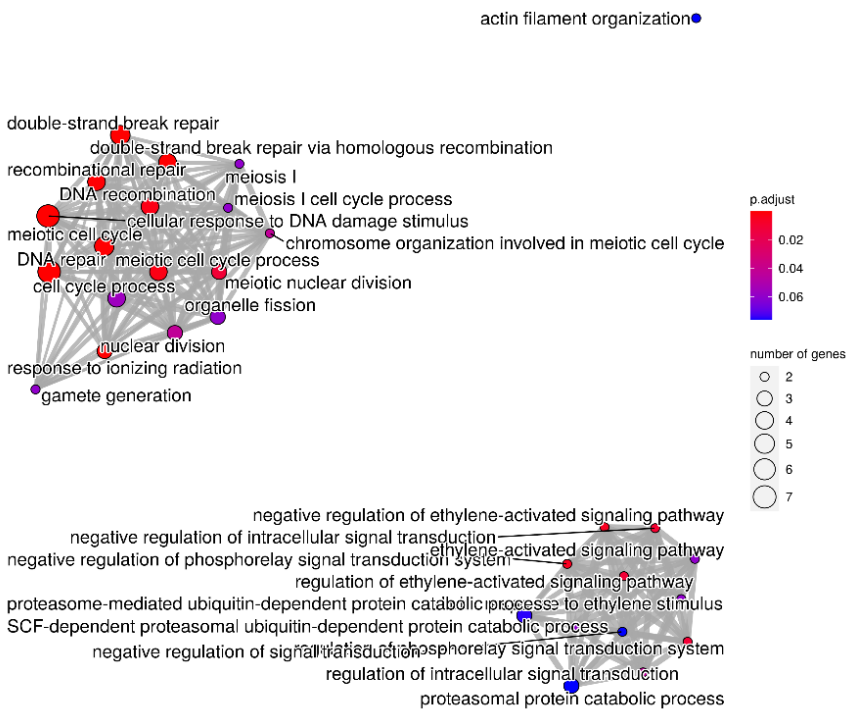


Figure 2.14 GO enrichment analysis of genes with a differential response to zeocin between WT and *brl3*. Network representation of GO categories enriched among the genes with a differential response to zeocin treatment in *brl3* and WT backgrounds (lineal model accounting for interaction). Colors represent the values for scaled data.

HOMOLOG 2, also known as ATRAD21), RPA1E (REPLICATION PROTEIN A 1E), GR1 (GAMMA RESPONSE 1) and RAD51 (Figure 2.15 B). In summary, all the deregulated genes involved in the response to DNA damage are upregulated in the *brl3* mutant, indicating that BRL3 would be inhibiting the DNA response and repair, specifically via downregulating the homologous recombination machinery.

In parallel, we identified genes that respond to BR signaling specifically in the BRL3 domain. A total of 1376 genes were significantly differentially regulated, being 654 upregulated and 722

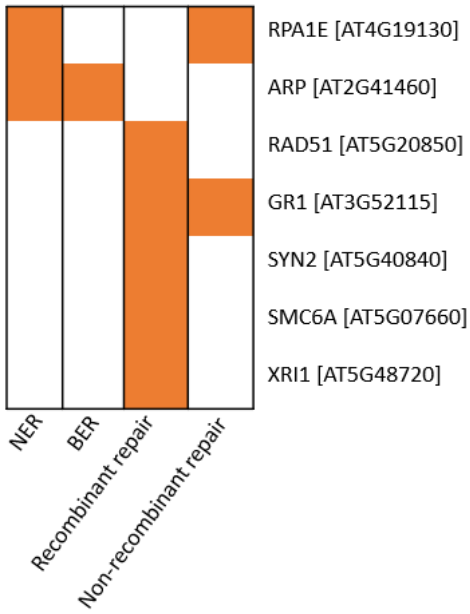


Figure 2.15 BRL3 inhibits DNA repair by homologous recombination during DDR. Deployment of genes within "Cellular response to DNA damage stimulus" (GO:0006974) and classify them as related to nucleotide excision repair (NER), base excision repair (BER) and recombinant or non-recombinant repair.

downregulated in response to BL (p-value < 0.05 and FC > |1|, see methods). GO enrichment analysis showed that, the most representative upregulated GO terms when applying BL were "Root morphogenesis" (GO:0010015) and "Polysaccharide metabolic process" (GO:0005976) (Figure 2.16), and the most representative downregulated GO terms in the same conditions were "Cellular response to hypoxia" (GO:1900039) and "Lignin biosynthetic process" (GO:0009809) (Figure 2.16). To refine our search the differentially expressed genes we intersected the data with the list of genes annotated in the GO "Regulation of cell cycle" (GO: 0051726), identifying 34 genes (Figure 2.17 A) or in the GO "Response to DNA damage" (GO:0006974), identifying 12 genes (Figure 2.17 B). These

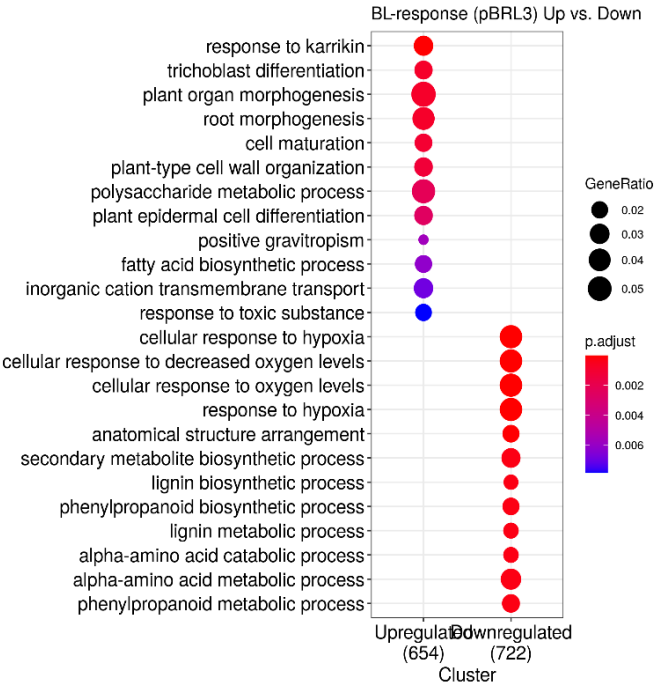


Figure 2.16 BR signaling controls root morphogenesis and response to oxygen in the BRL3 domain. Most representative GO categories enriched in pBRL3 domain roots responding to 2 h of BL, based on their biological function. Colors in the heatmap represent the values for scaled data.

results show that mainly CYCB and CDKB genes are being downregulated by BL. CYCBs transcripts accumulate periodically during the late S to G2 phase in the cell cycle, before being destroyed during the mitosis (Pines and Hunter, 1990). This downregulation of CYCB upon BL treatment has been previously shown and supports that BRs accelerates the cell cycle progression in the root meristem (González-García et al., 2011). Thus, those cells enter faster to the mitosis phase, where these cyclins are being degraded. Moreover, other machinery involved in the cell division are being activated. TUB1 (TUBULIN BETA-1 CHAIN) and TUB5 (TUBULIN BETA-1 CHAIN), and tubulin is known to be accumulated during division

(Dumontet et al., 1996). Also, CPI1 (CYCLOPROPYL ISOMERASE) functions during and just after division (Men et al., 2008).

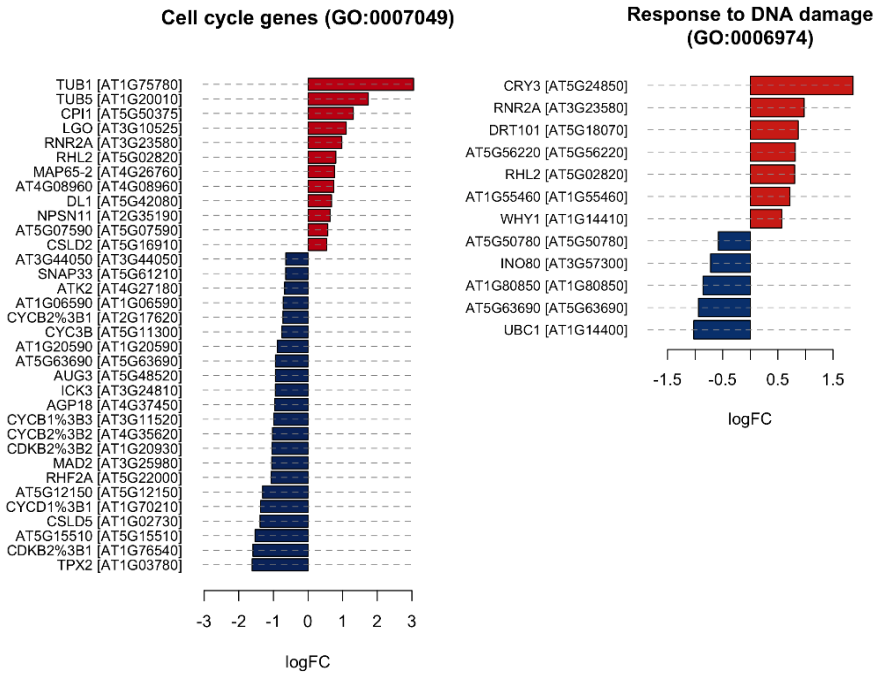


Figure 2.17 BR signaling controls cell cycle genes in the BRL3 domain. (A-B) Deployment of deregulated genes within in pBRL3 domain roots responding to 2 h of BL annotated as "Cell cycle genes" (GO:0007049) (A) and/or "Response to DNA damage" (GO: 0006974) (B). Colors in the heatmap represent the values for scaled data.

As these results are in concordance with the inhibitor role in the DNA repair machinery found for BRL3 receptor we would expect that BL would promote the cell cycle progression. If the DNA repair machinery is blocked by the activity of BRL3 the plants would not need to inhibit the cell cycle when this signaling is activated, as DNA repair will not be acting. Moreover, when analyzing the genes annotated in the response to DNA damage some genes that are involved in the DNA repair such as UBC1 (UBIQUITIN CARRIER

PROTEIN 1) and INO80 (Poli et al., 2017; Seufert et al., 1990), were downregulated. However, other genes involved in DNA repair were also being upregulated, such as CRY3 (CRYPTOCHROME 3) and DRT101 (DNA-DAMAGE-REPAIR/TOLERATION 101, DRT101), but those genes are more involved in repair photodamage (Fujimori et al., 2014; Pokorny et al., 2008).

Conversely, the results of both transcriptomic profiles point out that BRL3 has a role inhibiting the DNA repair (Figure 2.14, Figure 2.15). At the same time, BRL3 may also control the cell cycle progression during the DDR, as BR signal is important for this process at least in control conditions (Figure 2.17). During the DDR there are pathways that coordinate the DNA repair with the cell cycle progression. Normally, the cell cycle is blocked and the factors involved in the DNA repair machinery are stimulated, so these two processes are tightly coordinated (Campos and Clemente-Blanco, 2020). In this sense, if the lack of BRL3 is enhancing the DNA repairing machinery there should be a decrease in the cell cycle progression.

To confirm the role of BRL3 in modulating the DNA repair we performed a TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay, which marks the DSBs by labeling the 3-terminal ends of nucleic acid (Negoescu et al., 1996). We analyzed the DNA repair machinery in WT seedlings and in two knock-out mutants for BRL3 receptor, *brl3-1* and *brl3-2*. The TUNEL results show that *brl3* mutants are having a faster DNA repair, as after 24 h zeocin plus 12 h of recovery both alleles have a reduced amount of

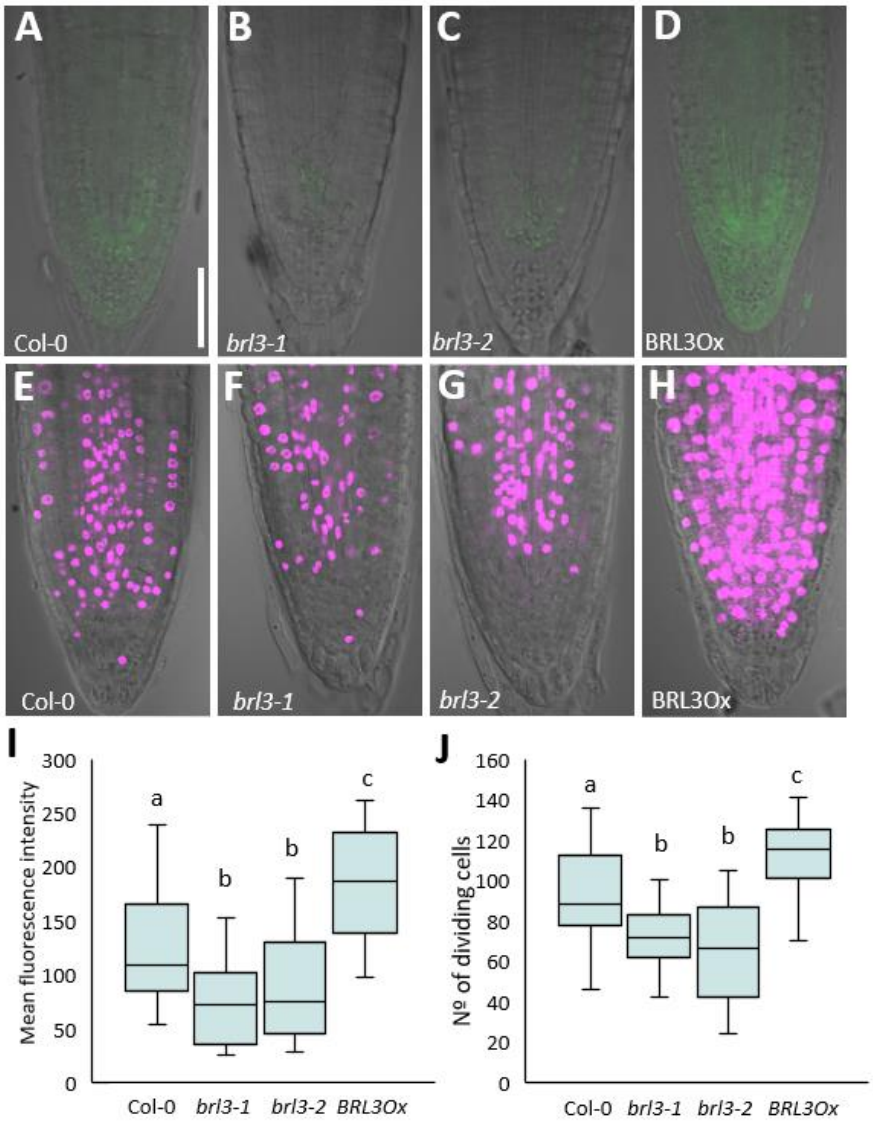


Figure 2.18 BRL3 inhibits DNA repair and activates cell cycle progression during DDR. (A-D) 6-day old seedlings confocal imaging fixed and stained with the TUNEL assay after 24 h zeocin plus 12 h of recovery. **(E-H)** 6-day old seedlings confocal imaging fixed and stained with EdU staining after 24 h zeocin plus 12 h of recovery. **(I)** TUNEL assay mean fluorescence intensity quantifications. Different letters mean significant differences assessed with a two-tailed t-test ($p<0.05$). Data are generated from two independent replicates ($n > 21$). Green for Alexa488. **(J)** Number of dividing cells during the 12 h of recovery. Different letters mean significant differences assessed with a two-tailed t-test ($p<0.05$). Data are generated from three independent replicates ($n > 29$). Magenta for Alexa488. Scale bar: 50 μm.

fluorescence in the TUNEL assay (Figure 2.18 A-C,E), which is caused by a lower accumulation of non-repaired DSBs. We also analyzed the overexpressor BRL3 line (35S:BRL3-GFP, *BRL3Ox* from now (Fàbregas et al., 2013)). When overexpressing BRL3 there is an increase of fluorescence in the TUNEL assay (Figure 2.18 A,D,I), indicating that it has a higher accumulation of DSBs, confirming the inhibitor role of BRL3 receptor in the DNA repair mechanisms.

To confirm the role of the receptor in mediating the cell cycle progression during DDR, the same genotypes were treated with zeocin during 24 h, then, the seedlings were passed to free-zeocin media to recovery. In this case the plants were recovered for 12 h, and the media was supplemented with EdU (5-ethynyl-2'-deoxyuridine), a thymidine analogue that is incorporated into actively dividing cells (Salic and Mitchison, 2008). The results show that both alleles of *brl3* mutants are having a slower cell cycle progression during the recovery after the damage, as they have less cells in the meristem that divide during the recovery time when comparing to the WT (Figure 2.18 E-G,J). Moreover, in agreement with the mutant data, *BRL3Ox* line has an increased cell division frequency as it has more dividing cells in the meristem during the recovery time (Figure 2.18 E,H,J). Indeed, further experiments confirmed that *brl3* mutants were showing shorter roots after facing a DNA damage treatment (Figure 2.19 A,B).

5-day-old seedlings were treated with zeocin during 24 h and then let them recover for another 5 days. The *brl3* mutants exhibited shorter roots compared with the WT (Figure 2.19 A,B), which is probably caused by its slower cell cycle, as indicated in the EdU staining (Figure 2.17 G-I,K). Strikingly, the *BRL3Ox* line shows a similar phenotype as the mutants, as their primary root cannot grow properly neither (Figure 2.19 A,B). As *BRL3Ox* is having a faster cell cycle (Figure 2.18 G,J,K) we could expect to have longer roots, however,

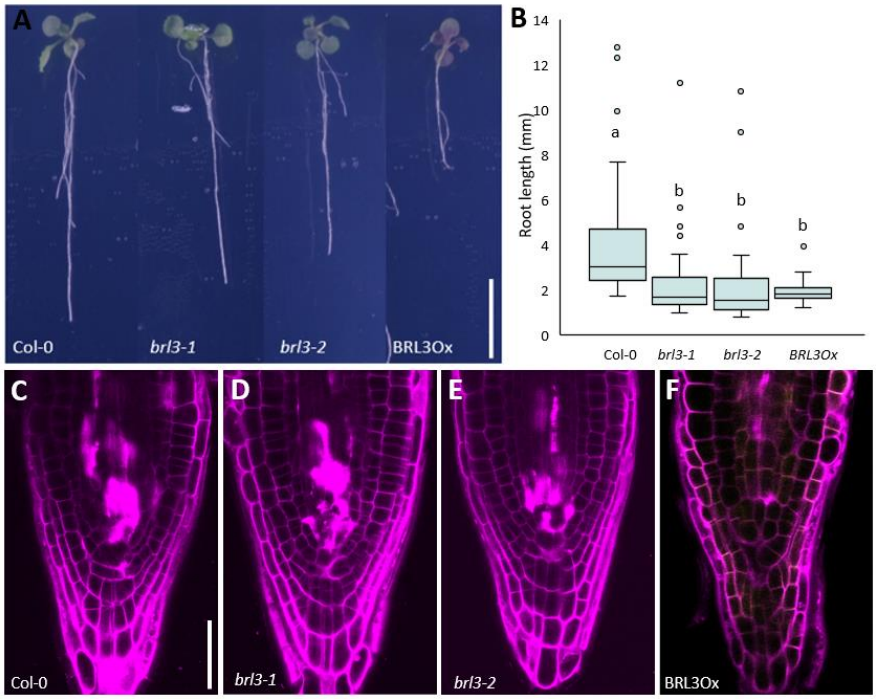


Figure 2.19 BRL3 levels are key for the root survival after DNA damage events. A) Representative 10-day-old seedlings treated with 24 h zeocin at 5-day-old and let them recover for 5 days more. Scale bar: 1 cm **(B)** Quantification of the root primary length of 10-day-old seedlings treated with 24 h zeocin at 5-day-old and let them recover for 5 days more. Different letters mean significant differences assessed with a two-tailed t-test ($p<0.05$). Data are generated from three independent replicates ($n > 36$). **(C-F)** 6-day old seedlings confocal imaging of WT (C), *brl3-1* (D), *brl3-2* (E) and *BRL3Ox* (F) upon 24 h zeocin. Scale bar: 50 µm. Magenta for PI, yellow for GFP.

this also comes with an inhibition of the DNA repair mechanisms (Figure 2.18 A,D,E) which could be detrimental to it. In order to test this hypothesis, we analyzed the roots stained with propidium iodide (PI), a clear start of root exhaustion in the *BRL3Ox* after 24 h of recovery can be observed (Figure 2.19 F), which was not observed neither in WT nor in *brl3* mutants (Figure 2.19 C-E). These results show how a proper balance between DNA repair and cell cycle progression are key to ensure the proper root growth and the survival of the plants after facing DNA damage events.

2.6 BRL3 promotes the triggering of QC division upon DNA damage

As BRL3 is regulating the cell cycle progression and it is enriched in the SCN we wanted to know if its expression was required for promoting the QC divisions during the recovery after a DNA damage. We analyzed the QC divisions of *brl1*, *brl3* and *bri1-301* (a knock-down of BRI1) and found that all the mutants were having a similar ratio of PCD after 24 h of zeocin. However, *bri1-301* mutant showed a smaller area of cells going to PCD (Figure 2.20 A-D,I), similar to the one previously observed in *bri1-116* (Figure 2.5A,E,I), probably caused by a reduced cell cycle progression. When analyzing the triggering of QC divisions, whereas WT, *brl1* and *bri1-301* showed around a 75% of QC division, *brl3* mutant was having a lack of QC divisions, presenting only a 50% (Figure 2.20 E-H,J). These results

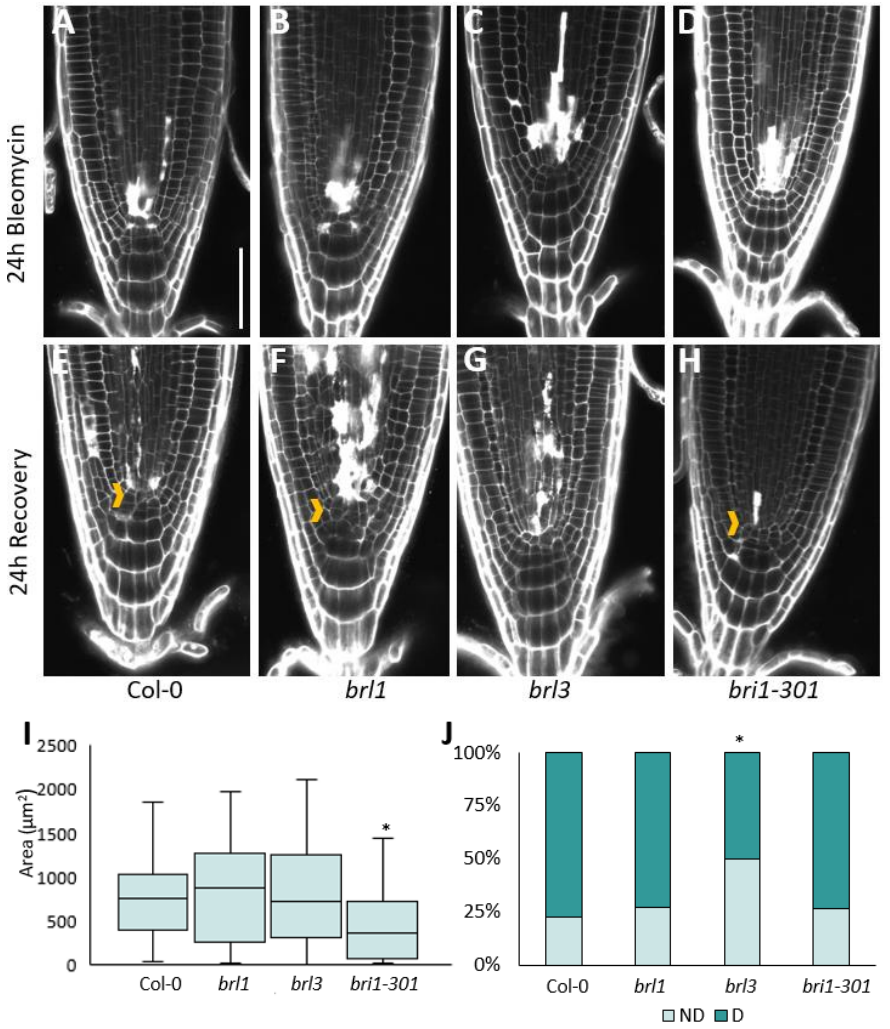


Figure 2.20. BRL3 promotes the triggering of QC divisions upon DNA damage. (A–D) Confocal images of 5-day-old seedlings treated with bleomycin for 24 h. **(E–H)** Confocal images of 5-day-old seedlings subjected to 24 h of bleomycin treatment and a subsequent 24 h of recovery. **(I)** The area of the root showing cell death in the root apex after 24 h of bleomycin treatment. Differences in the area were assessed with a two-tailed t-test (*$p<0.05$). Data are generated from three independent replicates ($n > 25$). **(J)** Quantification of QC divisions after 24 h of zeocin treatment and 24 additional hours of recovery. ND, QC non-divided; D, QC divided. Differences in the QC division frequencies were assessed with a two-sided Fisher's test (*$p<0.05$). Data are generated from three independent replicates ($n > 33$). Yellow arrows indicate QC division. White for PI. Scale bar: 50 µm.

indicate that BRL3 is additionally involved in the activation of the cell cycle progression of the QC cells, apart from its activity in the root meristem in response to DNA damage. Indeed, it indicates a more prominent role of BRL3 receptor in regulating this process, as in the single mutant of *brl3* lack of QC divisions can be observed upon DDR, just caused by the lack of this specific receptor.

In summary, in this chapter we uncover that in normal conditions active BES1 is a key factor for cell-autonomous QC divisions, likely transduced by BRI1 signaling, specifically in the SCN area. Moreover, the BR hormone itself is the limiting factor for BR-induced QC divisions, and under specific circumstances, such as after a DNA damage event, the BR biosynthesis can be activated to promote the accumulation of BR hormones. When BR concentration reaches a threshold in the SCN it acts as a paracrine signal. The paracrine signal is received by BR receptors in the SCN area, and their presence is key to sense the hormones and transduce the signal. During DDR, when this increased BR biosynthesis happens, BRL3 expression is also activated directly by SOG1, the master key regulator of the DDR. Both events are crucial to activate the BRL3 signaling downstream pathway, which leads to the specific activation of BZR1. The BRL3 signaling mediated by BZR1 is crucial to control different aspects of the DDR, as the inhibition of DNA repair mechanisms, especially the homologous recombination machinery. This inhibition of the DNA repair leads to an activation of the cell cycle progression, including the promotion of QC divisions, and the

balance between these two processes is key to ensure the proper growth and survival of the roots in stressful conditions.

Chapter 3

Results

Brassinosteroids control a stem cell specific RNR enzyme

Brassinosteroids control a stem cell niche specific RNR enzyme

3.1 Introduction

Stem cells are undifferentiated cells which have the potential of providing precursors of more-specialized cells, ensuring the survival and sustaining the growth of the organisms (Sánchez Alvarado and Yamanaka, 2014). In plants, stem cells are organized in niches and, specifically, the SCN of the root is located in its apex (Dolan et al., 1993; Heidstra and Sabatini, 2014). In the middle of the root SCN the QC is located, acting as a cell reservoir. The QC can divide upon a set of different stimuli, such as hormone stimulation, or stem cell damage caused by stresses, such as genotoxic one, in order to replenish the surrounding stem cells (Sablowski, 2004; Scheres, 2007).

The decision of divide or maintain the quiescence of the QC is a key process that is regulated by many hormones and transcription factors. For example, it is known that auxins displays a concentration gradient along the longitudinal axis of the root with a maximum at the QC cells (Petersson et al., 2009; Sabatini et al., 1999; Sarkar et al., 2007), which is needed to maintain the homeostasis of the SCN. Cytokinins have an antagonistic function to auxin in many developmental processes, including the control of QC divisions, as plants with

increased CK signaling display ectopic division of the QC cells (Zhang et al., 2011b; Zhang et al., 2013). ABA has also been reported as an inhibitor of the QC divisions, as exogenous ABA treatment induced the quiescence of the QC cells while ABA-deficient and ABA-insensitive mutants presented a higher percentage of QC divisions (Zhang et al., 2010). BRs also act as an antagonistic part to ABA, as exogenous treatment of BR induces the division of the QC cells (Fàbregas et al., 2013; González-García et al., 2011).

Despite all the knowledge acquired of how different hormone pathways are regulating the QC divisions little has been discovered about the downstream components that are being regulated by those hormones. A lot of reports have been centered in understanding which transcription factors are involved in the regulation of the QC divisions. It is known that WOX5 is induced by auxin in the QC (Sarkar et al., 2007), and that BRAVO is normally expressed there to inhibit QC divisions. Both of them interact (Betegón-Putze et al., 2021) and could be regulating together different genes involved in the control of the cell cycle of the SCN, such as the CYCD3;3 (Forzani et al., 2014). It is also reported that the activation of ERF115 regulates the expression of PSK5 (PHYTOSULFOKINES 5), a hormone peptide that induces a promotion of QC divisions (Heyman et al., 2016). However, a part from these downstream components that are involved in the regulation of QC divisions little is known about the specific enzymes that are being controlled directly or indirectly by these transcription factors or by BR signaling per se.

To further understand how BR are regulating the QC divisions we decided to use all the transcriptomic data available at the laboratory to find genes that may be regulated by BRL3, involved in the DDR and QC-specific. With this approach, in this chapter we found that upon DNA damage BR signaling ends with the activation of the RIBONUCLEOTIDE REDUCTASE 2A (RNR2A), an enzyme that is specifically located in the SCN area and that is in charge of replenish the dNTPs (deoxynucleotide triphosphates) of the cells. Controlling the levels of dNTPs is a key process to allow DNA repair and to progress in the cell cycle when needed. Specifically, we found that (*i*) RNR2A has a specific expression in the QC and columella cells. (*ii*) The regulation of RNR2A enzyme is mediated by the signaling coming from BRL3 and BZR1. (*iii*) RNR2A controls the cell cycle progression in the root meristem, and (*iv*) BRL3 and RNR2A activities during DDR are keys to trigger the QC division needed to replenish the surrounding stem cells.

3.2 Brassinosteroids upregulate a QC specific RNR enzyme

Controlling the cell cycle of QC cells is key to ensure the proper survival of the primary root, as BRL3 is involved in regulating the cell divisions upon DDR in the root meristem we focused in analyzing the role of this receptor in these specific cells located at the root apex. For that, and taking into account that many of the genes involved in regulating the QC divisions are having a QC-specific or

enriched expression, such as BRAVO and WOX5 (Betegón-Putze et al., 2021; Sarkar et al., 2007; Vilarrasa-Blasi et al., 2014), we crossed the 14 genes identified as BL-regulated in the BRL3 region and involved in DDR found in the previous chapter (2), with the QC enriched genes list (Brady et al., 2007). Only one gene was found; an enzyme called RNR2A (Figure 3.1 A).

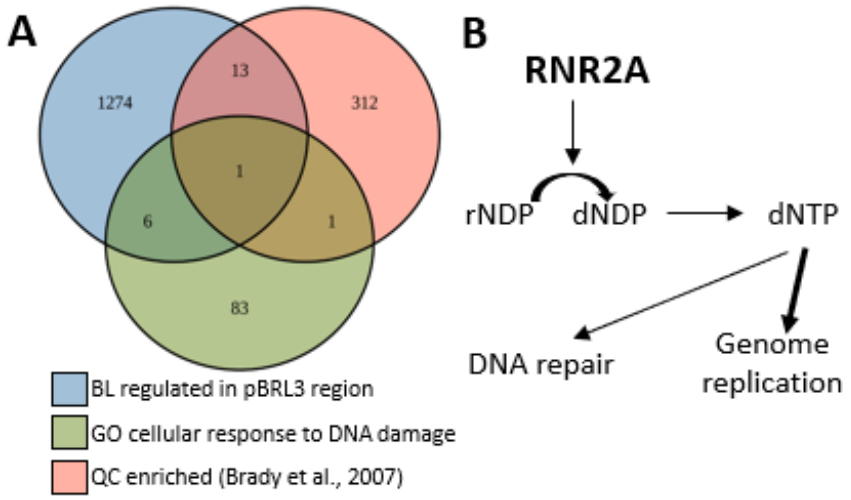


Figure 3.1 BRs regulate a QC specific RNR enzyme (A) Venn diagram of the deregulated genes by BL in pBRL3:nlsGFP region, enriched in the vascular initials/QC together with genes related with the response to DNA damage; only one gene fit all criteria, i.e., RNR2A. **(B)** Schematic representation of the enzymatic activity of RNR2A.

RNR2A is an enzyme that belongs to RNR family (Wang and Liu, 2006). RNR enzymes catalyze the reduction of the four ribonucleotide diphosphates (NDPs) into their deoxyribonucleosides (dNDPs) (Figure 3.1 B), in order to provide deoxyribonucleotide triphosphate (dNTP) precursors for DNA synthesis, and acting as a rate-limiting factor in DNA synthesis (Figure 3.1 B), not only in plants, but also in yeast and mammals (Kolberg et al., 2004; Torrents, 2014). RNR consists of two large subunits (R1) and two small

subunits (R2). The R2 subunit houses the di-iron tyrosyl radical cofactor essential for the reduction of NDP to dNDP (Kolberg et al., 2004). In arabidopsis, three small subunits of the RNR enzymes are present; the TSO2, the RNR2A and the RNR2B. While TSO2 is ubiquitously expressed in the root and has been widely characterized, RNR2A was found to be redundant to TSO2. Moreover, RNR2B is annotated as a pseudogene, as it has a frameshift at position 140 that results in a truncated protein. The constitutive expression of RNR2A could recover *tso2* phenotypes, but the *rnr2a* single mutant showed no apparent phenotypes (Wang and Liu, 2006). However, previous studies were never focused in the root SCN, so we decided to deeply study the role of this enzyme.

As RNR2A transcript appeared to be specific for the QC and upregulated by BL in our transcriptomic analysis, we established stable homozygous lines for pRNR2A:GFP and pRNR2A:RNR2A-GFP constructs to study the expression, dynamics and regulation of this enzyme. In the primary root, both, the transcript and the protein, appeared specifically localized at the QC cells and at the columella cell layers in the root apex (Figure 3.2 A,C), in agreement with the QC-enriched genes from Brady et al., 2007. Exogenous BL application was found to increase both, the transcript and the protein levels (Figure 3.2 A-F), being the transcriptional regulation in agreement with the data obtained in the microarray of FACS cells expressing pBRL3:nlsGFP, in which BL was upregulating RNR2A transcription with just 2 h treatment.

Although RNR enzymes were already known to be upregulated during DDR, there were no evidences of RNR2A responding to DSBs (Roa et al., 2009). This is probably due to a lack of resolution caused by its restricted expression which in the past also did not permit the localization of its mRNA in arabidopsis roots (Wang and Liu, 2006). To check RNR2A upregulation in response to DSBs we treated the transcription marker line with 24 h zeocin and found an increase under this condition (Figure 3.3 A,B,F, Figure 3.4). As in the

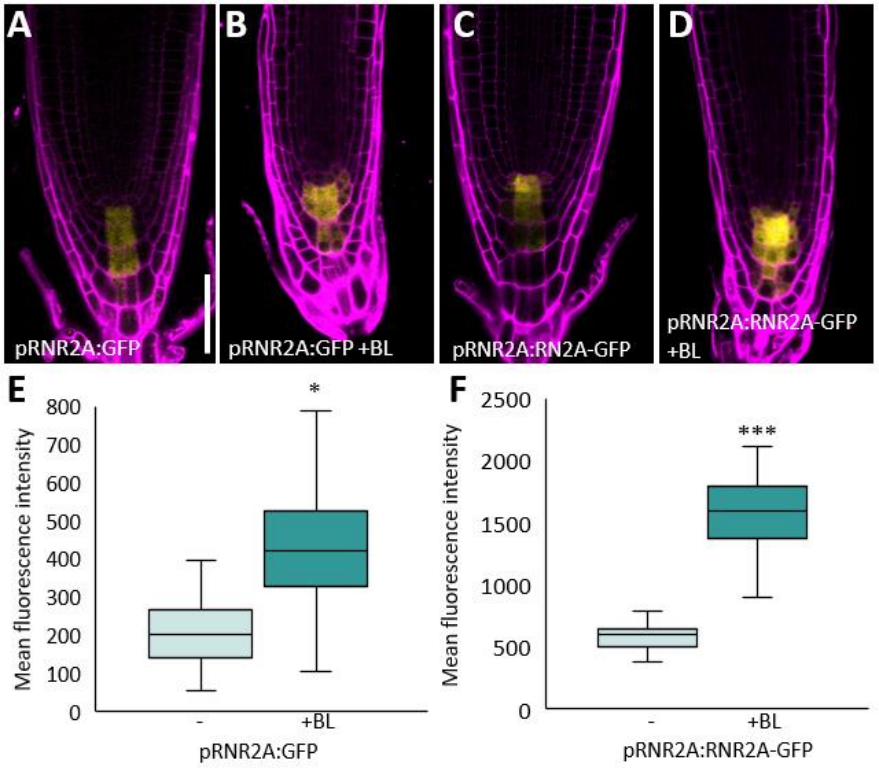


Figure 3.2 RNR2A is localized in the QC and in the columella cells and it is upregulated by BL (A-D) 6-day-old seedlings showing the expression and localization of RNR2A transcript in control conditions (A) and upon 4 Nm BL (B), and of RNR2A protein in control conditions (C) and upon 4 nM BL **(E)** Mean fluorescence levels of RNR2A transcriptional line in control conditions and upon BL. **(F)** Mean fluorescence levels of RNR2A translational line in control conditions and upon BL. Data are generated from three independent replicates (n > 29). Different letters mean significant differences assessed with a Student t test ($p<0.05$). Magenta for PI, yellow for YFP. Scale bar: 50 µm.

previous chapter (2), BRL3 signaling was found to be important to control, not only the DNA repair and cell cycle progression in the meristem, but also to trigger the QC divisions upon DNA damage we wondered whereas the regulation of RNR2A enzyme could be directly mediated by our proposed BRL3-BZR1 signaling module. In order to find it out we crossed the transcriptional reporter of RNR2A with *brl3* and *bzr1-d* mutants and analyzed its expression pattern. Our results indicate that, in the absence of BRL3, RNR2A expression cannot be increased upon DNA damage, in contrast to what happens in WT background (Figure 3.3 A-D,F, Figure 3.4). Moreover, the

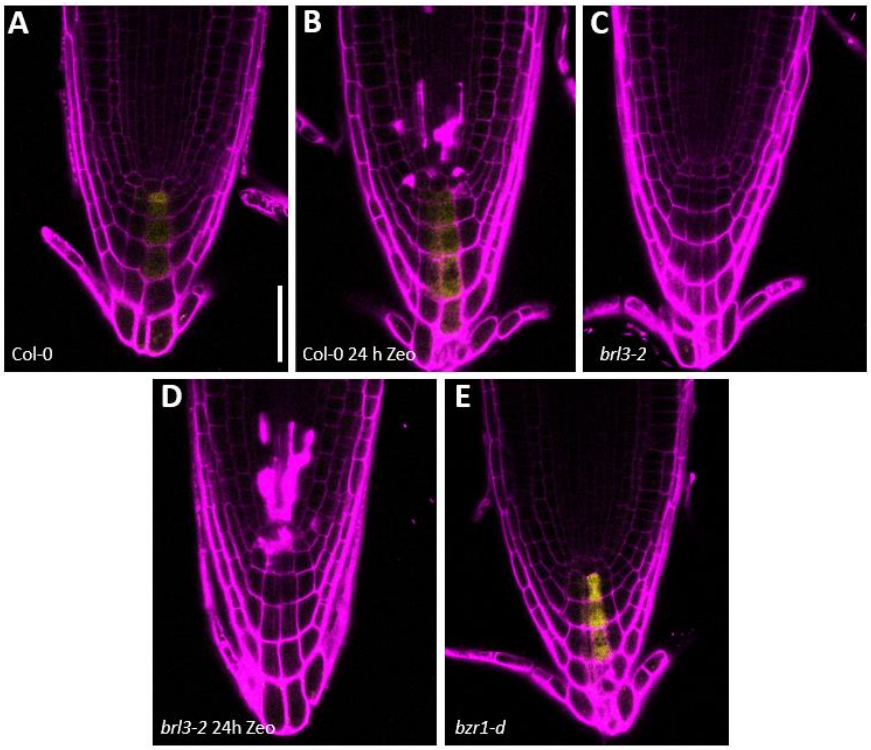


Figure 3.3 RNR2A expression is controlled by BRL3 receptor and downstream BR-response regulator BZR1. 6-day-old seedlings showing the expression and localization of RNR2A transcript in control conditions (A,C,E) and upon 24 h Zeocin (B,D) in WT (A-B), *brl3-2* (C-D) and *bzr1-d* (E) backgrounds. Magenta for PI, yellow for YFP. Scale bar: 50 µm.

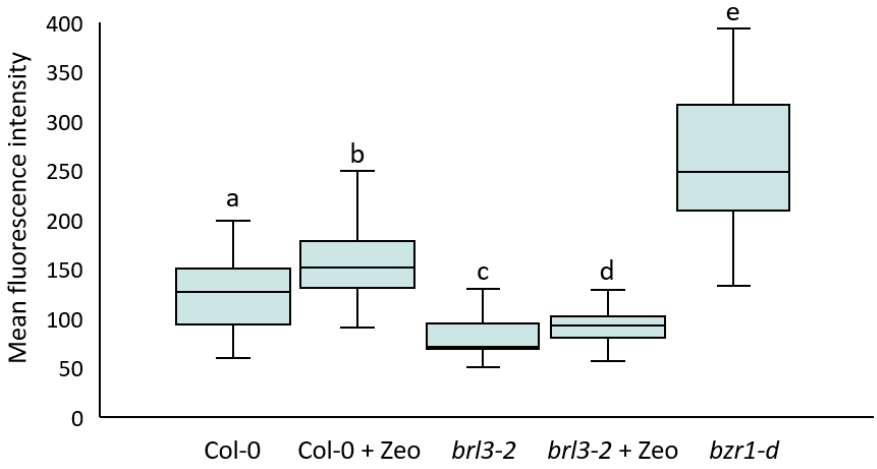


Figure 3.4 Quantification of RNR2A transcriptional activation upon DNA damage. Mean fluorescence levels of RNR2A translational line in control conditions and upon 24 h zeocin in different backgrounds. Data are generated from three independent replicates (n > 27). Different letters mean significant differences assessed with a Student t test ($p<0.05$).

cross of RNR2A transcriptional reporter with *bzr1-d* shows that having a constitutively active BZR1 is enough to promote the increase of RNR2A expression (Figure 3.3 A,E,F, Figure 3.4). Strikingly, the upregulation observed in the *bzr1-d* background is much higher than the one observed upon 24 h zeocin (Figure 3.3 F, Figure 3.4), indicating that during DDR BZR1 would be activated via BRL3 in a more specific and balanced manner.

3.3 RNR2A is key for a normal cell cycle progression in the root meristem

First, we analyzed the general root phenotypes of *rnr2a* knock-out mutant. The root length was measured along 8 days in control

conditions and the *rnr2a* mutants did not show any significant root growth phenotype (Figure 3.5). To test if RNR2A is involved in BR-regulated cell division, mutant seedlings were treated with two different concentrations. At low concentrations (0.04 nM), which normally promotes root growth (González-García et al., 2011), and a higher concentration (4 nM), which normally represses root growth as the cells are exiting to fast the MZ and cannot elongate properly (González-García et al., 2011).

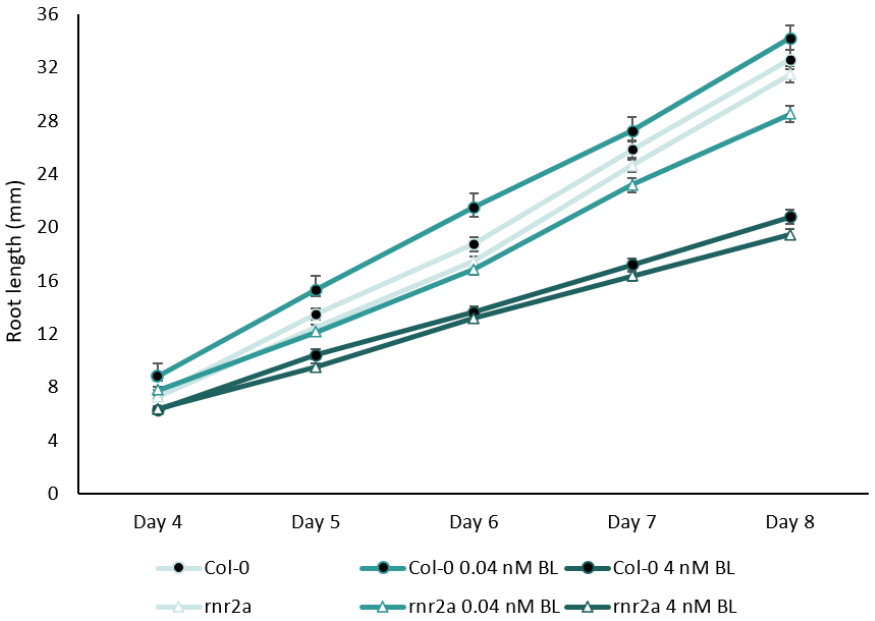


Figure 3.5 RNR2A is needed for a normal response to BR in terms of root growth. Root growth dynamics of WT and *rnr2a* mutants under two different BL concentrations. Differences were assessed with a two-tailed t-test (*p<0.05). Data are generated from three independent replicates (n > 56)

The *rnr2a* mutant showed an inhibition of root growth even at low doses of BL, while WT was longer than in control conditions (Figure 3.5). In the case of the highest concentration of BL, the *rnr2a* mutant did not show any difference with the WT, and both of them had a

reduced root length compared to control conditions (Figure 3.5). This result may indicate that *rnr2a* has a compromised cell cycle, as the addition of low doses of BL would force the cells to go through the cell cycle prematurely, causing a lack of elongation, as happens with high doses of BL (González-García et al., 2011). To confirm whereas RNR2A is important for the cell cycle progression in the root mediated by BR we analyzed the length of epidermal mature cells and found that even low concentrations of BL cause a reduction on the cell size (Figure 3.6). This indicates that *rnr2a* mutant is having a compromised cell cycle progression and that when BL accelerates this process the cells are not prepared for it.

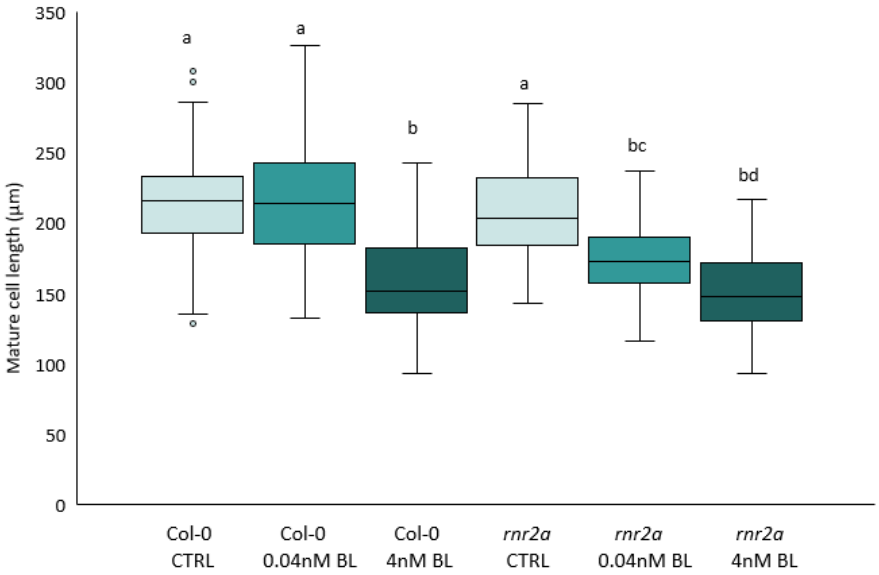
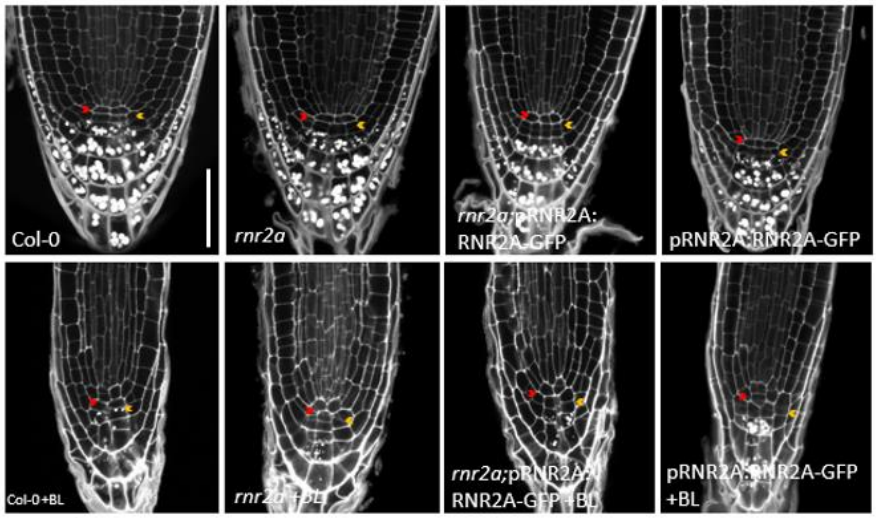


Figure 3.6 RNR2A is involved in the optimal regulation of elongation upon BL. Averages of the mature cell length (um) of 6-day old seedlings of the WT and *rnr2a* mutant in control conditions and upon 0.04 nM or 4 nM BL. Different letters mean significant differences assessed with a two-tailed t-test ($p<0.05$). Data are generated from three independent replicates ($n > 68$).

Given that the expression of RNR2A is restricted to the QC and the columella cells, we decided to analyze those cells in detail in the

rnr2a mutant and in a mild overexpressor of the RNR2A using the protein reporter, which has an extra copy of the gene. We used a 4 nM BL continuous treatment to promote QC division and the results showed that RNR2A is necessary to promote BR-dependent QC divisions, as the *rnr2A* mutant has a lack of QC divisions when treated with BL, and the overexpressor is already having more divisions in control conditions (Figure 3.7 A-C, Figure 3.8). Moreover, the lack of QC divisions in the mutant can be restored by expressing the RNR2A protein under their own promoter (Figure 3.7 A,B,D, Figure 3.8), reinforcing that the lack of QC divisions in the mutants are due to the expression lost. As RNR2A controls the dNTP formation and the enzyme is upregulated upon BR treatment it is likely that BR may control the dNTP pool needed for the progress of the cell-cycle on the QC. As in the mutant this cannot happen, it



shows a lack of cell cycle progression on those QC cells when adding

Figure 3.7 RNR2A mediates QC division and CSC differentiation mediated by BR signaling. (A-H) 6-day old fixed seedlings confocal imaging in control conditions (A-D) and upon 4 nM BL (E-H). Red arrows mark QC position, yellow arrows mark CSC position. White for PI. Scale bar: 50 µm.

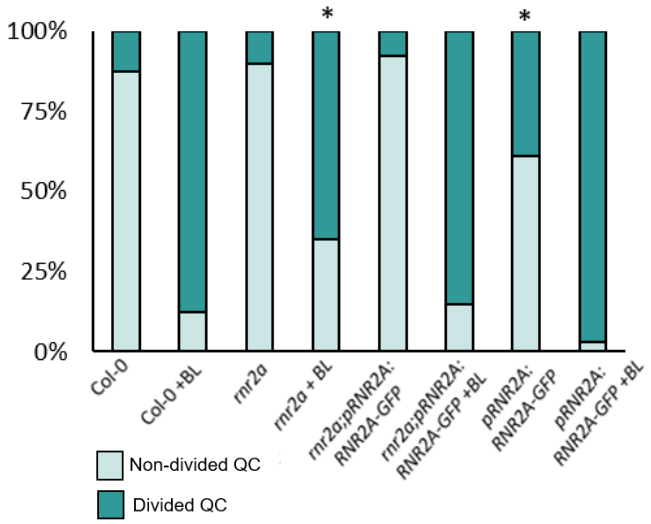


Figure 3.8 Quantification of QC division upon BL in *rnr2a* mutants. Quantification of QC upon BL. Differences in the QC division frequencies were assessed with a two-sided Fisher's test (*p<0.05). Data are generated from three independent replicates (n > 26) Red arrows indicate the position of QC cells; yellow arrows indicate the position of CSC.

BL, supporting our hypothesis. Next, we also checked the columella differentiation, as BR also promotes the differentiation of the CSC (González-García et al., 2011; Lee et al., 2015). The mutant showed a reduced differentiation upon BL (Figure 3.7 A-C, Figure 3.9), while the mild overexpressor showed a higher differentiation in control conditions (Figure 3.9). Moreover, as in the case of QC divisions the phenotype of the CSC differentiation was restored when expressing RNR2A in its native domain (Figure 3.9). The lack of division in *rnr2a* mutant could lead to a delayed cell differentiation, as the transition through mitosis and G1 phase is a key step for exiting the pluripotency status and initiating the cellular differentiation (Soufi and Dalton, 2016).

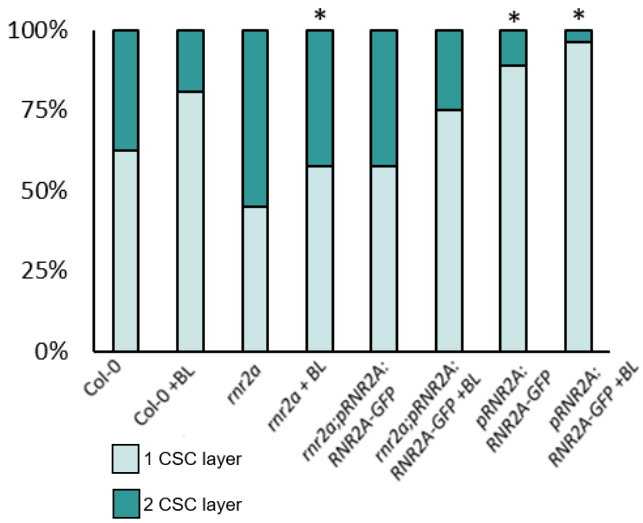


Figure 3.9 Quantification of CSC differentiation upon BL in *rnr2a* mutants. Quantification of CSC differentiation upon BL. Differences in the CSC differentiation frequencies were assessed with a two-sided Fisher's test (*p<0.05). Data are generated from three independent replicates (n > 26).

3.4 BRL3-mediated dNTP pool in the SCN is key to promote QC division during DDR

Finally, we analyzed the QC division frequency after 24 h bleomycin plus 24 h recovery, when the QC starts dividing to replenish the damaged stem cells (Lozano-Elena et al., 2018). As we already found that RNR2A is important to promote the QC divisions upon BL treatments we thought that probably it would also be key to promote the same process during the DDR. The *rnr2a* mutants present a lower frequency of division compared to the WT (Figure 3.10 A,B,I). This lack of QC divisions is similar to the one observed when treating the plants with BL (Figure 3.7, Figure 3.8), and as previously can be

complemented by expressing the RNR2A in its native domain in the *rnr2a* mutant background (Figure 3.10A-C,I). This supports that RNR2A is also needed to promote the cell cycle progression in the QC cells under these conditions.

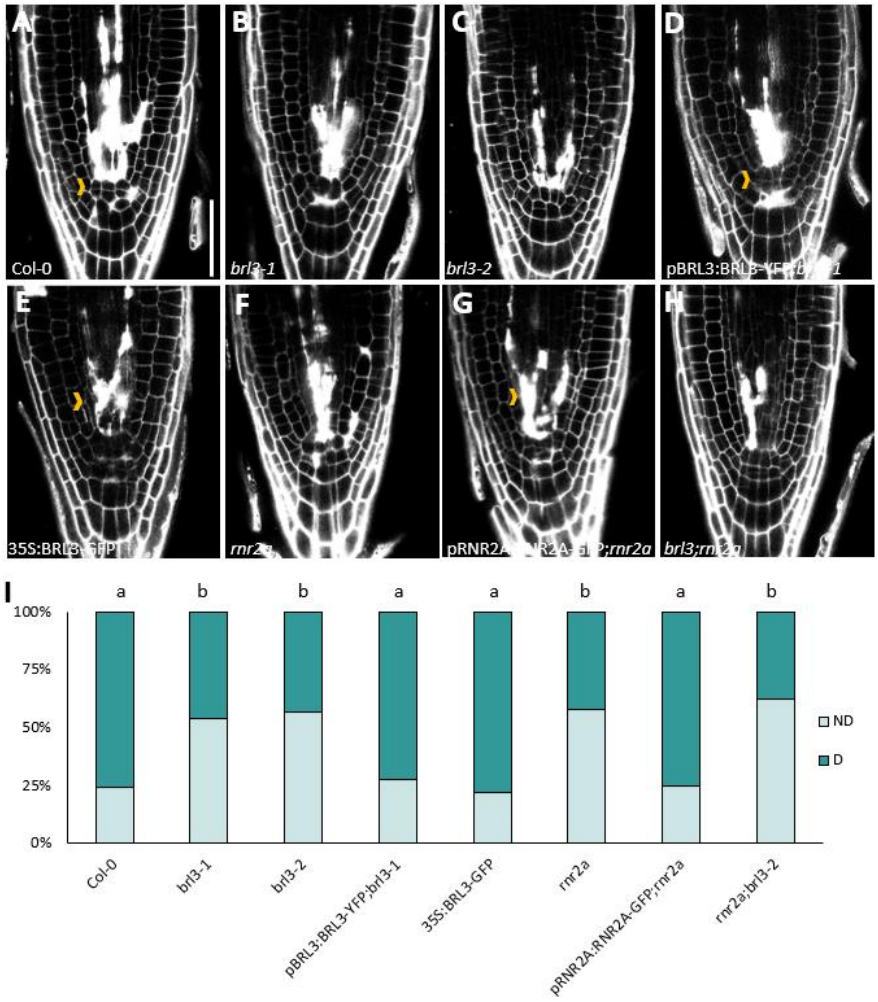


Figure 3.10 RNR2A and BRL3 are controlling the triggering of QC cell division during the DDR. (A-H) 6-day old seedlings stained with PI confocal imaging after 24 h zeocin and 24 h recovery in different genotypes. Scale bar: 50 µm. Yellow arrows indicate QC cells being divided. **(I)** Quantification of QC division rate. Different letters mean significant differences assessed with a Fisher's test ($p < 0.05$). Data are generated from three independent replicates ($n > 32$).

In the previous chapter (2), *brl3* mutant was already found to be required to trigger the QC division upon DNA damage events (Figure 2.20). Here we analyzed again *brl3-1* and *brl3-2* alleles and found that a lower frequency of QC divisions during DDR compared to the WT (Figure 3.10 A,C,D,I), and that it was very similar to the one of *rnr2a* mutant (Figure 3.10 A-D,I). The phenotype of the *brl3* mutants was restored when expressing BRL3 in its native domain (Figure 3.10 A,C-E,I), similar to what happened when complementing the *rnr2a* mutant. *BRL3Ox* line has the same phenotype as the WT (Figure 3.10 A,F,I), indicating that the normal upregulation of BRL3 that is happening in the WT upon DNA damage is already reaching the maximum threshold of BRL3 expression needed to promote the normal QC divisions in response to the DNA damage. Moreover, the double mutant *brl3;rnr2a* maintained the same frequency of QC divisions in response to DNA damage as the single mutant ones (Figure 3.10 A-E,F,I). These results indicate that the lack of QC divisions in both *brl3* and *rnr2a* mutants is due to the lack of function of RNR2A enzyme in these specific cells, which would lead to a deficit of dNTPs in the SCN.

To further prove that the lack of QC divisions in the *rnr2a* mutant were due to a detrimental dNTP synthesis and a consequential lack of cell cycle progression and DNA repair we performed the TUNEL assay. We analyzed the DNA damage accumulation after 24 h of bleomycin treatment plus 12 h of recovery. As expected, *rnr2a* showed a higher frequency of DNA damage, as it is having a higher accumulation of fluorescence in the TUNEL assay (Figure 3.11 A-

C). This result indicates that RNR2A is needed to repair de DSBs caused by the bleomycin treatment, probably due to a detrimental dNTP synthesis in the SCN area. It also supports that the differential phenotypes of QC division and CSC differentiation of *rnr2a* could be caused as a consequence of a cell cycle arrest, provoked by a lack of DNA repair, as have been proved before for other RNR enzymes such as TSO2 (Wang and Liu, 2006).

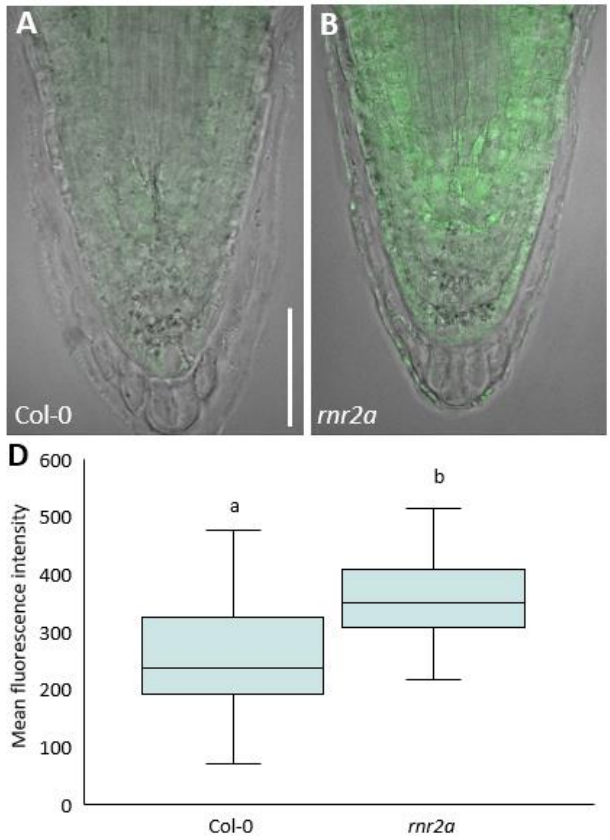


Figure 3.11 RNR2A is needed to repair DSBs in the SCN. (A-B) 6-day old seedlings confocal imaging fixed and stained with the TUNEL assay after 24 h zeocin plus 12 h of recovery. Scale bar: 50 µm. **(C)** TUNEL assay mean fluorescence intensity quantifications. Different letters mean significant differences assessed with a two-tailed t-test ($p<0.05$). Data are generated from three independent replicates ($n > 24$).

In summary, in this chapter, we begin to dissect the mechanism by which the QC divides upon DNA damage. While the majority of studies focus on transcription factor networks, our study innovates on the analysis of a previously unnoticed enzyme involved in the regulation of QC divisions. We found that RNR2A enzyme is specifically located in the QC and columella cells, and that its activity control the onset of QC divisions upon different stimuli, such as BR treatments or DNA damage events. The RNR activity of RNR2A is key replenish the dNTP pool of the stem cells, a key process to repair the DNA and cell cycle progression. Moreover, the RNR2A is specifically upregulated when needed, as for example during DDR.

Collectively, our study advances on the signaling mechanisms for stem cell renewal in plants. Upon DNA damage, the specific regulation of RNR2A during these conditions is finely mediated by signals transduced from BRL3 receptors and required downstream BZR1 activation. For the first time, we have deciphered the direct link of DNA damage and QC division triggering. From the upstream components of the pathway, including SOG1 and BRL3, to the final regulation of the RNR2A enzyme.

Chapter 4

Results

Brassinosteroid receptors control redox status of roots by interacting with RBOHD

Brassinosteroid receptors may control redox status of roots through RBOHD

4.1 Introduction

ROS, particularly H_2O_2, act as effective signaling molecules at low levels, regulating crucial plant biological aspects. H_2O_2 signaling works upstream and downstream from many other secondary messengers such as Ca^{2+}, NO, MAPKs, antioxidant enzymes and transcription factors (Mittler, 2017). Additionally, H_2O_2 interplays synergistically or antagonistically with several phytohormones, including BRs hormones, to regulate plant developmental and physiological processes and stress responses (Xia et al., 2015). Therefore, the hormonal and ROS networks cannot be regarded as independent mechanisms, as multiple components of phytohormones signaling pathways are modification targets of ROS.

On the one hand, H_2O_2 accumulation affects positively in BR signaling through the oxidation and activation of the main transcription factors, BES1 and BZR1 (Tian et al., 2018). This oxidative modification regulates plant primary root growth through enhancing root tip stem cell activity. On the other hand, BR signaling can trigger the accumulation of H_2O_2 (Tian et al., 2018; XIA et al., 2014). Several studies have reported that BR-induced H_2O_2 accumulation is necessary for numerous BR-mediated biological

processes, including QC division (Tian et al., 2018), stomatal movement (Shi et al., 2015; XIA et al., 2014), salt tolerance (Zhu et al., 2016) and responses to heat and oxidative stresses (Nie et al., 2013; Zhou et al., 2014).

Although the fact that BRs promote H_2O_2 accumulation is a non-doubting reality, little is known about the mechanism behind. Recent results indicate that BRI1 may trigger the production of H_2O_2 through a NADPH-dependent pathway (Tian et al., 2018). Interestingly, recent evidences showed RBOHD as a possible interactor of BRI1 and BRL3 receptors by mass-spectrometry (Fàbregas et al., 2013, Fàbregas et al., unpublished). This evidence, together with RBOHD as the major NADPH, makes it a good candidate to unveil the cross-talk between BR signaling and oxidative pathway. To further investigate how BR signaling is promoting the accumulation of ROS in arabidopsis, (*i*) we first deeply analyzed the available transcriptomic data produced in chapter 2, as BRL3 seemed to have a role in redox status. Then, (*ii*) we characterized BRI1-like receptor mutants to test if BR receptors were involved in the previously described BR-mediated H_2O_2. Also, (*iii*) *rbohd* mutants were used to test whether RBOHD could be directly activated by the interaction of BRI1 and BRL3 in response to BRs. (iii) Finally, we investigate the relevance of the BR-mediated H_2O_2 production in terms of QC division and root hair elongation.

Altogether, the results in this chapter uncover that: (*i*) BRL3 and BRI1 are required to trigger the BR-mediated H_2O_2 accumulation, (*ii*)

RBOHD is in charge of producing the H_2O_2 triggered by BR signaling, (*iii*) RBOHD is key to mediate BR-mediated responses in the primary root, such QC divisions, and (*iv*) vascular BRI1 and BRL3 receptors contribute to root hair elongation.

4.2 BRL3 is involved in root hair differentiation and in peroxidase activity

BRI1 is a key receptor to promote the BR-mediated H_2O_2 triggering (Tian et al., 2018), however, little is known about the role of BRL receptors in this process. Unpublished data in our laboratory and data reported in Fàbregas et al., 2013 support that both BRI1 and BRL3 receptors could be direct interactors of RBOHD, the major NADPH in arabidopsis. Moreover, in chapter 2 BRL3 was already related to H_2O_2 catabolism and to root hair development (Figure 2.12), a process that is known to be dependent on H_2O_2 signaling (Mangano et al., 2017; Tsukagoshi, 2016).

In order to address if BRL3 could be involved in the mechanism of triggering BR-mediated H_2O_2, we revisited the RNAseq data shown in chapter 2, comparing the differences between WT and *brl3-2* mutant in control conditions. GO enrichment analysis categorized all annotated genes in 15 different GO terms according to their biological function (GO domain) (Figure 4.1). Among them, the most representative deregulated GO term was “Trichoblast differentiation” (GO: 0042758) (Figure 4.1), which refers to the process in which a

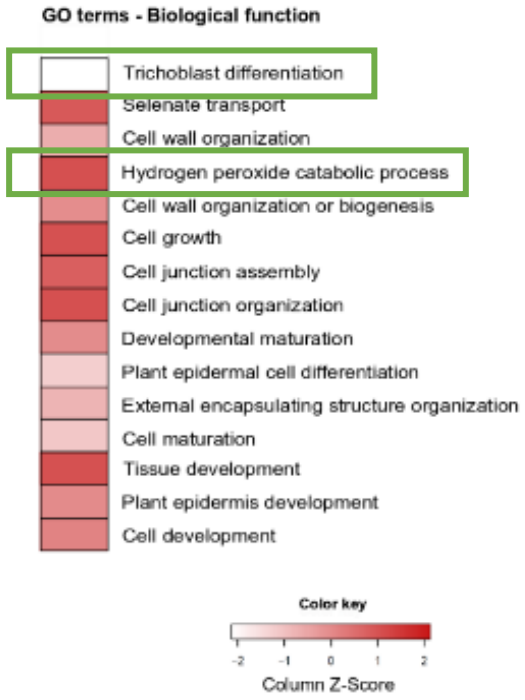


Figure 4.1 BRL3 is involved in trichoblast differentiation and hydrogen peroxide catabolism. Most representative enriched GO categories from the 156 deregulated genes in root tips of *brl3* vs WT 6-old-day seedlings, based on their biological function. Colors in the heatmap represent the values for scaled data.

relatively unspecialized cell acquires specialized features of a root hair cell. Thirteen genes belong and were annotated within this GO term (Figure 4.2 A). Within those genes, there are some of them that have been directly connected to root hair elongation, such as EXPA7 (EXPANSIN A7) (Lin et al., 2011), (RSL4 ROOT HAIR DEFECTIVE 6-LIKE4) (Datta et al., 2015), which are downregulated in *brl3* mutant, indicating that BRL3 would be activating their expression and therefore promoting root hair elongation. Moreover, there is a direct connection between ROS and root hair elongation, as root hair polar growth is endogenously regulated by oscillating levels of ROS, and particularly RSL4 is able of upregulating the expression of genes encoding NADPH oxidases

and class III peroxidases which catalyze ROS production (Mangano et al., 2017).

"Hydrogen peroxide catabolic process" (GO: 0042744) was another categorized GO term within biological function GO domain that was significantly affected in *brl3* mutant roots (Figure 4.2 B). This group included ten genes that are involved in the chemical reactions and pathways resulting in the breakdown of H_2O_2. However, many of them are still unknown, as the most deregulated one. In this case, the catabolism of H_2O_2 seems to be upregulated in *brl3* mutant, indicating that it should have a lower accumulation of H_2O_2 molecules.

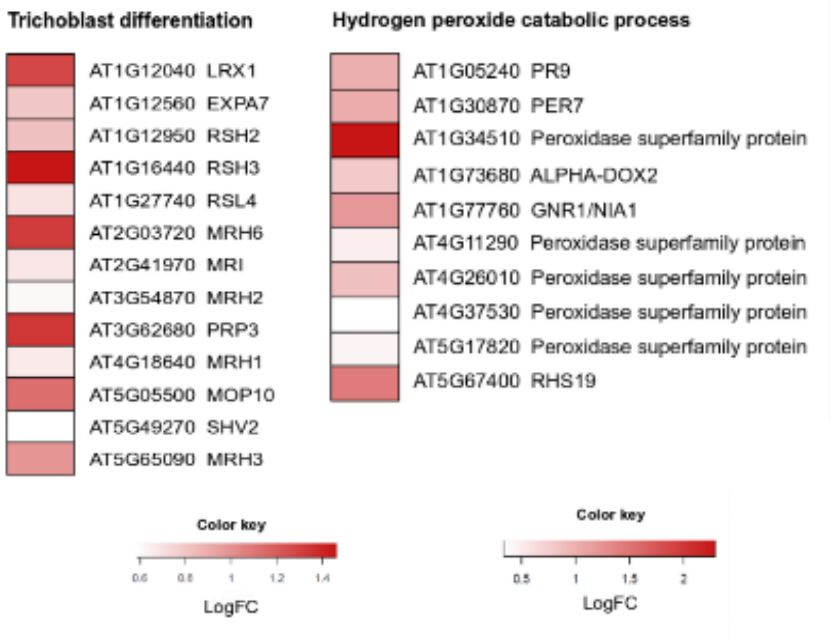


Figure 4.2 BRL3 deregulated genes involved in trichoblast differentiation and hydrogen peroxide catabolism. (A) Deployment of annotated genes classified within "Trichoblast differentiation" (GO: 0010054) **(B)** Deployment of annotated genes classified within "Hydrogen peroxide catabolic process" (GO: 0042744). Colors in the heatmap represent logFC of *brl3* vs. WT.

Based on their molecular function, all annotated genes were categorized in six GO terms (Figure 4.3 A). Among them, the most representative deregulated GO terms were “Peroxidase activity” and “Oxidoreductase activity, acting on peroxide as acceptor”, in which the same eight annotated genes were classified within the two terms (Figure 4.3 B). Most of the genes are peroxidases that are again downregulated in the *brl3* mutant, observed in the previous analysis (Figure 4.2 B). Interestingly, one of the most downregulated genes is part of the RBOH family, ATRBOHE, which is known to produce H_2O_2 in response to Ca^{2+} (Kaya et al., 2019). This suggests that BRL3 is not only involved in inhibiting the catabolism of H_2O_2 but also promotes its production.

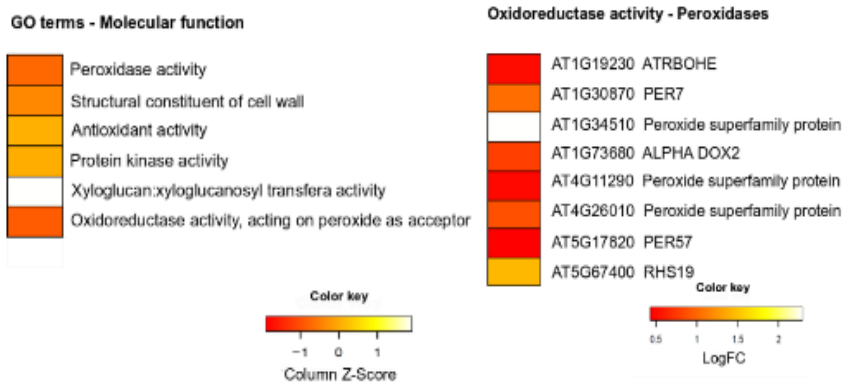


Figure 4.3 BRL3 controls peroxidase activity. (A) Most representative enriched GO categories from the 156 deregulated genes in root tips of *brl3* vs WT 6-old-day seedlings, based on their molecular function. Colors in the heatmap represent the values for scaled data. **(B)** Deployment of annotated genes classified within “Peroxidase activity” (GO: 0004601) and “Oxidoreductase activity, acting on peroxide as acceptor (GO:0004612). Colors in the heatmap represent logFC of brl3 vs. WT.

4.3 BR-mediated triggering of H2O2 is BRI1 and BRL3 dependent

Previously, it has been demonstrated that high levels of BRs increase prolonged H_2O_2 in a BRI1-dependent manner, as this triggering was not happening in *bri1-116* mutants (Tian et al., 2018). However, the specific contribution of each BR receptor in BR-mediated ROS production is not deciphered yet, and transcriptomic data indicates that at least BRL3 receptor may be also involved in this process. Here, to determine the role of BRLs receptors in BR-mediated H_2O_2 triggering we measured the amount of H_2O_2 in the roots of WT and BR receptor mutants in control conditions and after being treated with BL for 24 h. H_2O_2 was detected via confocal microscopy using 2',7'-dichlorodihydrofluorescein (H_2DCFDA) staining. This cell-permeable fluorogenic probe provides a reliable measurement of H_2O_2 levels. Particularly, H_2DCFDA is deacetylated by cellular esterases and in the presence of ROS, it is rapidly oxidized to a highly fluorescent 2',7'-dichlorofluorescein (DCF).

These results showed that even in control conditions, *brl3* and *bri1-116* mutants ex,hibit a lower H_2O_2 quantity compared to the WT (Figure 4.4 A-D,K), while *brl1* does not present any difference (Figure 4.4 A,E,K). Upon BL treatment, H_2O_2 triggering and accumulation occurs in the WT (Figure 4.4 A,F,K), as previously reported (Tian et al., 2018). In the case of *bri1-116* mutant, this triggering upon BL does not happen, as the levels of H_2O_2 remains

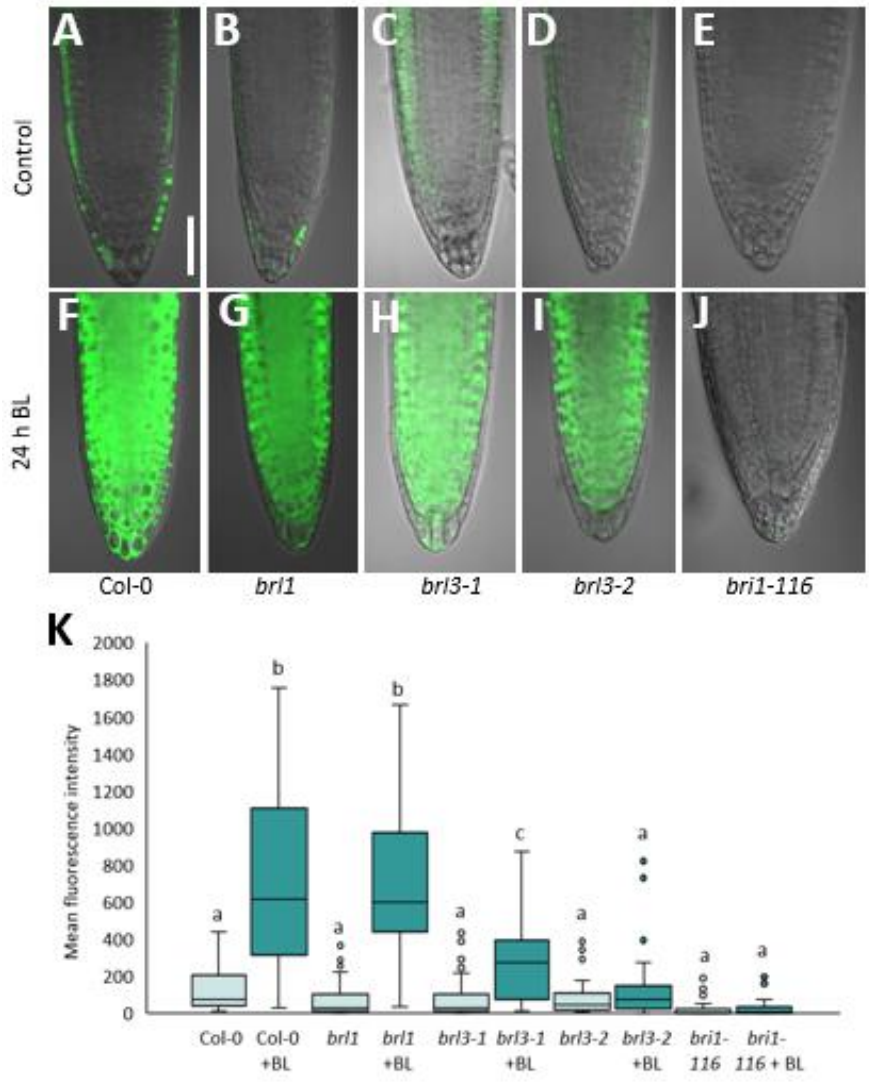


Figure 4.4 BRL3 and BRI1 control BR-mediated H2O2 triggering. (A–J) Confocal images of 6-day-old WT and mutant Arabidopsis roots stained with H_2DCFDA to visualize H_2O_2 accumulation in control conditions (A-F) and supplemented with 1 nM BL for 24 h (G-L). Scale bar: 50 µm. **(K)** Mean fluorescence intensity quantification referring to ROS accumulation in control conditions and upon BL treatment. Data from three independent biological replicates (n > 26). Statistical differences were detected by ANOVA test plus post-hoc Turkey HSD test, and significant differences were represented by different letters (p-value<0.05).

similar to the ones in control conditions, in agreement with Tian et al., 2018 (Figure 4.4 B,G,K). Strikingly, the two *brl3* mutant alleles showed a decreased H_2O_2 triggering when compared to the WT (Figure 4.4 A,C,D,F,H,I,K). In contrast, no differences with the WT were observed in *brl1* mutants (Figure 4.4 A,B,F,G,K), as the triggering of H_2O_2 upon BL happened normally. Altogether, these results indicate that both, BRI1 and BRL3 receptors, appear to be involved in the triggering and accumulation of H_2O_2 mediated by BR signaling, whereas BRL1 appear not to have a specific role in this process. In agreement, both BRI1 and BRL3 were the ones found to interact with RBOHD in the mass-spectrometry data, while BRL1 was not (Fàbregas et al., 2013, Fàbregas et al., unpublished), supporting the idea that BRI1 and BRL3 may act through RBOHD.

4.4 RBOHD mediates BR-dependent responses

RBOHD is the best characterized NADPH oxidase in arabidopsis, and a central driving force of ROS signaling in cells upon biotic and abiotic stimuli. RBOHD is a transmembrane protein which can be phosphorylated in the N-terminal and C-terminal domains, which leads to its activation. When RBOHD is active it triggers the ROS production, as BRI1 and BRL3 receptors were found to be interacting or forming a complex with it, we further studied which was the relation between this gene and the BR signaling and response.

We decided to analyze the *rbohd* mutant in control and upon BL treatment, and see if RBOHD is needed for having a normal response when BR signaling is activated. First, we measured the BR-mediated H_2O_2 accumulation in the *rbohd* mutant. Similarly to what happens in *bri1-116* and *brl3* mutants (Figure 4.4), the triggering of H_2O_2 induced by BR cannot be observed in *rbohd* mutant (Figure 4.5 A,C,D,F,G). This result supports the hypothesis in which the BR-mediated triggering of H_2O_2 is produced by the activity of RBOHD, and that RBOHD may be activated by BRI1 and/or BRL3. Interestingly, *rbohd* mutant has a higher response to BL in terms of H_2O_2 accumulation when compared with *brl3-2* (Figure 4.5 B,C,E,F,G), indicating that BRL3 may control this process not only through RBOHD, but also by using other mechanisms.

Then, we wanted to test if *rbohd* mutants were having any differential response to BL treatments in terms of regulating QC cell divisions. Normally exogenous BL application accelerates the cell cycle progression, including the triggering of the division of the QC cells (González-García et al., 2011; Vilarrasa-Blasi et al., 2014). In *bri1* mutants these changes in the QC division rate are not observed, as the mutant is insensitive to BL (González-García et al., 2011). In the case of the *brl1brl3* double mutants they seem to have the same phenotype as the WT (Fàbregas et al., 2013), but it has never been reported what happens in the *brl3* single mutant. Here, we tested if BRL3 and RBOHD are also involved in the regulation of QC divisions mediated by BR, in order to test if the triggering of H_2O_2 is

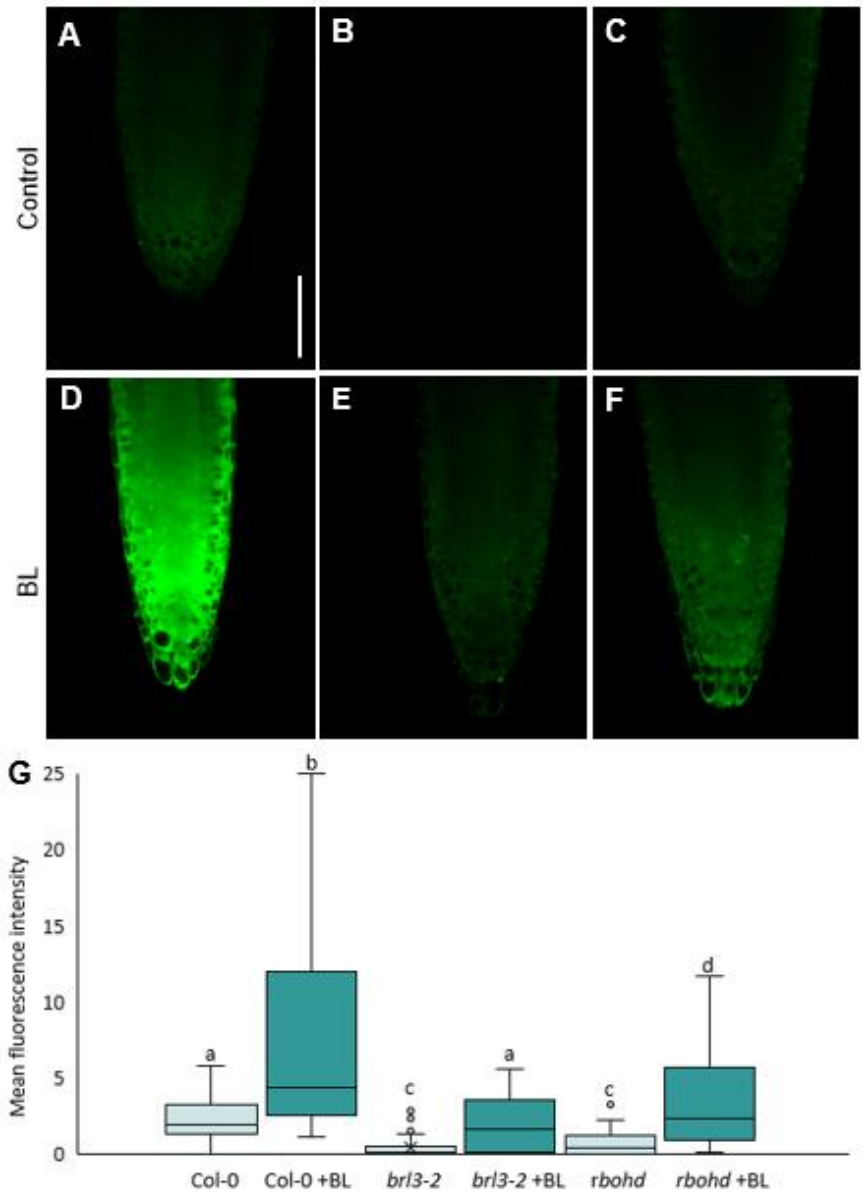


Figure 4.5 RBOHD is needed for the BR-mediated H2O2 triggering. (A–F) Confocal images of 6-day-old WT and mutant Arabidopsis roots stained with H_2DCFDA to visualize H_2O_2 accumulation in control conditions (A-C) and supplemented with 1 nM BL for 24 h (D-GF). Scale bar: 50 µm. **(G)** Mean fluorescence intensity quantification referring to ROS accumulation in control conditions and upon BL treatment. Data from three independent biological replicates (n > xx). Statistical differences were detected by ANOVA test plus post-hoc Turkey HSD test, and significant differences were represented by different letters (p-value<0.05).

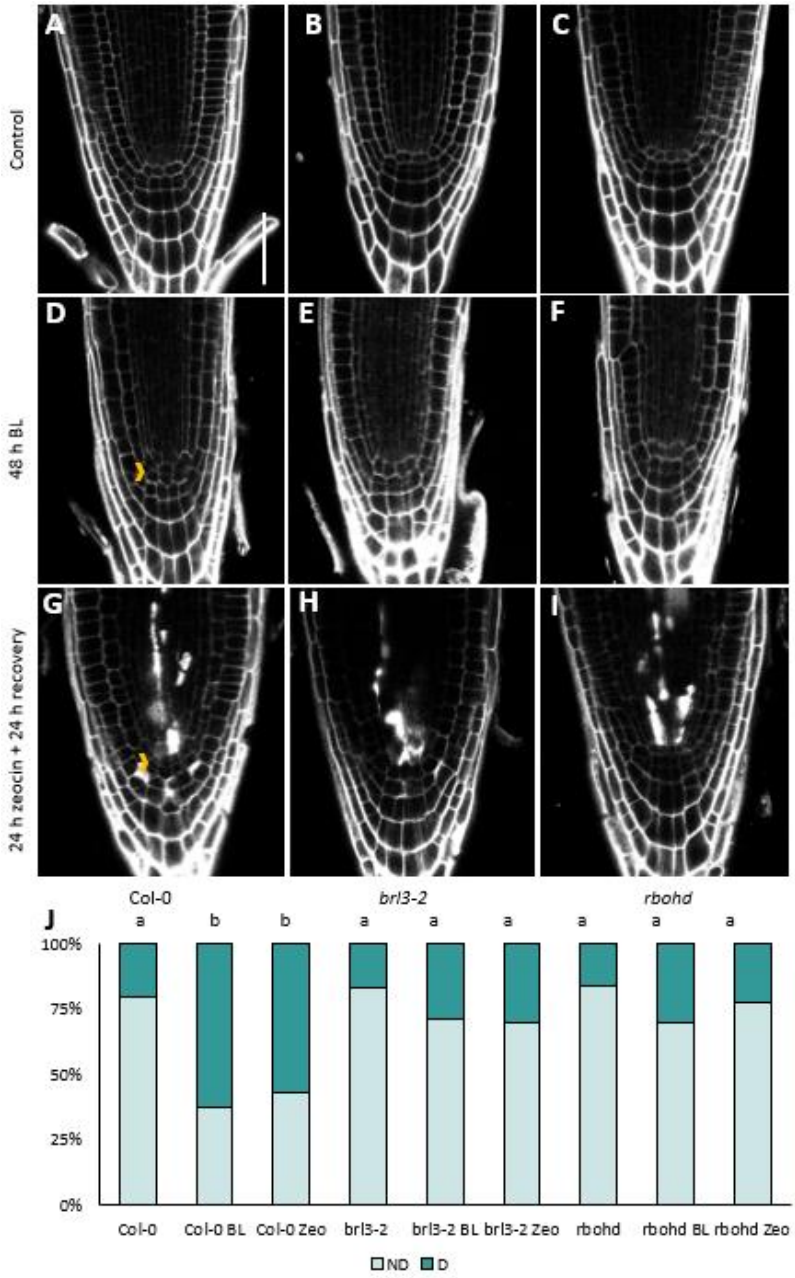


Figure 4.6 BRL3 and RBOHD control QC division triggering mediated by BRs. (A–I) 6-day old seedlings stained with PI confocal imaging in control conditions (A-C), after 48 h 4 Nm BL (D-F), and after 24 h zeocin and 24 h recovery (G-I) in different genotypes. Scale bar: 50 µm. Yellow arrows indicate QC cells being divided. **(J)** Quantification of QC division rate. ND = Non-divided QC, D = Divided QC. Different letters mean significant differences assessed with a Fisher's test ($p < 0.05$). Data are generated from two independent replicates ($n > 21$).

responsible of regulating this process, as the oxidation of BES1 and BZR1 transcription factors is important to trigger the QC divisions (Tian et al., 2018).

To analyze the QC division frequency, we forced its triggering by treating 4-day old seedlings for 48 h with 4 nM of BL or with 1 µM zeocin for 24 h (and let them recover for 24 h). In the WT both treatments promote the QC division (Figure 4.7), whereas the *bri1-116* mutant remains same as control conditions (Figure 4.7), being again insensitive to the treatments. In the case of *brl3* and *rbohd* mutants both of them show a lack of QC division in both treatments, indicating that they are needed to promote this process under stress conditions.

4.5 BR-induced root hair length and identity are mediated by BRL3-synthetized ROS

ROS accumulation in the root hair tip is essential for the normal root hair development and elongation (Mangano et al., 2017; Tsukagoshi, 2016). Knowing that BRs promote ROS production, and that BRL3 is also having a lot of genes involved in this process deregulated we further wanted to analyze whether the differences in ROS accumulation of different BR receptors detected previously could have any effect in root hair elongation.

We measured the root hair length of 6-day-old seedlings of the BR receptor mutants and of *BRL3Ox* line in control conditions and in BL supplemented media for 24 h. Our results showed that in control conditions only *bri1-116* exhibited a significant increase in root hair length comparing to WT (Figure 4.7 A,F,M)). On the contrary, neither *brl3* mutants, *brl1* mutant nor *BRL3Ox* showed any relevant difference compared to the WT in control conditions (Figure 4.7 A,E-M). Furthermore, upon 4 nM BL treatment, a general significant

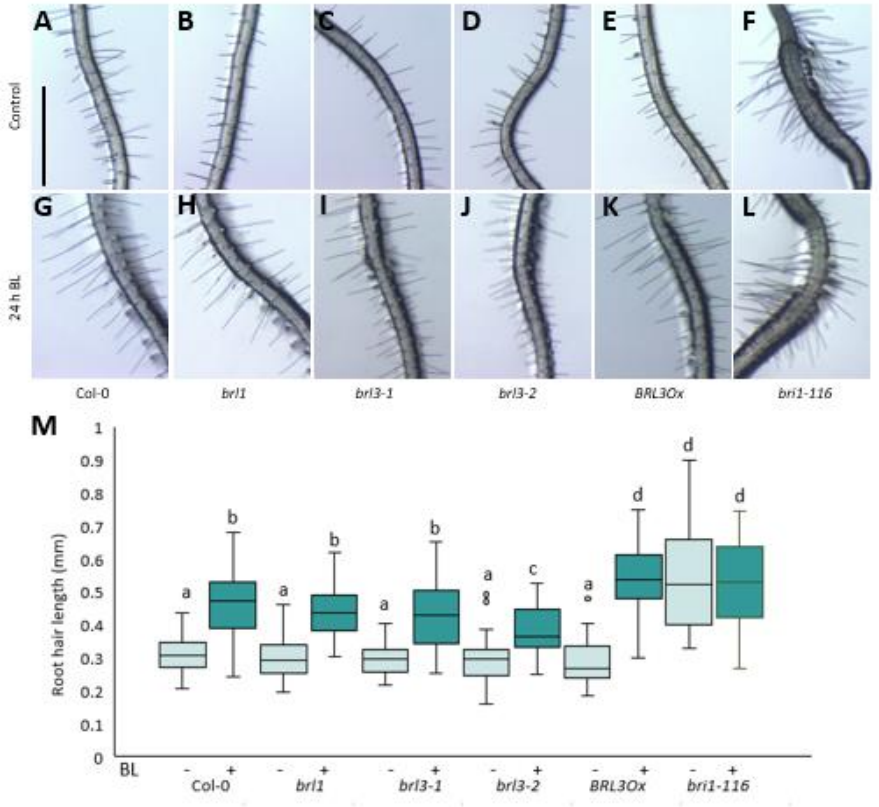


Figure 4.7 BRI1 and BRL3 control the root hair elongation process. (A-L) 6-day old roots in the stereomicroscope focused in the DZ to analyze the root hair elongation in control conditions (A-F) and upon 4 nM BL (G-L) of different genotypes. Scale bar: 1 mm. **(M)** Quantification of the root hair length in control conditions and upon BL in different BR mutants. Different letters mean significant differences assessed with a two-tailed t-test ($p<0.05$). Data are generated from two independent replicates ($n > 36$)

increase of root hair growth was observed in all the genotypes, except in *bri1-116* (Figure 4.7), which remained insensitive to the application of BL. Interestingly, the root hair length increase in *brl3* mutants was not as high as in *brl1* mutant and in the WT (Figure 4.7 M), being statistically significant in *brl3-2* allele (Figure 4.7 M). Moreover, the most prominent increase was seen in the *BRL3Ox*, as their root hairs elongated 2-fold times compared to the WT (Figure 4.7 M). Collectively, our results indicate that BRI1 and BRL3 are involved in BR-mediated root hair elongation, and that this process could be mediated by the BRL3-synthetized ROS.

Altogether we found that: (*i*) BRI1 and BRL3 receptors are both needed to trigger the H_2O_2 accumulation when sensing BR hormone. (*ii*) It seems that this H_2O_2 production will be directly led by RBOHD enzyme. Finally, (*iii*) BRI1 and BRL3 appear to control root hair elongation by different mechanisms, as they have antagonistic effects.

Chapter 5

General Discussion

General Discussion

5.1 BR signaling and DDR regulation

During last 25 years, it has been made a great effort on increasing our understanding of BRs signaling pathway, and its implications in plant growth and development as well as the adaptation to biotic and abiotic stresses (Planas-Riverola et al., 2019). Meanwhile *bri1* mutants display severe and highly pleiotropic phenotypes (Li and Chory, 1997), mutants of *brl1* and *brl3* did not displayed any noticeable phenotypes at normal growth conditions (Caño-Delgado et al., 2004), initially suggesting a maybe redundant function to the BRI1 receptor, and minor role in BR perception and signaling in the plant. However, a throughout analysis has led to discover cell specific functions for these receptors and started to shed light to the roles of BRL3 receptor, which, from the inner (vascular) tissues, seems to govern the plant adaptation to environmental stress, such as in drought (Fàbregas et al., 2018). In this thesis we support this idea of BRL3 receptor having an essential role, not in driving growth as BRI1, but in finely regulating growth and developmental adaptation to adverse climate conditions. Importantly, this thesis discloses that BRL3 receptor pathway is not only important to control adaptation to abiotic stress, but also to DNA damage, unraveling a new pathway.

In normal conditions, the root cells exit the SCN and start dividing in the MZ. When they arrive to the EZ they stop the divisions and

elongate. In all these processes BR signaling is key for a proper balance and regulation (González-García et al., 2011; Pavelescu et al., 2018; Planas-Riverola et al., 2019). Normally, the QC cells act as a reservoir for the surrounding stem cells (Dolan et al., 1993), dividing when needed, i.e. after DNA damage (Vilarrasa-Blasi et al., 2014). In this thesis, we report that the activation of BES1 in the QC is enough to promote the QC divisions, indicating that this process is controlled in an autonomous way (Figure 2.3). However, it would be interesting to check if a specific activation of BZR1 in the QC cells is also able to promote the division of the QC, as both *bes1-d* and *bzr1-d* mutants show the same phenotype of enhanced QC divisions (González-García et al., 2011; Lee et al., 2015).

Moreover, the presence of BR receptors in the SCN area is key to sense the signal of BR hormone, as if there is absence of the BR receptor in this area, the QC division cannot be activated (Figure 2.5). When we depleted BRI1 from the SCN using an amiR, we discovered that the receptor is needed in those cells to promote the QC division upon a DNA damage (Figure 2.5), sensing the signal, which we propose to be paracrine. However, as the amiR is also partially off-targeting the BRLs (Lozano-Elena et al., 2018), we cannot ensure that the absence of the QC divisions is only because of the lack of BRI1. Even if previous studies gave more importance to the role of BRI1 receptor, the fact is, that in the SCN area, BRL1 and BRL3 expressions are enriched, whereas BRI1 present a lower expression (Lozano-Elena et al., 2018; Wilma van Esse et al., 2011). Indeed, we discovered that, upon DNA damage, BRL3 receptor has an important

role regulating QC divisions during DDR (Figure 2.20). Taking everything into account, our current hypothesis is that, BRI1, from the epidermis, leads the normal root development in proper conditions, whereas BRL3, from the inner tissues, would be controlling the root development upon stressful environments, when plants need a balance between growth and adaptation to survive (Figure 5.1).

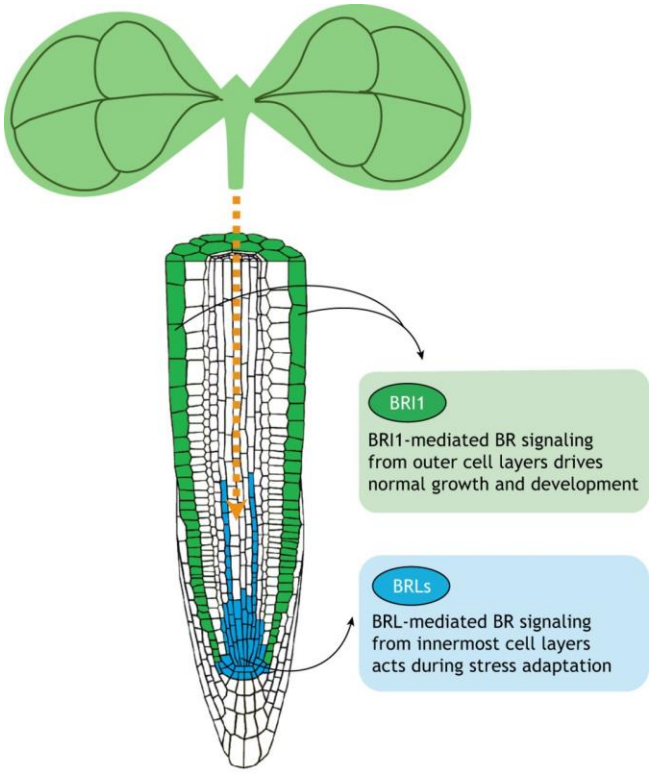


Figure 5.1 Model depicting the tissue-specific actions of BR receptors during growth and stress responses. Schematic of a scenario in which BRI1-based BR signaling from outer tissues (green) primarily regulates normal growth and development, whereas the signaling mediated by BRLs situated in the innermost cell layers (e.g. in the QC, the stem cell niche and vascular tissues; blue) controls stress adaptation responses. BRLs might also be involved in facilitating the mobilization of metabolic signals (orange arrow) from the shoot to root to provide stress tolerance.

DDR is composed by many mechanisms involving signaling pathways, hormones and transcription factors (Hu et al., 2016). For the past years, many studies have shown the direct interconnexion between DDR and different hormonal pathways, such as cytokinins and auxins (Takahashi et al., 2021). However, the link between BRs and DDR was still unknown. Interestingly both signaling pathways have very similar outputs, such as downregulation of BRAVO (Betegón-Putze et al., 2021; Vilarrasa-Blasi et al., 2014) and upregulation of ERF115 (Heyman et al., 2013; Heyman et al., 2016) transcription factors. In this thesis, in chapter 2, we have demonstrated that SOG1, the central regulator of DDR (Yoshiyama, 2016), is directly upregulating the BRL3 receptor expression by binding to its promoter (Figure 2.9, Figure 5.2). In addition, BR biosynthesis is also activated during DDR in the root meristem (Figure 2.6). We propose BR hormones to be the limiting factor for the BR signaling triggering, and that the increase of its biosynthesis will allow reaching a threshold to be sensed. The activation of BRL3 signaling pathway upon DNA damage will end with the modulation of DNA repair machinery and cell cycle (Figure 5.2).

Following DNA damage, cells detect the strand breaks and block the cell cycle progression to provide the cell the time required for repairing the DNA. The phosphorylation of SOG1 triggers its activation, which ends with the regulation of different processes, including the activation of the DNA repair machinery and the repression of the cell cycle (Yoshiyama et al., 2009). This is key to prevent genomic errors propagation. Indeed, if cells cannot repair the

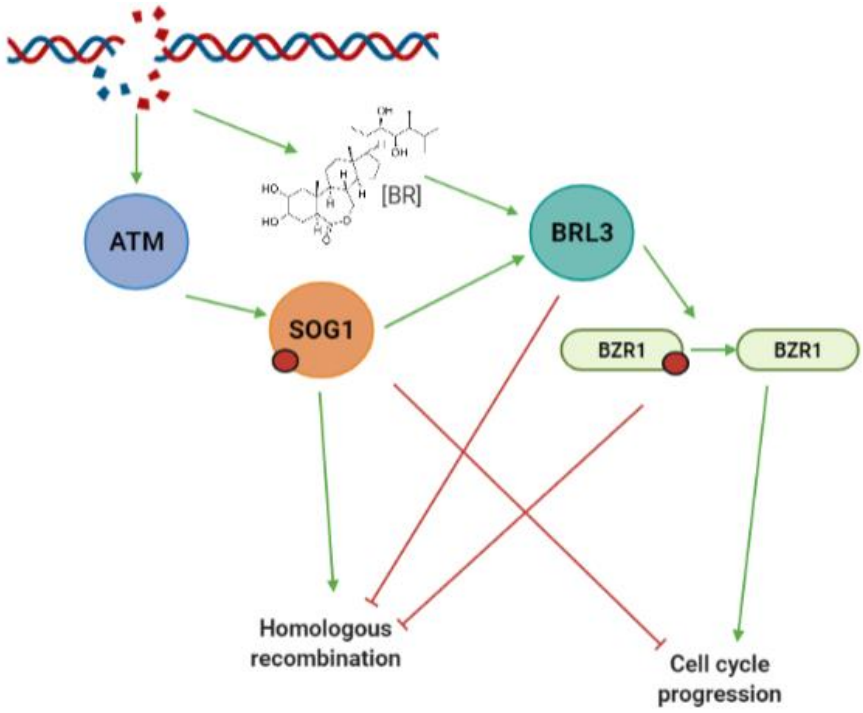


Figure 5.2 Working model of BRL3 cross-link with DDR pathway. Schematic representation of the crosslink between BR signaling and known components of the DDR. Upon DNA damage, and specifically upon DSBs, ATM will rapidly phosphorylate SOG1, the master key regulator of the DDR, which controls DNA repair machinery, such as homologous recombination, and stops cell cycle progression. SOG1 activates the expression of BRL3 and, at the same time, by unknown mechanisms BR biosynthesis is also activated. This leads to the activation of BR signaling pathways, which dephosphorylates and activates BZR1 to inhibit homologous recombination and activates the cell cycle progression.

DNA damage, SOG1 is also in charge of triggering the PCD to avoid genomic error propagation (Adachi et al., 2011; Furukawa et al., 2010). In order to repair the DNA damage SOG1 inhibits the cell cycle progression to allow the activity of the DNA repair machinery. However, cells must also prevent permanent exit from the cell cycle and actively allow cell cycle progression to resume to ensure the survival of the tissues (Shaltiel et al., 2015). In this sense, the activation of BRL3 during the DDR, mediated by SOG1 (Figure 2.7,

Figure 2.9, Figure 5.2), could be acting as a switch to stop repairing the DNA in order to re-activate the cell cycle progression, as BRL3 is inhibiting the DNA repair machinery and accelerating the cell cycle progression (Figure 2.18, Figure 5.2). Moreover, the upregulation of BR biosynthesis under this stress may raise the possibilities of activating the BR pathway mediated by BRL3 in the SCN. We also have specifically found that BRL3 signaling upon DNA damage is activating BZR1 transcription factor, and not BES1 (Figure 2.10, Figure 2.11). Our transcriptomic analyses have shown that BRL3 is involved in the regulation of the DNA repair mechanisms, specifically the homologous recombination one (Figure 2.14, Figure 2.15). We also found that BR signaling is controlling the cell cycle by accelerating its progression, in agreement with González-García et al., 2011. After BL treatment we can see how the CYCB genes are being repressed (Figure 2.17). These results indicate that BL is actually accelerating the cell cycle progression, thus, those cells enter faster to the mitosis phase, where these cyclins are being degraded (Pines and Hunter, 1990). Moreover, other machinery involved in the cell division are being activated, such as tubulins, which are accumulated during mitosis (Dumontet et al., 1996).

In mammals, it is known that tissues that have sustained transcriptional activity elicited by steroid hormones, such as prostate and breasts, are more prompted to have spontaneous DNA damage (Wengner et al., 2020). Regions where intense transcription takes place have a loosened chromatin structure and undergo large-scale changes which make them more liable to DNA damage (Haffner et

al., 2011). Persistent signaling by androgen and estrogen receptors requires numerous transcription factors and cofactors to interact with gene regulatory regions, leading to topoisomerase II-induced DSBs to loosen the chromatin (Ju et al., 2006; Morimoto et al., 2019). When BR signaling is activated, a similar increase in the transcription may take place, as many transcription factors are recruited to the nuclei by the action of BES1 and BZR1 to promote the signaling (Li et al., 2018). This would mean that the activation of BR signaling may trigger the accumulation of DSBs, making plants with an over-activated BR signaling more sensitive to DNA damage agents, such as what happens in *BRL3Ox* (Figure 2.19, Figure 2.20). Indeed, different topoisomerases genes such as BIN3 (BRASSINOSTEROID INSENSITIVE 3, also known as RHL3 (ROOT HAIRLESS 3) and BIN5 (also known as RHL2) are partially insensitive to BR treatments (Mittal et al., 2014; Yin et al., 2002b). Moreover, BIN5 is upregulated upon 2 h BL in the BRL3 native domain (Figure 2.17), indicating that the role of these enzymes is crucial for a normal BR signaling.

Altogether, chapter 2 of this thesis demonstrates for the first time that BRL3 has a key role in regulating the cell cycle and DNA repair upon stress. We have found that the balance between DNA repair and cell cycle progressions needs to be optimal in order to allow a proper root growth after DNA damage. A deeper study on which genes are being directly regulated by BZR1 upon DNA damage will provide insightful information in order to discern if BRL3 is actually directly inhibiting the DNA repair machinery, as some of the DNA repair

deregulated genes in the RNAseq of *brl3* with zeocin appear as putative direct targets of BZR1, but in the low-confidence data (Sun et al. 2010). Alternatively, it may be a consequence of the combination of DSBs accumulation to loosen the chromatin and allow BR signaling happening together with the acceleration of the cell cycle, which also would lead to a repression of the DNA repair mechanisms. Moreover, how BR biosynthesis is upregulated upon DNA damage is still an open question. Further experiments on this topic will reveal crucial information, as, for example, CPD and DWF4 do not seem to be directly controlled by SOG1 in its ChIP-seq (Ogita et al., 2018). In addition, it will also be fruitful to study which BR hormones (and levels) are being preferentially accumulated upon DNA damage, as BR receptors may have different affinity for different ligands. Future research in understanding the different pathways regulated by BRL3 in adverse environmental conditions will be key to produce plants adapted to different stresses without penalizing the growth, as already shown in Fàbregas et al., 2018 for drought.

5.2 Enzymatic regulation of the QC divisions

How the QC cells of arabidopsis roots decide to divide or to remain quiescent is a question that has been researched for the last 30 years (Dolan et al., 1993; van den Berg et al., 1997). Different studies permitted the discovery of transcription factors that are specifically inhibiting or promoting the divisions of the QC cells. However, little

was known about the downstream components of the network that is involved in the regulation of QC divisions. In this thesis, in chapter 3, we found thar RNR2A, a SCN specific enzyme with RNR activity is regulated by BR signaling and involved in controlling this process. The RNR2A enzyme from arabidopsis was discovered in 2006 and was found to be able to complement all the phenotypes of *tso2* mutants, indicating that it has the same RNR enzymatic activity (Wang and Liu, 2006). RNR activity is in charge of reducing the dNDPs to dNTPs, controlling the dNTP pool of the cells (Elledge et al., 1992), a key process to allow DNA repair and cell cycle progression.

Downstream components beyond BES1 and BZR1 have been found to be involved in the QC division and to have a BR-mediated regulation, such as BRAVO, WOX5 and ERF115 (Vilarassa-Blasi et al., 2014, Forzani et al., 2007, Heyman et al., 2013, Betegón-Putze et al., 2021). It is deeply reported that the lack of *bri1* makes plants that do not divide the QC (Fàbregas et al., 2013; González-García et al., 2011), but little is known about the role of BRL1 and BRL3 in this process. A little reduction in QC division mediated by BR was previously seen (Fàbregas et al., 2013), but no more information was known until now. In this thesis, in chapter 3, we found that, in specific conditions, such as upon DNA damage, BRL3 receptor plays an important role in the triggering of QC divisions (Figure 2.20). Indeed, we have linked how BR signaling, through BRL3 receptor, is actually directly controlling a specific root SCN RNR enzyme by directly controlling RNR2A transcription through the activation of BZR1

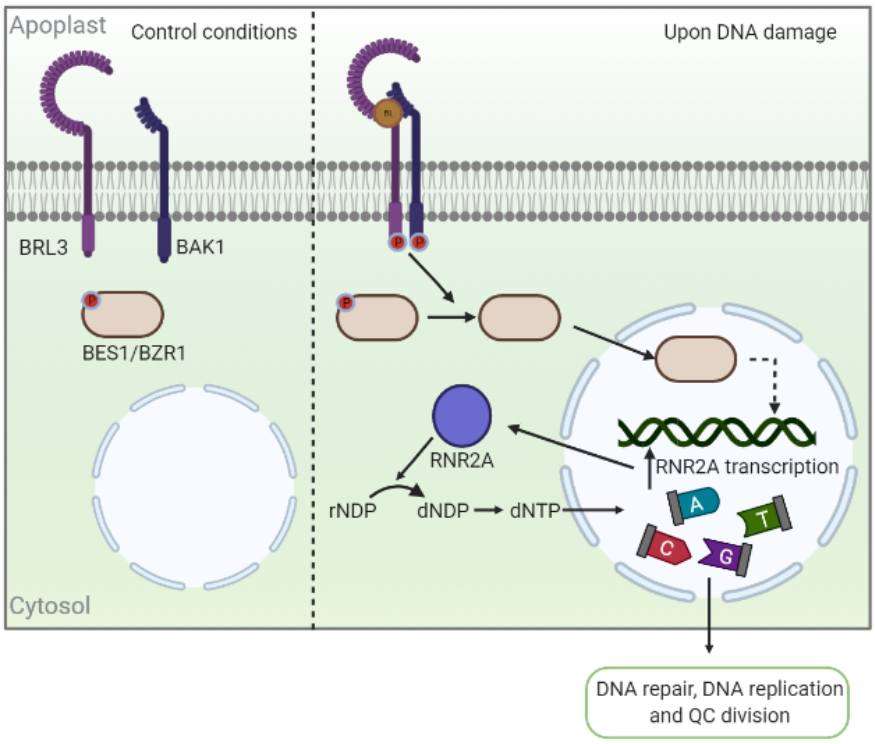


Figure 5.3 Working model of Model for RNR2A regulation and its impact on QC cells. Schematic representation of the RNR2A regulation by BRL3. Upon DNA damage conditions, the activation of BRL3 signaling through BZR1 will lead to an increase of RNR2A expression, needed for the replenishment of the dNTPs of the cell. This regulation of dNTP pool will be key to allow the DNA repair and replication of the QC cells, needed to allow the QC divisions for the replenishment of stem cells.

(Figure 3.3, Figure 5.3). To date, the role of enzymes being part of the regulation of QC divisions are just starting to be elucidated (Forzani et al., 2014), going beyond the transcription factor regulation layer. In order to promote the cell cycle progression in the QC cells during DDR, BRL3 signaling, through BZR1, would be increasing the dNTP levels of these cells, by activating the expression of the RNR2A enzyme (Figure 5.3). As the availability of dNTP is essential for the progression of the cell cycle, this can be a crucial and

specific mechanism of QC cells for controlling their divisions (Figure 3.10, Figure 5.3). The fact that root stem cells have a specific RNR enzyme apart from TSO2 indicates the complex and precise regulatory mechanism they have, which make them different from the rest of the cells conforming all root tissues.

RNR consists of two large subunits (R1) and two small subunits (R2). The R2 subunit houses the di-iron tyrosyl radical cofactor essential for the reduction of NDP to dNDP. In mammals the large subunit is encoded by the RRM1 (RIBONUCLEOTIDE REDUCTASE CATALYTIC SUBUNIT 1) gene, while for the small subunit there are two isoforms, encoded by the RRM2 and RRM2B genes. RRM1 expression is ubiquitous, while RRM2 expression is cell cycle dependent (Aye et al., 2015). In arabidopsis, the big subunit is encoded by RNR1 (Garton et al., 2007; Sauge-Merle et al., 1999) and the small subunits present three forms, TSO2, RNR2A and RNR2B (Wang and Liu, 2006). In contrast to what happens in mammals, both RNR subunits in plants have shown to be regulated in a cell cycle dependent manner, and that are regulated by the DNA response machinery (Culligan et al., 2004; Roa et al., 2009). The combination of the regulatory machinery of three genes involved in the RNR machinery in the QC, RNR1, RNR2A and TSO2 is possibly responsible of its fine-tune cell division, impacting in the SCN maintenance.

In animals, the maintenance of the pluripotent state of stem cells requires hypoxic conditions, whereas higher oxygen condition

promotes cell differentiation (Mohyeldin et al., 2010). In mammals, under these hypoxic conditions, the DNA-synthesis is almost completely inhibited, primarily because of the lack of RNR activity (Eklund et al., 2001). However, cells have a mechanism to maintain basal levels of dNTPs in hypoxic cells in order to ensure their survivals. While RRM1 and RRM2 are downregulated in this conditions RRM2B is upregulated to prevent the replication stress caused by hypoxia (Foskolou et al., 2017). In plants, it was recently discovered that hypoxic conditions are also needed to stablish the shoot apical meristem, converging with the animal stem cell evolution (Weits et al., 2019). If this hypoxic status is also conserved in the root apical meristem, RNR2A could be functioning as RRM2B in mammals, while TSO2 may be less expressed in the SCN. Moreover, BR signaling may be mediating the control of RNR activity by promoting the expression of RNR2A in order to promote the QC division when needed, acting as a mechanism to re-start the cell cycle of QC cells.

Future experiments will be key to better understand the control of dNTP in the SCN area. Radiolabeled dNTP measurements can be key to fully demonstrate that RNR2A is key to regulate the levels of this molecules in the cells it is expressed. In plants, dNTP pool measurement protocols are available (Martí et al., 2012). However, as RNR2A expression in the root is limited to a few cells (Figure 3.2), a challenging tissue-specific experiment should be planned in order to have a nice resolution an enough statistical power to find differences. Moreover, it will be interesting to further research

whether BZR1 can bind directly to RNR2A promoter, as it presents two E-boxes and one BR responsive element that could be recognized by BZR1, or if it is maybe directly controlled by other transcription factors mediated by BR signaling, such as BRAVO and/or WOX5.

In addition, experiments with hydroxyurea (HU) could be performed to better understand the role and regulation of RNR2A. HU is a chemotherapeutic agent that acts as a specific but reversible inhibitor of the R2 subunit (Cools et al., 2010). Strikingly, HU treatments in WT plants induce the triggering of QC divisions (Cruz-Ramírez et al., 2013), caused by a replication stress, which is characterized by replication fork stalling as a consequence of dNTP lack. As HU treatment is affecting both R2 subunits, RNR2A and TSO2, probably the plant is suffering this replication stress in all the dividing cells in the meristem, and this forces the QC divisions to try to survive. This seems to argue our present data, as lack of RNR2A seems to not have any effect in control conditions and to promote the QC divisions upon stress (Figure 3.10). However, in *rnr2a* mutants TSO2 will be acting normally, having only defects in the QC and not in the the rest of the meristem, then, the replication stress would be not happening. The study of *rnr2a* single mutant had allowed us to distinguish which is the specific role of RNR2A in the QC cells. Nevertheless, it will also be interesting to analyze the QC phenotypes in the *rnr2a;tso2* double mutants.

5.3 BR-mediated triggering of ROS burst

During recent years, the “ROS network” concept has emerged and gained special attention, as ROS as signaling molecule play indispensable roles in a broad range of plant biological processes. Research conducted in the last decades provided an insight into molecular mechanisms of the ROS signaling, which is interconnected with signaling pathways of other phytohormones and environmental cues. In the case of BR signaling there is a bidirectional cross-link as BR promote the triggering of ROS, and ROS is important to activate the BR signaling (Shi et al., 2015; Tian et al., 2018; XIA et al., 2014). BRI1 receptor is known to be key to promote the burst of ROS upon BL (Tian et al., 2018). However, BRL3 has been seen as a negative regulator of ROS burst. Specifically, the phosphorylation of a seven-transmembrane domain Regulator of G-protein Signaling 1 (AtRGS1) by BRL3 followed by the activation of G protein complex has been elucidated as a way to prevent an excess of ROS burst and control growth inhibition (Tunc-Ozdemir and Jones, 2017).

Our study, in chapter 4, has revealed that BRI1 directs BR-induced ROS production together with BRL3. Our results demonstrate that ROS production is induced by BR-mediated pathway driven from BRI1 and BRL3 receptors (Figure 4.4). Moreover, we proposed that BRI1 and BRL3 receptors may be using RBOHD machinery, as both receptors are forming complexes with RBOHD by a mass-spectrometry experiments (Fàbregas et al., 2013, Fàbregas et al., unpublished). RBOHD is the best characterized NADPH oxidase in

arabidopsis, and a central driving force of ROS signaling in cells upon biotic and abiotic stimuli. RBOHD is a plasma-membrane protein and it is expressed mainly in the cortex and the epidermis of the maturation zone, but also in the epidermal cells of the meristematic zone. Mutants of RBOHD also present a lack of H_2O_2 triggering upon BL (Figure 4.5). Moreover, they are also partially deficient to respond to BL in terms of QC division (Figure 4.7). It is known that H_2O_2 triggering is important for the promotion of QC divisions, as the oxidated forms of BES1 and BZR1 are needed to activate this process (Tian et al., 2018). Mutants lacking BRI1, BRL3 or RBOHD showed less QC divisions upon BL treatments (Figure 4.7). This is probably due to an inefficient H_2O_2 accumulation, that would lead to a not oxidated the BES1 and the BZR1 proteins (Figure 5.4). Interestingly, the BRI1 and the BRL3 receptors are located in the plasma-membrane, as the RBOHD protein, and their cytoplasmic kinase domains have the capacity to phosphorylate. These BR receptors could directly phosphorylate the conserved residues of the C or N-terminal regions of RBOHD to activate this protein, as they share subcellular localization, causing the ROS burst. In summary, these results point to a crosstalk between BRs and ROS. Further, we suggest that the accumulation of ROS could induce the oxidation of the BRs' signaling pathway main transcription factors, BES1 and BZR1; which will subsequently enhance their transcriptional activity and regulate BR-mediated biological processes (Figure 5.4). However, more biochemical studies, such as yeast-two-hybrid, FRET-FLIM (Förster resonance energy transfer by fluorescence lifetime imaging) and phosphorylation assays, will be necessary in

the future to confirm our working model and to fully elucidate the molecular mechanism of this process.

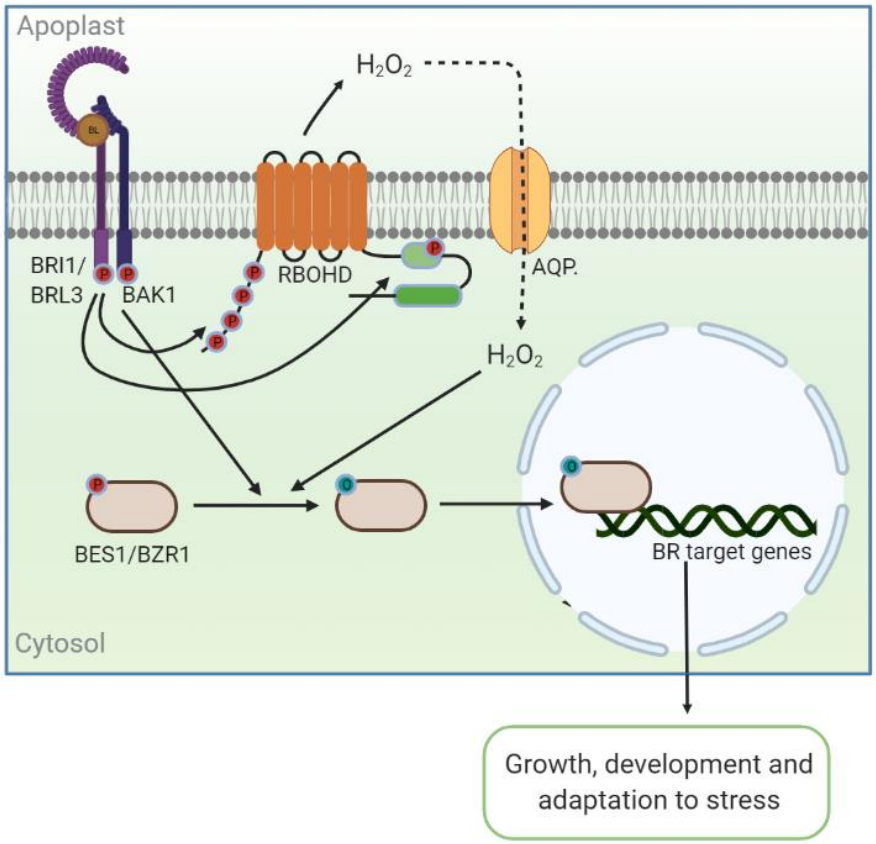


Figure 5.4 Working model for ROS triggering by BR signaling. Schematic representation of how BRI1 and BRL3 may activate RBOHD by direct phosphorylation to trigger H_2O_2 production under specific circumstances in which BR signaling is activated. Following the triggering of H_2O_2 accumulation, BES1 and BZR1 transcription factors will be dephosphorylated by the canonical BR pathway and oxidated by the accumulation of H_2O_2. This will finally end with the promotion of BR-responsive genes.

Regarding to the impact of ROS triggering mediated by BR, we focused in the development of root hairs. Root hairs are tubular-shaped extensions of epidermal cells located in the differentiation zone, and their presence greatly increases plant's ability to absorb water and mineral nutrients, which are essential for a proper growth

and development (Shibata and Sugimoto, 2019). It has been well established that root hair development is strictly regulated by intercellular and intracellular signal communications, being auxin- and ethylene-mediated pathways crucial in promoting root hair length (Shibata and Sugimoto, 2019). Our RNAseq analysis of *brl3* mutant in control conditions revealed that RLS4 (ROOT HAIR DEFECTIVE SIXLIKE 4) expression is down-regulated in *brl3* mutant roots. RSL4 is a central growth regulator that is activated by auxin, which in turns promotes ROS production by upregulating the expression of genes coding for NADPH oxidases (RBOHC/J) as well as for class III peroxidases (Mangano et al., 2017). This indicates that ROS also play an important role in root hair development.

In our study, no significant changes of root hair length in *brl3-2* mutants were observed comparing to WT at control conditions (Figure 4.7). However, the elongation of root hairs was slightly decreased in *brl3-2* compared to the WT when treated with BL (Figure 4.7). These results, in combination with previous findings, may suggest that (*i*) the small decrease of root hair elongation compared to the WT could be caused by the down-regulated RSL4 expression in *brl3-2* mutant, (*ii*) BRI1 follows another independent pathway to regulate root hair elongation process, as it has an antagonistic effect to BRL3. Consistently, *BRL3Ox* exhibited a prominent increased in root hair length upon BL treatment (Figure 4.8). These results corroborate what we have previously suggested; both BRL3-mediated ROS production, as well as RSL4-induced content could be promoting root hair growth, resulting in a higher

amount of ROS in *BRL3Ox* and consequently larger root hairs, which is an important trait for nutrient and water uptake, and may be important for adaptation to different adverse conditions, such as drought.

RBOHD has never been related to root hair elongation process. It is known to be important for (*i*) the closing of stomata (Kwak et al., 2003) (*ii*) for defense against pathogenic bacteria and fungi (Morales et al., 2016), (*iii*) to control lateral root development (Li et al., 2014), (*iv*) and to regulate many abiotic stresses, including temperature and salt among others (Ben Rejeb et al., 2015; Wang et al., 2014). It would be interesting to further study if the BRL3 role t in root hair elongation is dependent on RBOHD activity or if it is caused by other mechanisms, as we also found that BRL3 is inhibiting RBOHE (Figure 4.3), which is known to produce H_2O_2 in response to Ca^{2+} (Kaya et al., 2019). Moreover, as BRL3 is starting to be seen as a regulator of stresses it would also be very insightful to check if, BRL3, together with RBOHD, can regulate any of the biotic and abiotic stress responses in which RBOHD is known to be important.

In this thesis, in chapter 4, the specific role of BRL3 receptor in BR-mediated H_2O_2 production was established and a mechanism of how it does this by interacting with RBOHD is proposed. Moreover, we also studied the implication of this BR-mediated H_2O_2 production in arabidopsis developmental processes, such as in the triggering of QC divisions and in root hair elongation. However, additional studies are required to establish the complex and function of BR receptors with

RBOHD, and to unravel the molecular link between BR and H_2O_2-mediated polar root hair growth. As BRs play pivotal roles in cell growth, development and responses to adverse environments, once this crosslink is established, we could use the generated knowledge for manipulation to improve plant agronomic traits.

Conclusions

Conclusions

1. Brassinosteroids are required for the triggering of QC divisions upon DNA damage.

i. Local steroid levels are the main limiting factor for QC division, which are increased upon DNA damage conditions.

ii. QC divisions can be cell-autonomously promoted by the action of active BES1 transcription factor.

iii. BR receptors in the root SCN are required to perceive the steroid paracrine signal from neighbor damaged cells.

2. BRL3 regulation and signaling upon DNA damage is important to control the DNA repair and cell cycle progression in the root apical meristem. This is important for the adapted growth and development in adverse situations.

i. BRL3 is upregulated after a DNA damage by the direct union of the master regulator of DDR, SOG1, to its promoter.

ii. The presence of BRL3 is needed during the DDR to dephosphorylate and activate the downstream transcription factor BZR1.

iii. BRL3 signaling inhibits the DNA repair machinery, specifically, the homologous recombination one.

iv. BR signaling, through BRL3, accelerates the progression of the cell cycle.

v. A balance between DNA repair and cell cycle progression is crucial to ensure the plant survival upon a DNA damage.

3. Brassinosteroid receptor BRL3 pathway and downstream BZR1 effector control DNA synthesis of root stem cells in response to DNA-damage regulating a QC-specific RNR2A enzyme.

i. RNR2A enzyme is specifically located in the QC and columella cells in the root of arabidopsis.

ii. RNR2A can be upregulated by BR treatments and by DNA damage conditions to produce DNA in newborn cells after damage.

iii. The regulation of RNR2A transcription is controlled by BRL3 and BZR1 signaling pathway.

iv. The activity of RNR2A is important for a proper cell cycle in the root meristem.

v. RNR2A and BRL3 are key proteins to trigger the QC divisions both upon BR treatments and upon DNA damage conditions.

4. BRI1 and BRL3 receptors are key components to trigger the H_2O_2 accumulation mediated by BR, together with RBOHD enzyme. This response is important to modulate different developmental aspects, as QC divisions and root hair elongation.

i. BRL3 is involved in the regulation of redox activity in the root.

ii. BRI1 and BRL3 receptors are key components for the BR-mediated H_2O_2 accumulation in the root.

iii. RBOHD enzyme is also important for the BR-mediated H_2O_2 accumulation in the root and for the normal response to BL in terms of development aspects.

iv. BRI1 and BRL3 receptors are forming complexes with RBOHD enzyme.

v. A proper H_2O_2 accumulation in response to BR is important for QC divisions and root hair elongation processes.

Material and Methods

Materials and Methods

Plant material and growth conditions

All lines used in this study, along with their references are listed in Supplementary Table 7.1. We used *Arabidopsis thaliana (L.)* Heyhn, ecotype Columbia-0 (Col-0) as the control background line.

Seeds were surface sterilized using 35% bleach during 5 minutes, and subsequently washed five times with distilled sterile water. Seeds were vernalized at 4ºC in the dark for 48 h before sowing to synchronize germination. Then the seeds were sowed under sterility conditions in square plates (120 x 120 mm) containing half-strength Murashige and Skoog (MS) medium with vitamins but no sucrose supplements (0.5XMS-) and 0.8% plant agar with adjusted pH to 5.7. The plates with the seeds were sealed with Micropore tape (https://www.3m.com) to allow gas exchange. The plates with arabidopsis seeds were finally grown vertically in *in vitro* chambers with long day conditions (LD, cycles of 16 h light and 8 h dark) at 22ºC and 60% relative humidity. After 10 days of growth, seedlings were transferred to soil-containing pots with a mixture of soil, perlite and vermiculite with a proportion of 8:1:1 respectively and grown until desired age in growing chambers at the greenhouse with LD conditions and 22ºC and 60% relative humidity.

Table 7.1 Arabidopsis plant lines used in this thesis

Line name	Description	Reference	Chapter
Col-0 (WT)	Wild-type, ecotype Columbia-0	-	Chapter 2,3,4
bri1-116	BRI1 knockout allele, Q583STOP	Li and Chory, 1997	Chapter 2,4
bri1-301	BRI1 knockdown allele, 989G to 989I	Xu et al., 2008	Chapter 2
bzr1-D	BZR1 point mutation, P223L, gain of function	Wang et al., 2002	Chapter 3
brl1	T-DNA insertion mutant, SALK_005982	Kang et al., 2017	Chapter 2,4
brl3-1	T-DNA insertion mutant, SALK_031017	Caño-Delgado et al., 2004	Chapter 2,4
brl3-2	T-DNA insertion mutant, SALK_ 006024	Kang et al., 2017	Chapter 2,3,4
pBES1:BES1-GFP	BES1 protein reporter line	Yin et al., 2002	Chapter 2
pBRI1:BRI1-YFP	BRI1 protein reporter line	Geldner et al., 2007	Chapter 2
pBRL1:BRL1-GFP	BRL1 protein reporter line	Fàbregas et al., 2013	Chapter 2
pBRL3:BRL3-YFP	BRL3 protein reporter line	Fàbregas et al., 2013	Chapter 2,3
pBRL3:nlsGFP	BRL3 transcript reporter line with nuclear localization signaling	This work	Chapter 2,3
pBRL3(1796):GFP /GUS	BRL3 transcript reporter line (promoter length 1719 bp)	Salazar-Henao et al., 2016	Chapter 2
pBRL3(755):GFP/ GUS	BRL3 transcript reporter line (promoter length 755 bp)	Salazar-Henao et al., 2016	Chapter 2
pBZR1:BZR1-YFP	BZR1 protein reporter line	Wang et al., 2015	Chapter 2
pCPD:nlsGFP	CPD transcript reporter line with nuclear localization signal	Vukašinović et al., 2021	Chapter 2
pDWF4:nlsGFP	DWF4 transcript reporter line with nuclear localization signal	Vukašinović et al., 2021	Chapter 2
pRNR2A:GFP	RNR2A transcript reporter line	This work	Chapter 3

Line name	Description	Reference	Chapter
pRNR2A:RNR2A-GFP	RNR2A protein reporter line	This work	Chapter 3
pSOG1:SOG1-Myc	SOG1 protein reporter line	Yoshiyama et al., 2013	Chapter 2
pWOX5:*bes1-D*-GFP	Active BES1 overexpression in the QC	Vilarrasa-Blasi et al., 2014	Chapter 2
pWOX5:BRI1-amiR	QC-specific knockout of BRI1	Lozano-Elena et al., 2018	Chapter 2
pWOX5:BRI1-YFP	BRI1 overexpression in the QC	This work	Chapter 2
rbohd	T-DNA insertion mutant, SALK_109396	Gouhier-Darimont et al., 2013	Chapter 4
rnr2a	T-DNA insertion mutant, SALK_ 150365	This work	Chapter 3
sog1-1	SOG1 knockout allele, 155G to 155R	Preuss and Britt, 2003	Chapter 2
35S:BRL3-GFP (*BRL3Ox*)	BRL3 overexpression	Fàbregas et al., 2013	Chapter 2,3,4

Plant physiology

Pharmacological treatments

For Brassinolide (BL) treatment, BL ($C_{28}H_{48}O_6$; Wako, Osaka, Japan) previously dissolved in EtOH was added to the media adjusted to different final concentrations. In general, for physiological and imaging experiments BL was used either at 0.04, 1 or 4 nM in solid media either as a continuous treatment since the plating or just for some hours transferring the seedlings to solid media containing the desired BL concentration. In the case of the microarray assay 6-day-old seedlings were treated with 10 nM BL in liquid media for 2 h.

For DNA damage treatment, seedlings were transferred to vertical plates supplemented either with 0.6 µg/mL of bleomycin (Calbiochem) or with 2 µg/mL of zeocin (Duchefa) 4, 5 or 6 days after sowing. For recovery, plants were transferred back to control medium after 24 h of growth in bleomycin or zeocin containing medium, and grown for either 12, 24 or 72 h. In the case of the RNAseq assay 6-day-old seedlings were treated with 10 µM of zeocin in liquid media for 2 h.

Root length and root hair measurements

For root length measurements, images of seedlings were taken with a Nikon D7000 camera and root length measurement was performed with MyROOT software (Betegón-Putze et al., 2019) and ImageJ software (http://imagej.nih.gov/ij/).

For root hair measurements, images of seedlings were taken with a stereomicroscope and root hair length measurement was performed with ImageJ software (http://imagej.nih.gov/ij/).

QC division and CSC differentiation quantification

For QC division and CSC differentiation analysis, 6-day-old seedlings were fixed, clarified and counterstained using modified Pseudo Schiff-propidium iodide (mPS-PI) staining (Truernit et al., 2008). Briefly, seedlings were fixed overnight at 4ºC in 10% acid acetic and 50% methanol. Then seedlings were washed twice with

distilled water and incubated for 30 min in a 1% periodic acid solution. Next, seedlings were washed again twice with distilled water and stained during 1-2h with a mix of Schiff reagent (25 mg/ml of sodium bisulfite and 1'5% (v/v) pure HCl) with PI at a final concentration of 1ug/ml. Finally, seedlings were mounted onto a microscopy slide with a drop of Hoyer's solution (30 g gum arabic, 200 g chloral hydrate, 20 g glycerol and 50 mL water).

QC division was also quantified in vivo in DNA damage assays and, in this case, seedlings were just stained with propidium iodide as explained below. Images were obtained using a FV 1000 confocal microscope (Olympus, Tokyo, Japan) with a 60x water-submerged objective, exciting PI with a 488nm laser line and detecting it with a band-pass 570-670 nm filter.

The scoring of the QC division was as follow: QC divided (D) if any QC cell showed a division plane; QC non-divided (ND) if none of the QC cells showed a division plane. The scoring of the CSC differentiation was as follow: 0 layers when all columella layers presented starch accumulation, having no stem cells, 1 layer when only one cell layer of the columella did not present starch accumulation, 2 layers when at least 2 cell layers of the columella did not present starch accumulation.

Imaging

Propidium Iodide staining

Propidium Iodide for in vivo visualization was performed by staining the 6-day-old seedlings with 10 µg/ml PI for 1-2 minutes and were visualized after excitation by a Kr/Ar 488 nm laser line using a FV 1000 confocal microscope (Olympus, Tokyo, Japan). PI were detected with a band-pass 570-670 nm filter. Together with PI, in vivo detection of GFP and YFP were done with band-pass 570-670 nm, or 570-670 nm filters, respectively. Images were processed with ImageJ software (http://imagej.nih.gov/ij/).

GUS staining

For GUS detection, 6-day-old seedlings were immersed in ice-cold 90% (v/v) acetone, incubated for 20 min on ice, rinsed twice in dH2O, infiltrated with GUS [100 mM sodium phosphate buffer (pH 7.2), 10mM sodium EDTA, 0.1% Triton X-100, 1 mg ml–1 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Xgluc; Duchefa, Haarlem, The Netherlands), 10 mM potassium ferrocyanide and potassium ferricyanide] and incubated at 37 °C for 15 h in the dark. Samples were rinsed three times in dH2O and treated with 70% ethanol. Stained roots were visualized with an AxioPhot (Zeiss, Jena, Germany) microscope. Images were processed with ImageJ software (http://imagej.nih.gov/ij/).

TUNEL assay

Seedlings were fixed using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) during 8h. After samples were washed with PBS three times and next, they were stained with Click-iT™ Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor™ 488 dye (ThermoFisher) for 1 hour at 37ºC according to manufacturer's instructions. Then, seedlings were washed again with PBS five times and transferred to a slide using Vectashield Antifade Mounting. Consecutively, samples were visualized on a FV 1000 confocal microscope (Olympus, Tokyo, Japan). Alexa Fluor™ 488 was excited with a 488 nm laser line and detected with a band-pass 570-670 nm filter.

EdU staining

For evaluating EdU staining, we used the Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher). Six days after sowing, and after being treated for 24 h with zeocin, seedlings were transferred to vertical plates supplemented with 10 µg/ml EdU. After 12 h, seedlings were fixed in a solution containing 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 in 1× PBS for 1 h in a vacuum. After fixation, the seedlings were washed twice with 3% (w/v) BSA in 1× PBS, and subsequently incubated in the Click-iT reaction cocktail (as described in the protocol of Invitrogen EdU Click-iT Reaction Imaging Kit) for 1h in the dark. For counterstaining, seedlings were washed twice with 3% BSA in 1× PBS and incubated for 30 min with 1 µg/ml DAPI in 1× PBS in the

dark. Finally, the seedlings were washed a final time in 3% BSA in 1× PBS. Consecutively, samples were visualized on a FV 1000 confocal microscope (Olympus, Tokyo, Japan). Alexa Fluor™ 488 was excited with a 488 nm laser line and detected with a band-pass 570-670 nm filter.

DCFD-H2A assay

6-day-old seedlings were incubated in the dark with 25 μM 2,7-dichlorofluorescein diacetate (H_2DCF-DA, Sigma) for 30 min in dark for hydrogen peroxide (H_2O_2) detection. H_2DCF-DA is a common marker of oxidants and has an increased sensitivity for H_2O_2. After the incubation the seedlings were washed three consecutive times with double-distilled water. Then, the seedlings were mounted in slides with a drop of water and kept in dark. Finally, the seedlings were observed on a FV 1000 confocal microscope (Olympus, Tokyo, Japan) no later than 30 min after being mounted. H_2DCF-DA was excited with and detected with a band-pass.

Fluorescence quantification

For fluorescence quantifications, the mean pixels/area of fluorescence in the green channel, to quantify either GFP, YFP, Alexa488 or DCF-DA were quantified with ImageJ (http://imagej.nih.gov/ij/), either on complete images or by measuring only the region of interest (ROI).

Methods in molecular biology

DNA extraction and genotyping

For genotyping plants, DNA was extracted from arabidopsis leaves according to the following protocol: Frozen tissue was grinded in the Tissuelyser (Qiagen) at frequency of 30 s-1 for 30s. Then, 400 µl of extraction buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0 and 2% (v/v) SDS) were added and agitated with a vortex for 5 seconds. Samples were centrifuged for 5 min at 13000 rpm. 300 µl of the supernatant were transferred to a new tube and 300 µl of isopropanol were added to precipitate the DNA. Tubes were inverted 5 times and Samples were incubated 5 min and centrifuged 10 min at 13000 rpm. The supernatant was discarded and 500 µl of 70% ethanol were added to clean the pellet. Samples were centrifuged 10 min at 13000 rpm and the supernatant was discarded. Tubes were kept overnight to dry. DNA was resuspended in 50 µl of sterile-distilled water. DNA concentration and purity were then assessed with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

The different mutants described in this thesis were genotyped to select the homozygous plants. In the case of T-DNA insertion lines from SALK, primers were obtained from SALK, using the combination of LP+RP (left primer + right primer) to amplify the WT sequence, and the combination of LBB1.3+RP to amplify the knock-out sequence. LBB1.3 primer anneals with a common sequence of the T-DNA and is commonly used for the genotyping of these lines.

For *bri1-116*, which has a point mutation, after the PCR an enzymatic digestion was performed to differentiate between the WT (which will be digested) and the mutant (which will not be digested). In the case of *bzr1-d* mutants, homozygous plants were selected just by adult morphological traits, having more leaves and delayed flowering when compared to the WT (Wang et al., 2002). In the case of *bri1-301* mutants, homozygous plants were also selected by morphological traits, as they present dwarfism (Xu et al., 2008). *sog1* mutants were genotyped by Dr. Naoki Takahashi in Dr. Masaaki Umeda laboratory.

The sequences of the primers used for genotyping are described in Table 7.2.

Table 7.2 Primers used for genotyping.

Primer name	Sequence	Comments
bri1-116_fw	CATCGGAACCATTGTTATCAAACGTC	Genotype *bri1-116*, PCR followed by MseI digestion
bri1-116_rv	CATCGGACCCATTGTTATCAA	Genotype *bri1-116*, PCR followed by MseI digestion
brl1_LP	ATATGGATGTTGCCGAATCTG	Genotype *brl1*, LP
brl1_RP	CTGTAAAGCGCCATGACTAGC	Genotype *brl1*, RP
brl3-1_LP	GAAATCCCTGTAGGAATCGGAAAGCTTG	Genotype *brl3-1*, LP
brl3-1_RP	TTTAGGGTGAGCATGAGATCTCGTGGGC	Genotype *brl3-1*, RP
brl3-2_LP	CCAGTGAACTCGTTTGAGCTC	Genotype *brl3-2*, LP
brl3-2_RP	TTTATCGAACACTTTGTGGGC	Genotype *brl3-2*, RP
JMRB	GCTCATGATCAGATTGTCGTTTCCCGCCTT	Genotype T-DNA insertion of *brl3-1*
LBB1.3	ATTTTGCCGATTTCGGAAC	Genotype all T-DNA insertion lines except *brl3-1*
rnr2a_LP	GCCTTGCAGACAAACTCTGTC	Genotype *rnr2a*, LP
rnr2a_RP	TTCAGGCTCGTGCTTTCTATG	Genotype *rnr2a*, RP
rbohd_LP	CGATCTGTTTCACCAATGTCC	Genotype *rbohd*, LP
rbohd_RP	TTTGATGCCAAACTCCAAGTC	Genotype *rbohd*, RP

Molecular cloning and generation of transgenic lines

For the generation of the different constructs for plant transformation, we used the Gateway system technology (Invitrogen, Karimi et al., 2002), following manufacturer's instructions.

In the case of pWOX5:BRI1-YFP transgene, the BRI1 coding sequence was amplified from cDNA and was introduced into the pDONR221 vector. Then, we used plasmids including the WOX5 promoter and the YFP (Vilarrasa-Blasi et al., 2014), and together with BRI1 were recombined into the destination vector pB7m34GW. The RNR2A CDS was amplified by PCR from cDNA and the promoter region (650 bp upstream RNR2A start codon, corresponding to the whole intergenic region) was amplified by PCR from genomic DNA. These fragments were introduced into the pDONR221 and pDONR-P4P1r vectors (Invitrogen) respectively, through a BP reaction (Gateway, Invitrogen). For the generation of pRNR2A:RNR2A-GFP and pRNR2A:GFP-GUS, the entry clones were subcloned into binary vectors R4pGWB604 and R4L1pGWB632 (Tsuyoshi Nakagawa, Shimane, Japan) through a LR reaction (Gateway, Invitrogen).

The constructs were transferred to Agrobacterium tumefaciens strain GV3101 by electroporation. Transgenic arabidopsis lines were generated by Agrobacterium-mediated transformation (Zhang et al., 2006) and homozygous transgenic T3 lines carrying a single insertion were used.

Table 7.3 Primers used for molecular cloning.

Primer name	Sequence	Comments
BRI1_fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGAAGACTTTTTCAAGCTTCTTTCTC	Cloning the coding sequence of BRI1 in pDONR221
BRI1_rv	GGGGACCACTTTGTACAAGAAAGCTGGTATAATTTTCCTTCAGGAACTTCTTTTATACTC	Cloning the coding sequence of BRI1 in pDONR221
RNR2A_fw	GGGGACAACTTTGTATAGAAAAGTTGTCTTGTTTTCGCCGAACC	Cloning the coding sequence of RNR2A in pDONR221
RNR2A_rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAAGTCCTCTTCAGTCGTGAATT	Cloning the coding sequence of RNR2A in pDONR211
pRNR2A_fw	GGGGACTGCTTTTTTGTACAAACTTGTATCTCGAATTCGTCTCTTA	Cloning the promoter of RNR2A in pDONR-P4P1r
pRNR2A_rv	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTATGGGTTCGCTTAAGGAAGG	Cloning the promoter of RNR2A in pDONR-P4P1r

RNA extraction

Sample collection and RNA extraction for real time qPCRs, microarrays and RNAseqs was done as follow: Root tips of 6-day old seedlings was collected with sterile razor blades and rapidly flash-frozen in liquid nitrogen. Frozen samples were grinded in the in TissueLyser (Quiagen) at frequency of 30 s-1 for 30 s, and then kept in liquid nitrogen until the RNA extraction. RNA was extracted with the Maxwell RSC Plant RNA Kit (Promega) using the Maxwell RSC instrument (Promega) according to the manufacturer's recommendations. Final elution was done in 40 µl of RNAse-free

water. Concentrations and purity were checked using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Real time quantitative PCRs

Real time quantitative PCRs (qPCRs) experiments of BRL3 expression presented in chapter 2 were performed by Dr. Naoki Takahashi in Dr. Masaaki Umeda laboratory.

For RT-qPCRs cDNA was obtained from RNA samples by using the NZY First-Strand cDNA Synthesis Kit (NZYtech) according to the manufacturer's recommendations. RT-qPCR amplifications were performed from 10 ng of cDNA using SYBR Green I master mix (Roche) in 96-well plates according to the manufacturer's recommendations. The RT-qPCR was performed on a LightCycler 480 System (Roche). Three different biological replicates were performed for each region of interest. ACTIN2 (AT3G18780) was used as housekeeping gene for relativizing expression, according to the formula:

$$\text{Relative expression} = 2^{(\text{Cp-housekeeping} - \text{Cp-transcript})}$$

Where Cp (Crossing point) is the double derivative of the logistic function best fitted to the amplification curve (fluorescence vs. cycles).

Primers used for real time qPCR are described in Table 7.4.

Table 7.4 Primers used for real-time qPCR.

Primer name	Sequence	Comments
BRL3_fw	CCAACTGTAGTAATCTGCGAGTTC	Amplification of BRL3 cDNA
BRL3_rv	CATTTACCAAGCTCTACAGGGACAG	Amplification of BRL3 cDNA
ACT2_fw	CTGGATCGGTGGTTCCATTC	Amplification of ACT2 cDNA, housekeeping
ACT2_rv	CCTGGACCTGCCTCATCATAC	Amplification of ACT2 cDNA, housekeeeping

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments presented in chapter 2 were performed by Dr. Naoki Takahashi in Dr. Masaaki Umeda laboratory.

1-2 mg of 10-day-old seedlings were collected. The experiments were performed as explained elsewhere (Gendrel et al., 2005) with minor modifications. pSOG1:SOG1-MYC seeds were germinated in 100 mL liquid MS medium and cultured under continuous light at 23°C with gentle shaking (50 rpm). After a 14-day culture period, zeocin was added to the medium giving a final concentration of 15 μM, and the seedlings were cultured for 2 h. Chromatin bound to the SOG1-Myc fusion protein was precipitated with an anti-Myc antibody (clone 4A6, Millipore).

Detection of PCR products was performed on a LightCycler 480 System (Roche) using SYBR Green I master mix (Roche) in 398-

well plates according to the manufacturer's recommendations. It was performed on Three different biological replicates were performed for each region of interest.

Primers used for ChIP-PCR are described in Table 7.5.

Table 7.5 Primers used for ChIP-PCR

Primer name	Sequence	Comments
ACT2_fw_ChIP	ACTCGTTTCGCTTTCCTTAGTGTTAGCT	Amplification of UBC30, negative control
ACT2_rv_ChIP	AGCGAACGGATCTAGAGACTCACCTTG	Amplification of UBC30, negative control
BRL3_1fw	GCTGTTGTCACGTCTGTTGCATTC	Amplification of BRL3, region 1
BRL3_1rv	GCATGAAACGTAAGTGTTATCCTG	Amplification of BRL3, region 1
BRL3_2fw	CTAAAGCTGTCACGAGAAAGCCAG	Amplification of BRL3, region 2
BRL3_2rv	GGAAGCGTGTTTAGTACGACCACTAC	Amplification of BRL3, region 2
BRL3_3fw	CCAACTGTAGTAATCTGCGAGTTC	Amplification of BRL3, region 3, negative control
BRL3_3rv	CATTTACCAAGCTCTACAGGGACAG	Amplification of BRL3, region 3, negative control

Bioinformatics

RNAseqs analysis

RNAseq raw data analysis was performed in collaboration with Dr. Fidel Lozano-Elena in Dr. Ana I. Caño-Delgado laboratory.

For the RNAseq described in chapter 2 and 3 6-day old roots of WT and *brl3-2* mutants were grown in vertical plates and transferred to liquid MS 0.5- media supplemented with 10 µM zeocin for 2h. Two biological replicates were performed. RNA from the root tips was extracted as explained above. Poly-A mRNA libraries were prepared using "TruSeq Stranded mRNA Sample Prep Kit" (Illumina) from 1-2 ug of good quality RNA (R.I.N. > 7). Further purification steps were performed with 0.81X Agencourt AMPure XP beads. Final RNA libraries were quantified with Qubit 2.0 Fluorometer (Thermo Fischer). Libraries were then processed with Illumina cBot for cluster generation on flowcell and sequenced on paired-end (2x75 bp, 45M reads per sample) at the multiplexing level requested on HiSeq2500 (Illumina). The CASAVA v1.8.2 of Illumina pipeline was used to process raw data and de-multiplexing. Raw reads were quality checked with FastQC software (v0.11.2) and trimmed 9 bp at 3' and quality filtered to a minimum average score of 25 minimum score quality of 25 (minium read length to keep of 60 bp) using Trimmomatic software (v0.38). Filtered reads were aligned to TAIR10 genome using HISAT2 aligner (v2.1). Features were quantified at gene level using Araport11 annotation file and FeatureCounts software (version 1.6.2). Raw counts were then normalized using TMM method and low-counts genes filtered-out.

For differential expression analysis, samples were TMM-normalized and statistical values calculated with the "EdgeR" package in R. Results were filtered for adjusted p-value (FDR) < 0.05 and FC > |2| in the pairwise comparisons. For the evaluation of differential

response to zeocin between WT and *brl3-2* roots, a lineal model accounting for the interaction genotype and drought was constructed with the "EdgeR" package (v4.1.1). The interaction term was evaluated. A gene was considered to be affected by the interaction if p-value (uncorrected) < 0.0025. Heatmaps and networks were performed in R.

GO enrichment analysis was performed in R using "clusterProfiler" (v4.0.5) package based on arabidopsis GO annotations from TAIR available in Bioconductor ("org.At.tair.db" v3.13.0), using all genes with GO annotations as background set.

Microarray analysis

Microarray and FACS experiments described in chapter 2 and 3 were performed by Dr. Mary-Paz González-García in Dr. Ana I. Caño-Delgado laboratory. Microarray raw data analysis was performed in collaboration with Dr. Fidel Lozano-Elena in Dr. Ana I. Caño-Delgado laboratory.

As a summary, 6-day-old roots expressing pBRL3:nls GFP marker were grown in vertical plates and transferred to liquid MS 0.5- media supplemented with 10 nM BL for 2 h. Two biological replicates were performed. Protoplasts of these roots were sorted by FACS to select GFP positive cells as described elsewhere (Vilarrasa-Blasi et al., 2014), and RNA from sorted cells was converted to cDNA and

hybridized to the Affymetrix ATH1-121501 GeneChip as described elsewhere (Birnbaum et al., 2005).

Raw intensities were read from .cel files. Intensities files were quality-checked and then converted to an expression dataset by correcting the background with Robust Multi-Array average expression measure (RMA, affy version >1.1) and normalized using quantile normalization. These steps were performed in R (v4.1.1) using "affy" package (v1.70.0) and its wrapper function *rma()*. Microarray annotation was retrieved from Bioconductor (ath1121501.db) and gene names and descriptions were retrieved from Araport11 annotation file. Differential expression was calculated by fitting to lineal models and running empirical Bayes method implemented in "limma" package (v3.48.3). GO enrichment analysis of microarrary DEGs was performed similarly as described above (RNAseq).

Heatmaps and networks were performed in R. Venn diagram presented in chapter 3 was created with InteractiVenn tools (Heberle et al., 2015, http://www.interactivenn.net/).

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Resume and Publications

AINOA PLANAS RIVEROLA

Email: a.planasriverola@gmail.es || Phone: +34 639927093
Twitter: @Ainoa_PR
Nationality: Spanish || Date of Birth: 01/07/1993

ACADEMIC TRAINING

April 2017 - currently: PhD in Genetics (Autonomous University of Barcelona) at Center for Research in Agricultural Genomics (CRAG). Severo Ochoa fellowship.

Thesis supervised by Dr. Ana I. Caño-Delgado. "Brassinosteroid-mediated root stem cell divisions upon stress".

Sept 2015 - July 2016: Master in Advanced Genetics. Autonomous University of Barcelona.

Master thesis supervised by Dr. Ana I. Caño-Delgado at CRAG. "Brassinosteroid regulation of stem cell function in the Arabidopsis primary root".

Sept 2011 - June 2015: Genetics degree. Autonomous University of Barcelona.

Undergrad project supervised by Dr. Eva Colàs at Vall Hebron Research Institute (VHIR). "Ovarian and endometrial cancer".

RESEARCH STAYS

June 2018 – Sept 2018: Internship in Dr. Masaaki Umeda group at Nara Institute of Technology (Japan). Ministry of Education, Culture, Sports, Science and Technology-Japan grant.

SCIENTIFIC PUBLICATIONS

1. Martin A. Mecchia*, Mariano García-Hourquet*, Fidel Lozano-Elena, **Ainoa Planas-Riverola**, David Blasco-Escámez, Mar Marquès-Bueno, Santiago Mora-García*, Ana I. Caño-Delgado*. (2021) The BES1/BZR1-family transcription factor MpBES1 regulates cell division and differentiation in Marchantia polymorpha. **Current Biology.** *Equal contribution

2. Isabel Betegón-Putze*, Josep Mercadal*, Nadja Bosch, **Ainoa Planas-Riverola**, Mar Marquès-Bueno, Josep Vilarrasa-Blasi, David Frigola, Rebecca Corinna Burkart, Cristina Martínez, Yvonne Stahl, Salomé Prat, Ana Conesa, Rosangela Sozzani, Marta Ibañes* and Ana I. Caño-Delgado*. (2021) Precise transcriptional control of cellular quiescence by BRAVO/WOX5 complex in Arabidopsis roots. **Molecular Systems Biology**. *Equal contribution

3. **Ainoa Planas-Riverola**, Enara Markaide, Ana I. Caño Delgado. (2020) New conception on ROS sensing by LRR-receptor kinases. **Trends in Plant Science.**

4. **Ainoa Planas-Riverola***, Aditi Gupta*, Isabel Betegón-Putze, Nadja Bosch, Marta Ibañes and Ana I. Caño-Delgado. (2019) Brassinosteroid signaling in plant development and adaptation to stress. **Development**. *Equal contribution.

5. Irina Pavelescu, Josep Vilarrasa-Blasi, **Ainoa Planas-Riverola**, Mary-Paz González-García, Ana I. Caño-Delgado*, Marta Ibañes*. (2018) A Sizer model for cell differentiation in Arabidopsis thaliana root growth. **Molecular Systems Biology**. *Equal contribution

6. Fidel Lozano-Elena*, **Ainoa Planas-Riverola***, Josep Vilarrasa-Blasi, Rebecca Schwab, Ana I. Caño-Delgado. (2018) Paracrine brassinosteroid signaling at the stem cell niche controls cellular regeneration. **Journal of Cell Science**. *Equal contribution.

CONFERENCES AND MEETINGS

September 2021. XXXIII Argentinian meeting of Plant Physiology (RAFV2021).

November 2020. XVRBMP – XV Reunión de Biología Molecular de Plantas. Oral communication: “Precise transcriptional control of cellular quiescence by BRAVO/WOX5 complex in Arabidopsis roots”.

May 2019. Joint Meeting for Young Researchers on Frontiers in Imaging Probes and Technologies. Flash Talk: “Brassinosteroid signaling in plant development”.

May 2019. At the Forefront of Plant Research 2019.

June 2017. XXII Reunión de la Sociedad Española de Fisiología Vegetal. XV Spanish Portuguese Congress of Plant Physiology. Poster: “Paracrine Brassinosteroid signaling at the stem cell niche controls cellular regeneration upon damage”.

COURSES

December of 2021. RNAseq analysis course, on-line course at CRAG.

May of 2019. Functional live imaging of plants EMBO course at Nagoya University, Japan.

April of 2019 - Data visualization, storytelling and scientific principles of design course at CRAG.

February and March of 2019. Teamwork techniques and conflict management course at CRAG.

March of 2018 - Introduction and advanced tools in Genevestigator course at CRAG, on-line webinar by Nebion.

OUTREACH ACTIVITIES

November of 2019. Volunteer in the Mutant Plants workshop offered by CRAG to schools (kids of 10 years old).

September of 2019. Oral communication to biology students in the high school (teenagers of 16 years old). “An unknown world: molecular plant biology research”.

March of 2019. Organized the visit of Escola Canigó (kids of 9 years old) to CRAG. Oral communication and workshops.

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RESEARCH ARTICLE

SPECIAL ISSUE: PLANT CELL BIOLOGY

Paracrine brassinosteroid signaling at the stem cell niche controls cellular regeneration

Fidel Lozano-Elena[1,§], Ainoa Planas-Riverola[1,§], Josep Vilarrasa-Blasi[1,*], Rebecca Schwab[2,‡] and Ana I. Caño-Delgado[1,¶]

ABSTRACT

Stem cell regeneration is crucial for both cell turnover and tissue healing in multicellular organisms. In Arabidopsis roots, a reduced group of cells known as the quiescent center (QC) act as a cell reservoir for surrounding stem cells during both normal growth and in response to external damage. Although cells of the QC have a very low mitotic activity, plant hormones such as brassinosteroids (BRs) can promote QC divisions. Here, we used a tissue-specific strategy to investigate the spatial signaling requirements of BR-mediated QC divisions. We generated stem cell niche-specific receptor knockout lines by placing an artificial microRNA against BRI1 (BRASSINOSTEROID INSENSITIVE 1) under the control of the QC-specific promoter WOX5. Additionally, QC-specific knock-in lines for BRI1 and its downstream transcription factor BES1 (BRI1-EMS-SUPPRESOR1) were also created using the WOX5 promoter. By analyzing the roots of these lines, we show that BES1-mediated signaling cell-autonomously promotes QC divisions, that BRI1 is essential for sensing nearby inputs and triggering QC divisions and that DNA damage promotes BR-dependent paracrine signaling in the stem cell niche as a prerequisite to stem cell replenishment.

KEY WORDS: Brassinosteroid, Quiescent center, Cell division, Stem cell, DNA damage, Paracrine

INTRODUCTION

Brassinosteroids (BRs) are plant steroid hormones that were originally discovered in *Brassica napus* pollen for their ability to promote growth when exogenously applied to other vascular plants (Mitchell et al., 1970). Impaired BR biosynthesis or signaling causes reduced organ growth and abnormal development, and thereby limits plant fertility and yield (Li and Chory, 1997; Wei and Li, 2016). Despite parallels between the functions of plant and animal steroid hormones (Li and Chory, 1997; Thummel and Chory, 2002), substantial differences exist with respect to their perception and signal transduction mechanisms. Whereas animal steroid perception is mainly mediated by transcription factors inside the cell (Aranda and Pascual, 2001), plant steroids are perceived by leucine-rich repeat (LRR) receptor kinases located at the plasma membrane (Kim and Wang, 2010).

BR signaling is initiated by the direct binding of the steroid molecule to a 93 amino acid region located within the extracellular domain of the LRR receptor kinase BRI1 (BRASSINOSTEROID INSENSITIVE 1) (Hothorn et al., 2011; Kinoshita et al., 2005; Wang et al., 2001). Upon BR binding, the heterodimerization of BRI1 with BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) is enhanced, and a cytoplasmic cascade of phosphorylation and dephosphorylation events is initiated (Li and Nam, 2002; Russinova et al., 2004). These events lead to the degradation of BIN2 (BRASSINOSTEROID INSENSITIVE 2) kinase (Li and Nam, 2002; Peng et al., 2008), and a consequential increase in the dephosphorylated forms of the BZR1 (BRASSINAZOLE RESISTANCE 1) (Wang et al., 2002) and BES1 (BRI1-EMS-SUPRESSOR 1) (Yin et al., 2002) transcription factors. Dephosphorylated BZR1 and BES1 are translocated into the nucleus where they modulate the transcription of thousands of genes by directly interacting with DNA and other transcription factors (He et al., 2002). In fact, BZR1 and BES1 are known to bind specific DNA sequences: the BR-response element (BRRE, CGTGC/TG) and E-boxes (CANNTG) (He et al., 2005; Sun et al., 2010; Yu et al., 2011). Furthermore, recent work has revealed that these transcription factors are subjected to post-transcriptional regulation in response to external stimuli such as light (Kim et al., 2014) and environmental stress (Nolan et al., 2017). In this way, BR-mediated transcriptional responses are also controlled by an additional regulatory layer.

In addition to BRI1, Arabidopsis contains three BRI1-like (BRL) receptor kinase homologues. Interestingly, however, only BRL1 and BRL3 (BRI1-LIKE 1 and 3) are functional BR receptors capable of binding the hormone (Cano-Delgado et al., 2004). Although BRI1 is present in the majority of plant cells (Friedrichsen and Chory, 2001), the BRL1 and BRL3 receptors are enriched in vascular tissues and the stem cell niche (Cano-Delgado et al., 2004; Fàbregas et al., 2013; Salazar-Henao et al., 2016).

By providing a continuous supply of precursor cells, stem cells are primarily involved in sustaining growth and replacing damaged tissues (Sablowski, 2004). Root stem cells, also known as initials, are located at the root apex and surround the quiescent center (QC) (Dolan et al., 1993) (Fig. 1A,B). The QC, which comprises a small group of cells with very low mitotic activity, not only acts as a cell reservoir for the surrounding actively dividing stem cells (Scheres, 2007; Dolan et al., 1993), but is also responsible for maintaining the stem cells in their undifferentiated state (Sabatini et al., 2003; van den Berg et al., 1997). However, upon cellular damage, the QC loses its quiescence and enters into a state of cell division to enable stem cell replenishment (Cruz-Ramírez et al., 2013; Heyman et al., 2013; Vilarrasa-Blasi et al., 2014).

Hormonal stimulation also plays an important role in governing cell division in the QC (Gonzalez-Garcia et al., 2011; Heyman et al.,

[1]Department of Molecular Genetics, Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Barcelona E-08193, Spain. [2]Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA. [§]These authors contributed equally to this work *Present address: Carnegie Institution for Science, Department of Plant Biology, 260 Panama St. Stanford, CA 94305, USA. [‡]Present address: Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany.

[¶]Author for correspondence (ana.cano@cragenomica.es)

A.I.C-D., 0000-0002-8071-6724

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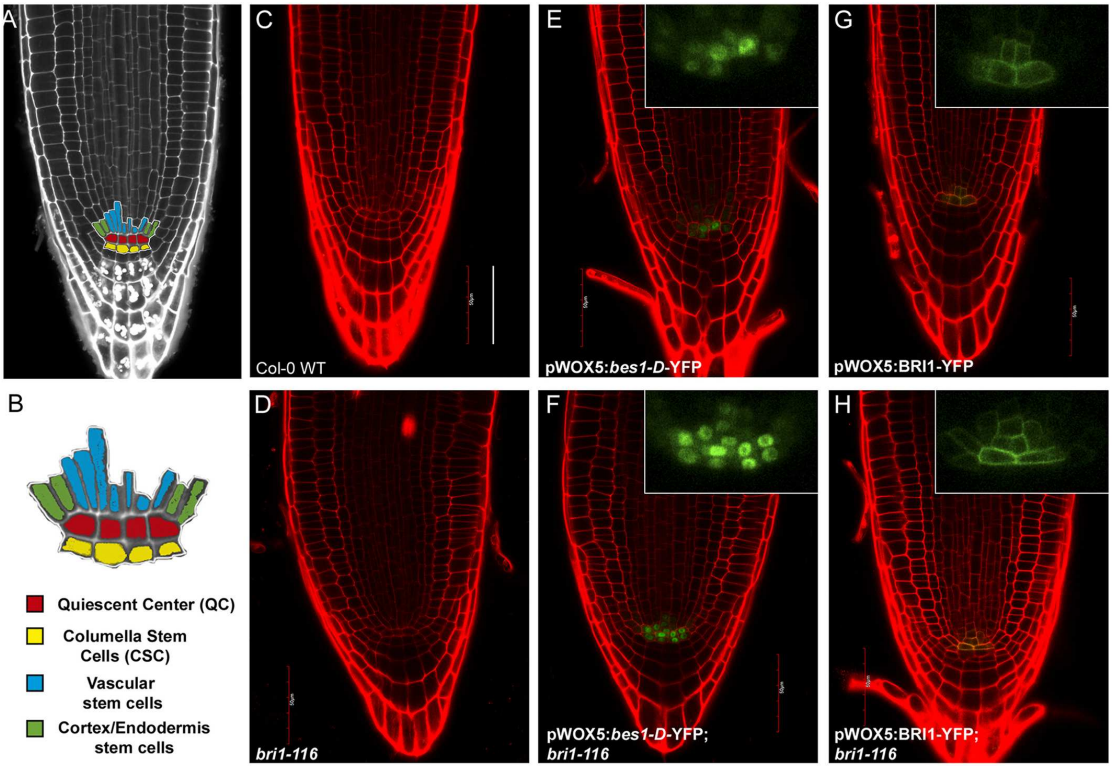

Fig. 1. The stem cell niche of Arabidopsis roots and QC-specific expression of BR pathway components. (A) A stereotypical Arabidopsis WT primary root under confocal microscopy. The root stem cell niche is highlighted in color. (B) Detailed representation of the root stem cell niche. (C–H) Confocal images of 6-day-old WT and mutant Arabidopsis roots in control conditions. Green represents YFP-tagged pathway components. Red is PI counterstaining. Insets show the YFP channels at higher magnification. Scale bar: 50 µm.

2013; Zhang et al., 2010). For instance, BRs are known to promote both cell division in the QC and differentiation of the surrounding columella stem cells (Fàbregas et al., 2013; Gonzalez-Garcia et al., 2011; Vilarrasa-Blasi et al., 2014). More specifically, the ERF115 transcription factor, which is activated by BRs, promotes QC divisions and stem cell regeneration after DNA damage (Heyman et al., 2016, 2013). In contrast, BRAVO (BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER), an R2R3-MYB transcription factor identified using cell-specific transcriptomics, acts as a repressor of QC divisions (Vilarrasa-Blasi et al., 2014). Interestingly, BRAVO is a direct transcriptional target of and interacts with the BR-regulated transcription factor BES1 at the protein level, forming a feedback loop that antagonistically regulates QC divisions (Vilarrasa-Blasi et al., 2014). Despite the importance of these transcription factors for locally safeguarding QC divisions, it is still unknown whether BR-regulated QC function is maintained in a cell-autonomous fashion or requires external signaling. Moreover, although BR receptors collectively modulate QC cell division and differentiation of surrounding stem cells under normal conditions (Fàbregas et al., 2013), the specific contribution of each receptor within the stem cell niche is not known.

These questions prompted us to investigate BR-mediated regulation of quiescence and its impact on stem cell regeneration after DNA damage at the local level. Accordingly, we used a tissue-specific approach in order to determine the ability of QC cells to integrate exogenous steroid signals. For this purpose, we specifically overexpressed two BR signaling components – the BRI1 membrane receptor and the BES1 transcription factor – in QC cells, and specifically knocked out BRI1 in the stem cell niche using an artificial microRNA (amiRNA) (Dolan et al., 1993; Schwab et al., 2006). Altogether, we demonstrate that: (1) active BES1 is necessary for cell-autonomous QC divisions; (2) the BR hormone itself (i.e. not the receptors) is the limiting factor for BR-induced QC divisions in the root apex; (3) BRI1 is required at the stem cell niche for mediating BR-dependent QC divisions; and (4) upon stem cell death, paracrine BR signaling is required for QC divisions. Overall, our results establish a hierarchy for the different BR receptors within the stem cell niche, indicating that under normal conditions the BRI1 receptor acts as the principal player controlling QC divisions, rather than its homologous.

RESULTS

Active BES1 promotes cell-autonomous QC division

We first wanted to elucidate whether the BR-induced division signals of the QC were transduced in a cell-autonomous manner through the canonical BR signaling cascade. To this end, we used the gain-of-function BES1 mutant, *bes1-D*, which is known to be constitutively active (Yin et al., 2002). Previously, we cloned *bes1-D* under the control of the promoter of the QC-specific gene

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WOX5 (Sarkar et al., 2007), and fused YFP to its C-terminus (Vilarrasa-Blasi et al., 2014). This construct, pWOX5:*bes1-D*-YFP, was transformed into both Col-0 wild-type (WT) and the null BRI1 mutant *bri1-116* (Li and Chory, 1997) (Fig. 1C–F).

Confocal microscopy of 6-day-old roots revealed an increase in the number of QC divisions in both the WT and the *bri1-116* mutant upon expressing *bes1-D* under the *WOX5* promotor (Fig. 2A,D,F,M; Table S1). This indicates that active BES1 locally promotes division at the QC in a cell-autonomous manner. Interestingly, however, the QC division rates in the *bri1-116* background were lower than those in the WT background (Fig. 2M; Table S1), suggesting that BR signaling from surrounding tissues also participates in activation of QC divisions.

In addition, treatment of WT plants harboring the pWOX5:*bes1-D*-YFP construct with brassinolide (BL) did not result in a significant increase in cell division rates (Fig. 2D,J,M; Table S1). This is probably due to a saturated BRs signal contributed also by basal receptor-transduced signaling. Conversely, upon BL treatment, a significant increase in cell division rate was observed for the *bri1-116* plants that contained pWOX5:*bes1-D*-YFP (Fig. 2F,L,M; Table S1).

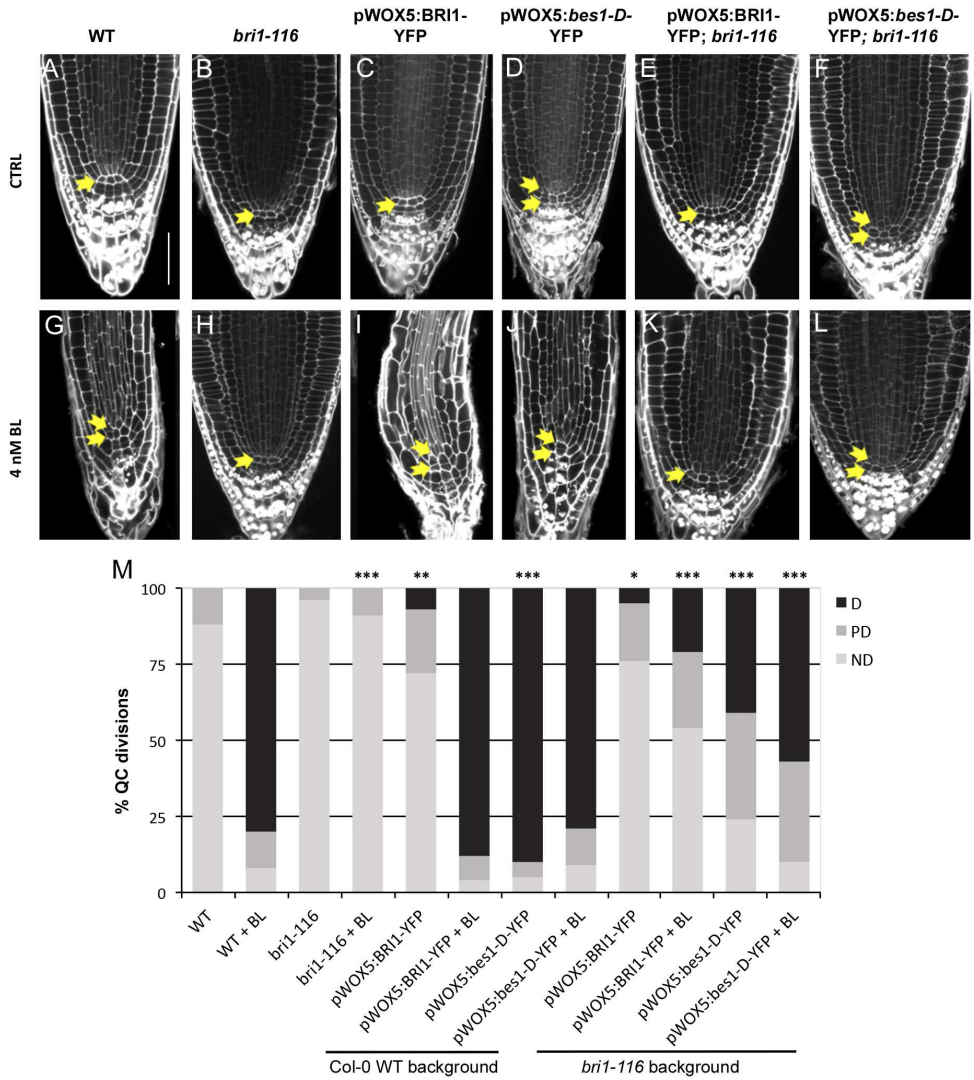

Fig. 2. The BR-regulated transcription factor BES1 promotes QC division in a cell-autonomous manner. (A–F) Confocal images of fixed 6-day-old WT and mutant Arabidopsis roots in control conditions. (G–L) Root anatomy of 6-day-old seedlings grown in medium supplemented with 4 nM BL. Arrows indicate the number of QC cell layers identified. (M) Quantification of QC division rate. ND, QC non-divided; PD, QC partially divided; D, QC totally divided. Asterisks indicate statistically significant differences due to genotype, comparing against WT either in control or 4 nM BL conditions. Frequencies in QC divisions were assessed with a two-sided Fisher's test. Values for all pairwise comparisons are provided in Table S1. Data are generated from three independent replicates (n>21). *P<0.05, **P<0.01, ***P<0.005. Scale bar: 50 µm.

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This suggests that the signal is not saturated in these plants, and that the BRL receptors are also contributing factors.

The local BR hormone level is the main limiting factor for QC division

Next, by introducing the pWOX5:BRI1-YFP transgene into both WT and *bri1-116* backgrounds, we evaluated the local contribution of the BRI1 receptor to QC division (Fig. 1C,E). As the *WOX5* promoter drives relatively high expression compared with the endogenous *BRI1* promoter, *WOX5*-controlled expression of the BRI1 receptor resulted in its local overexpression in the QC. Confocal images comparing BRI1 expression under its endogenous promoter (Geldner et al., 2007) with BRI1 expression in the pWOX5:BRI1-YFP lines are shown in Fig. S1.

When BRI1 is locally overexpressed using the *WOX5* promoter, a small increase in QC division rate was observed in both the WT and the *bri1-116* backgrounds (Fig. 2C,E,M; Table S1). This increase, however, was substantially smaller than that observed upon expression of *bes1*-D using the same promoter (Fig. 2D,F,M; Table S1). Upon application of exogenous BL, we observed a dramatic increase in the QC division rate for those plants expressing pWOX5:BRI1-YFP in the WT background but not in the *bri1-116* background (Fig. 2C,E,I,K,M; Table S1). This implies that BRI1 signaling in the QC alone is not sufficient to promote QC divisions, but rather additional external signaling is required. The fact that overexpression of BRI1 in the QC did not result in a large increase in QC division until exogenous BL was applied, indicates that the BR hormone itself is the limiting factor of QC division. Furthermore, only after applying BL to the pWOX5:BRI1-YFP; *bri1-116* roots could a dramatic reduction in meristem cell number be observed (Fig. S2A). This typical effect of exogenous BL application was not seen when just BRI1 is overexpressed. Together, these results suggest two possible scenarios: (1) there is an insufficient level of BRs in the root stem cell niche to promote QC division, or (2) BRI1-like receptors (i.e. BRL1 and BRL3) act as competitors for BR ligand binding.

To address the second scenario, we crossed the pWOX5:BRI1-YFP plants with double and triple mutants lacking two (*brl1brl3*) or all receptors (*bri1-116brl1brl3*), respectively, and assessed the occurrence of spontaneous QC divisions or an increased sensitivity to BL. Application of BL to the *brl1brl3* double mutant backgrounds yielded similar effects to those in the WT background, showing that the loss of these genes does not affect QC division rates even when applying lower concentrations of BL (0.04 nM) (Fig. S3, Table S2). With respect to the triple mutant, we obtained results similar to those found in the *bri1-116* background (Fig. S3, Table S2). Altogether, these results indicate that the BRL1/3 receptors do not compete with the BRI1 receptor for hormone binding. Interestingly, a lack of BRL receptors attenuates the slight increase in QC division that is observed upon overexpressing BRI1 in the QC (Fig. 2M; Fig. S3K, Table S2). In agreement with previously reported data (Fàbregas et al., 2013), this supports a marginal role for the BRL1and BRL3 receptors in promoting BR-mediated QC divisions in normal conditions. These results, together with the previous ones, exclude the possibility that BRL receptors compete with BRI1 for ligand binding. Thus, we conclude that the BR hormone concentration must be the limiting factor for promoting QC division.

BRI1 is required in the stem cell niche for BL-triggered QC division

To more thoroughly understand the receptor requirements that drive BES1-mediated QC division, we specifically knocked out BRI1 expression in the WOX5 domain. For this, we designed and cloned an amiRNA against BRI1 (see Materials and Methods; Fig. S4A,B). To validate the ability of our amiRNA to knock out BRI1 expression, we first placed it under the control of the constitutive promoter CaMV35S. This resulted in dwarf plants similar to null *bri1* mutants (Li and Chory, 1997) (Fig. S4C). Next, cell-specific knockouts were generated by placing the amiRNA under the control of the QC-specific promoter *WOX5*. As seen by crossing pWOX5:BRI1-amiR plants with plants expressing BRI1-GFP under the control of the endodermis-specific promoter scarecrow (SCR) (Hacham et al., 2011), inhibition of BRI1 expression was not limited to the QC cells, but also occurred in nearby surrounding cells (Fig. 3A,B). This implies that the small size of the mature amiRNA enables it to diffuse to adjacent cells. Importantly, YFP signals observed in plants that overexpressed BRI1-YFP in the QC completely disappear when crossed with pWOX5:BRI1-amiR plants, indicating that our amiRNA is indeed effective at attenuating BRI1 expression (Fig. 3C,D). Finally, genetic crosses between the pWOX5:BRI1-amiR line and the translational reporter lines pBRL1:BRL1-GFP and pBRL3:BRL3-GFP (Fàbregas et al., 2013), showed that the BRI-amiR is partially depleting *BRL1* and *BRL3* transcripts, as consequence of sequence similarity (Fig. 3E–H). A GFP intensity reduction of ∼40% could be detected in the crosses (Fig. S5A,B).

Next, we analyzed two independent pWOX5:BRI1-amiR lines in terms of their sensitivity towards exogenous BL. Based on root length, meristem cell number and stele width, we found that both lines expressing the amiRNA retained a BL sensitivity closely similar to that of WT plants. In contrast, the null *bri1-116* plants were insensitive to hormone application (Fig. S2C–E), thereby suggesting that the effect of the mature amiRNA is strongly limited to a local level. Interestingly, both pWOX5:BRI1-amiR lines were completely insensitive to BL application in terms of QC division (Fig. 4A–G; Table S3). Taken together, these results indicate that the presence of BRI1 receptors in the QC is essential for QC division. Additionally, pWOX5:BRI1-amiR lines exhibited impaired root growth, having slightly, but significantly shorter roots than WT plants starting from 5 days after germination (Fig. 4H; Fig. S2C), suggesting that the presence of BR receptors in root stem cell niche contributes for optimal root growth.

We next asked whether the reduction in QC divisions in the pWOX5:BRI1-amiR lines was a consequence of a slower cell cycle progression in the meristem. To answer this question, we stained roots with 5-ethynyl-2′-deoxyuridine (EdU), a thymidine analogue that is incorporated into actively dividing cells (Salic and Mitchison, 2008). In WT plants, we observed a uniform EdU staining in the entire root meristem except for in the QC, which owing to its quiescence, barely incorporates EdU (Fig. 5A). The same results, which are indicative of a normal cell cycle in the meristem, were also obtained for the pWOX5:BRI1-amiR lines (Fig. 5B,C). Thus, the QC remains quiescent because of the absence of BRI1, and not because of a meristem-wide deceleration of the cell cycle. In contrast, the *bri1-116* mutant showed a much lower extent of EdU incorporation, thereby confirming that it has a slower cell cycle compared with WT plants (Fig. 5D). Fluorescence intensity quantification confirmed that pWOX5:BRI1-amiR lines incorporate EdU at the same levels as in the WT, whereas *bri1-116* does so at lower rates (Fig. S5C) and it agrees with the previously reported slow cell cycle progression of *bri1-116* (Gonzalez-Garcia et al., 2011).

Furthermore, we treated both WT and pWOX5:BRI1-amiR lines with BL in order to evaluate whether BL promotes QC cell division. Upon BL treatment, WT roots incorporated EdU into the QC

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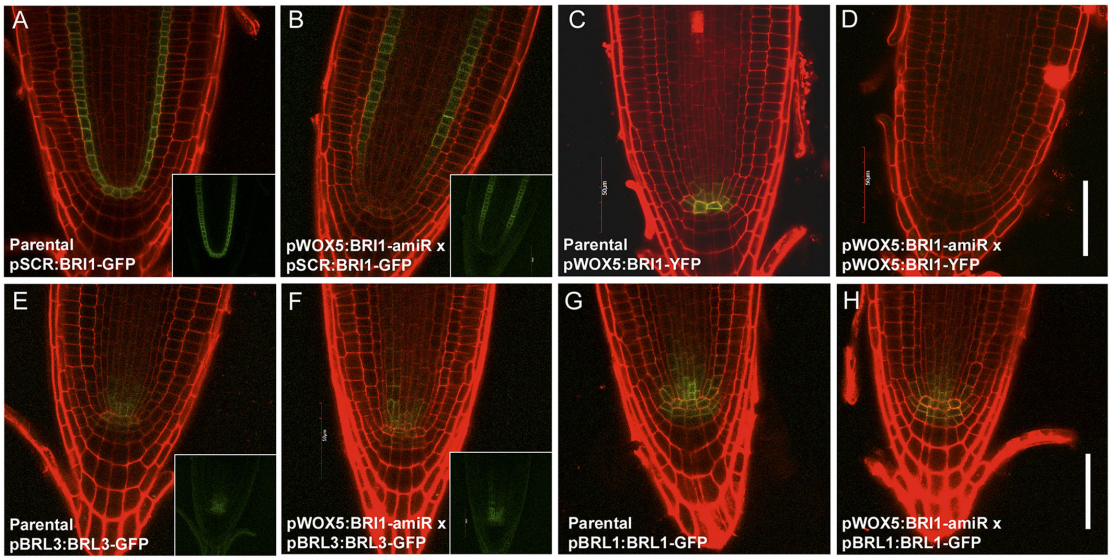

Fig. 3. The pWOX5:BRI1-amiR construct targets BRI1 and downregulates its transcription in the root stem cell microenvironment. Confocal images of 6-day-old Arabidopsis roots. (A,B) Genetic crosses between pWOX5:BRI1-amiR and pSCR:BRI1-GFP lines reveal that BRI1 is knocked down in the stem cell microenvironment. (C,D) Genetic crosses between pWOX5:BRI1-YFP and pWOX5:BRI1-amiR lines show that the amiRNA completely depletes BRI1 around the QC domain. (E–H) Genetic crosses of pWOX5:BRI1-amiR lines with pBRL1:BRL1-GFP and pBRL3:BRL3-GFP lines. Insets show the GFP channel separately. All crosses are F3 double homozygous plants. Scale bar: 50 µm.

(Fig. 5E), thereby confirming that the QC cells were undergoing cell division. In contrast, however, the pWOX5:BRI1-amiR lines did not incorporate EdU into the QC after being subjected to identical BL treatment (Fig. 5F,G). This clearly supports the hypothesis that pWOX5:BRI1-amiR lines are insensitive to BR-mediated signals in the QC. Along the same lines, the plant that has a constitutively dividing QC due to overexpression of active BES1 (i.e. the pWOX5:*bes1-D*-YFP line), also exhibited EdU incorporation in the QC (Fig. 5H). This, in effect, mimics the results obtained with exogenous BL treatment, and confirms that activated downstream components of BR receptors are capable of triggering QC division in a cell-autonomous manner.

Stem cell regeneration upon DNA damage entails the local action of BR receptors

Since the QC has been proposed to act as a stem cell reservoir and is known to divide in the face of environmental stresses, we decided to evaluate whether the BR receptors are essential for carrying out such stress-induced division. For this purpose, we decided to use bleomycin, a chemotherapeutic drug that has been described to preferentially harm root vascular stem cells and induce QC division (Fulcher and Sablowski, 2009; Vilarrasa-Blasi et al., 2014). As such, this system triggers QC division independently of BR treatment. We compared the local knockout lines (i.e. pWOX5:BRI1-amiR) against both the null *bri1* mutant and WT roots. While the pWOX5:BRI1-amiR lines were damaged at the same rate as the WT plants (Fig. 6A,B,C,I; Table S4), the *bri1* mutant remained free of any visible damage (Fig. 6D,I; Table S4). As previously described, this is probably due to its slow cell cycle progression (Gonzalez-Garcia et al., 2011; Vilarrasa-Blasi et al., 2014). Interestingly, in contrast to what was observed for the WT roots, the QC of the pWOX5:BRI1-amiR lines remained undivided following 24 h of bleomycin treatment plus 24 h of recovery (Fig. 6E,F,G,J; Table S5). In the case of *bri1*, the QC also remained undivided, but as previously mentioned, the roots were not damaged by bleomycin (Fig. 6H,J). Given that the pWOX5:BRI1-amiR lines and WT show similar levels of provascular cell death after 24 h of bleomycin treatment (Fig. 6A,B,C,I; Table S4), as well as the same amount of EdU staining (Fig. 5A–C; Fig. S5C), our results argue that the absence of QC divisions in bleomycin-treated pWOX5:BRI1-amiR lines is due to neither an inherent resistance against DNA damage nor a slow cell cycle progression. Interestingly, our results reveal the paracrine nature of this DNA damage response: a signal that emerges from damaged stem cells triggers cell division in the adjacent QC. Moreover, according to our data, this signal must be a type of steroid molecule that is locally and mainly transduced by BRI1 in the stem cell niche.

DISCUSSION

The slow-dividing nature of the cells in the QC enable it to act as a cell reservoir and organizer for surrounding stem cells (Fulcher and Sablowski, 2009; Pi et al., 2015; Sarkar et al., 2007; van den Berg et al., 1997; Vilarrasa-Blasi et al., 2014). Although recent studies have started to shed light on the molecular components behind QC quiescence, the exact mechanisms that are responsible for ensuring such a low rate of cell division remain largely unknown. One fairly recent study discovered that the interaction between RETINOBLASTOME-RELATED (RBR) and SCARECROW (SCR) is required for quiescence maintenance (Cruz-Ramírez et al., 2013). Nonetheless, rather than being completely static, the QC is in fact regulated by plant hormone signaling. For instance, while it has been shown that abscisic acid (ABA) reinforces the quiescence of this group of cells (Zhang et al., 2010), ethylene (Ortega-Martinez et al., 2007) and cytokinin (Zhang et al., 2013) are

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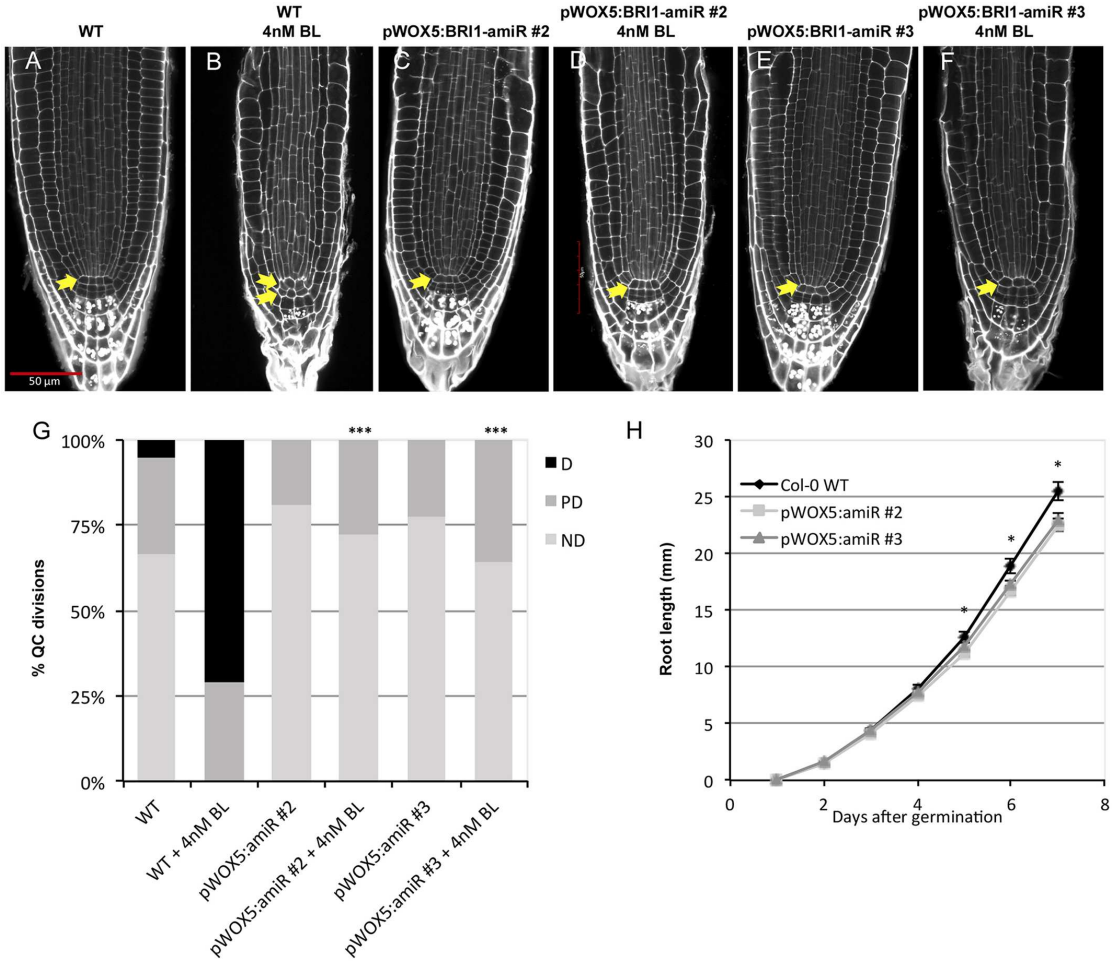

Fig. 4. BRI1 in the stem cells niche is required to promote QC divisions. (A,B) Confocal images of 6-day-old WT Arabidopsis roots grown in either control conditions or 4 nM BL show the change in QC division and organization. (C–F) pWOX5:BRI1-amiR transgenic lines grown in control conditions or in medium supplemented with 4 nM BL. Arrows indicate the number of QC cell layers identified. (G) Quantification of the QC divisions of WT and pWOX5:BRI1-amiR plants. ND, QC non-divided; PD, QC partially divided; D, QC totally divided. Asterisks indicate statistically significant differences due to genotype, comparing against WT either in control or 4 nM BL conditions (***P<0.005). Frequencies in division occurrence were assessed with a two-sided Fisher's test. Values for all pairwise comparisons are provided in Table 3. Data generated from three independent replicates (n>39). (H) Root growth dynamics of WT and pWOX5:BRI1-amiR lines. Asterisks denote significant differences with respect to the WT in a two-tailed t-test (*P<0.05). Data are generated from three independent replicates (n>46). Scale bar: 50 µm.

known to disrupt their quiescence and promote division. With respect to BR hormones, they have been shown to promote QC divisions while maintaining regular cell cycle progression in the rest of the root meristem (Gonzalez-Garcia et al., 2011). The mechanisms underlying BR-mediated QC divisions are slowly being uncovered with the identification of BR-regulated and QC-specific transcription factors such as ERF115 (Heyman et al., 2013) and BRAVO (Vilarrasa-Blasi et al., 2014). However, how these signaling mechanisms are locally confined to the stem cell niche of the root is still controversial. In fact, although it has been proposed that BR action at the epidermis (Hacham et al., 2011) and vascular tissues (Kang et al., 2017) can similarly regulate meristem size and plant growth, it is unknown whether these local signals are also capable of driving QC divisions. Here, our findings show that QC activities at the stem cell niche require the presence of BR receptors in both the QC cells themselves and nearby surrounding cells.

Activated BES1 can trigger cell-autonomous QC division but needs membrane support

Physiological analysis of QC-specific overexpression of BES1 revealed that active BES1 has the potential to trigger QC division in an autonomous manner. However, as the same QC division rates were not observed when the transgene was introduced into the *bri1* mutant background (Fig. 2M; Table S1), it became apparent that BRI1 was also required for this process. It is important to note that BRI1 might also activate other downstream components besides

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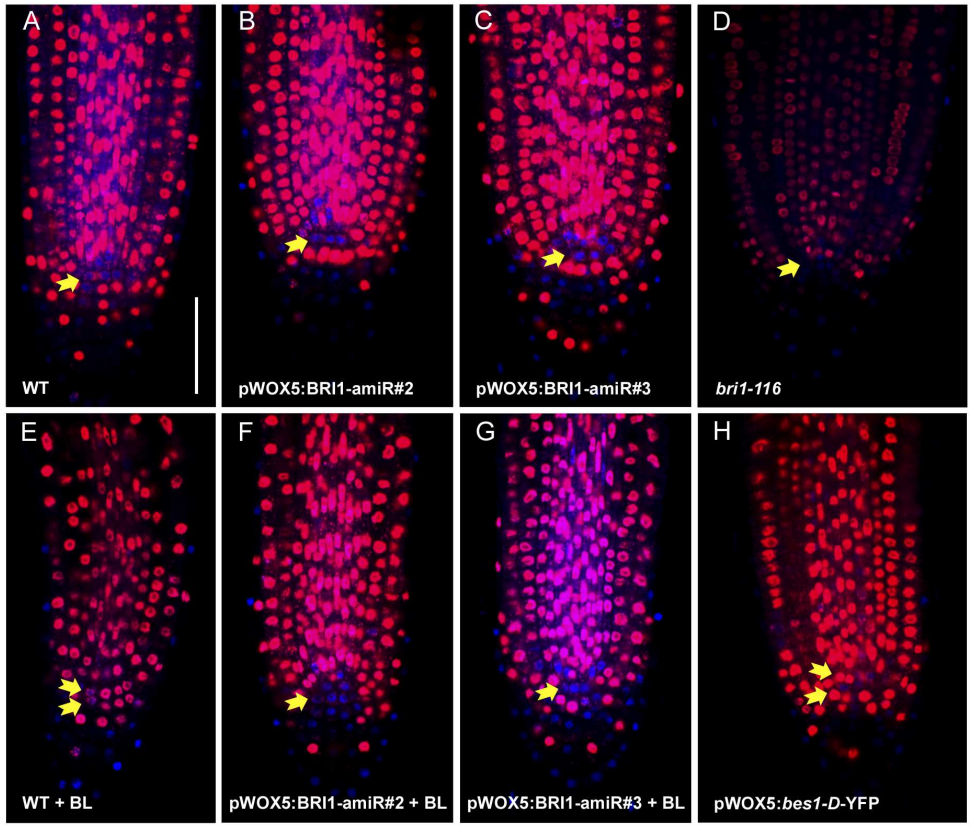

Fig. 5. pWOX5:BRI1-amiR seedlings exhibit normal meristem divisions. Confocal images of fixed and EdU-stained 6-day-old Arabidopsis roots. (A–C) WT, pWOX5:BRI1-amiR#2 and pWOX5:BRI1-amiR#3 lines grown in control conditions. (D) *bri1-116* line grown in control conditions as a negative control for QC division. (E–G) WT, pWOX5:BRI1-amiR#2, and pWOX5:BRI1-amiR#3 lines grown for 4 days in control conditions and 2 days in medium supplemented with 4 nM BL. (H) pWOX5:*bes1-D*-YFP line grown in control conditions as a positive control for QC division. Arrows indicate the number of QC cell layers identified. Scale bar: 50 µm.

BES1. For example, one potential downstream target could be the transcription factor BZR1, which has been shown to promote autonomous QC division when activated (Chaiwanon and Wang, 2015; Lee et al., 2015). Interestingly, in the *bri1* background lines, we detected an increase in QC division frequency upon BL application (Fig. 2M; Table S1). This increase could be attributed to BRL receptors compensating for the lack of BRI1 and activating other downstream components.

The hormone is the limiting factor for promoting QC divisions

Surprisingly, when the plants that overexpressed BRI1 in the QC (pWOX5:BRI1-YFP) were assessed in terms of QC division rates, we found only a limited increase in both the WT and *bri1* backgrounds (Fig. 2M; Table S1). The fact that the roots showed signs of recovery in the *bri1* background line (i.e. longer roots) however, confirmed that BRI1 was still functional when fused to YFP (Fig. S2B). Upon BL treatment, the QC division frequency of pWOX5:BRI1-YFP plants is similar to that in WT plants treated with BL (Fig. 2M; Table S1), thus revealing that an excess of receptor has no effect until the ligand is added. As the plants overexpressing pWOX5:BRI1-YFP displayed no dramatic phenotype until exogenous hormone was applied, we concluded that the stem cell niche microenvironment must be characterized by an excess of BRI1 and a limited amount of free hormone. We discounted competition for the ligand between BRI1 and BRLs as the reason for this (Fig. S3, Table S2), and hypothesize that, in the root stem cell niche, a threshold of available hormone has to be reached in order to promote QC divisions.

BRI1 is necessary but not sufficient to promote QC division

According to our results, the presence of BRI1 in the QC is not the limiting factor for the QC division process. In fact, very low amounts of BRI1 receptor are present within these cells (Wilma van Esse et al., 2011). Furthermore, BRL1 and BRL3, both of which bind the hormone with a higher affinity than BRI1, are also present in these cells (Cano-Delgado et al., 2004; Fàbregas et al., 2013). Accordingly, we wondered whether BRI1 was absolutely necessary in this domain. Our results show that WT lines expressing the amiRNA against BRI1 in the stem cell niche (pWOX5:BRI1-amiR) are completely insensitive towards BL-induced QC divisions (Fig. 4E). At the same time, however, BRI1 acting exclusively in the QC (i.e. pWOX5:BRI1-YFP; *bri1-116* line) is not enough to recover BL-induced QC divisions to WT levels (Fig. 2M). Taken together, these results suggest that the effects of BRI1 are reinforced

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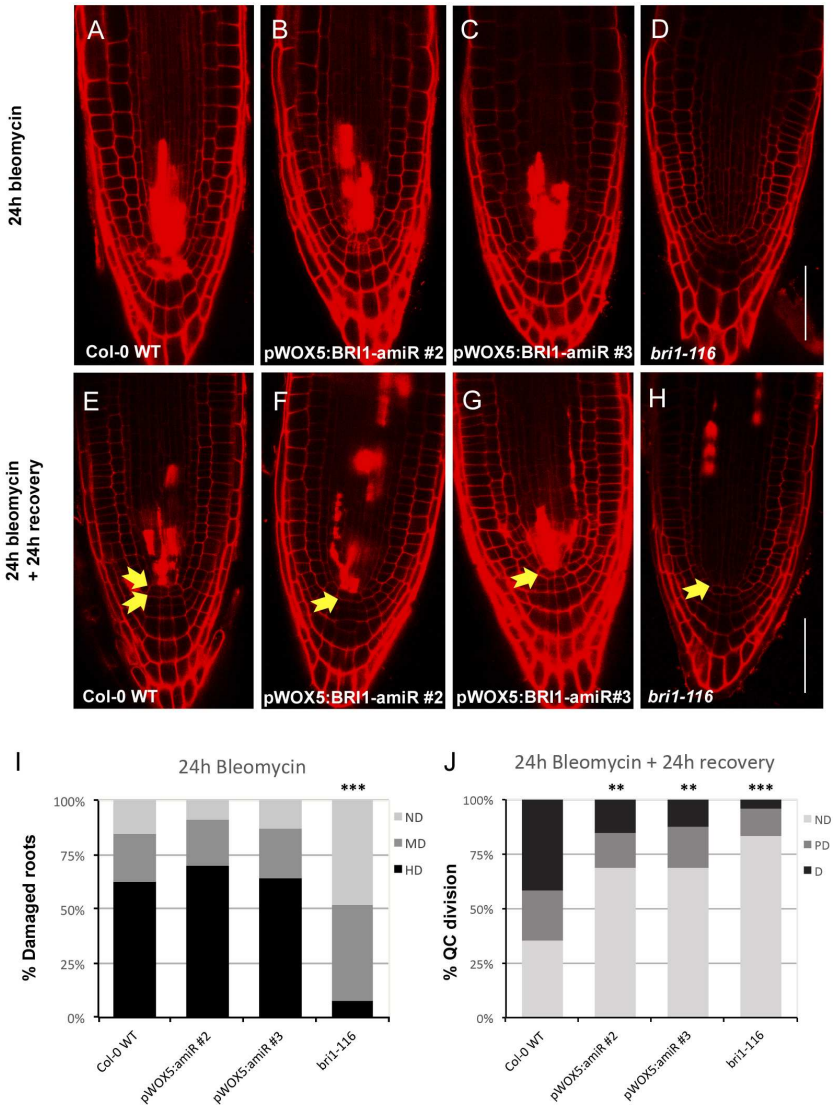

Fig. 6. BR receptors in the stem cell niche modulate QC divisions upon DNA damage. (A–D) Confocal images of 5-day-old seedlings treated with bleomycin for 24 h. (E–H) Confocal images of 5-day-old seedlings subjected to 24 h of bleomycin treatment and a subsequent 24 h of recovery. (I) The proportion of roots showing cell death in the root apex after 24 h of bleomycin treatment. HD, hard damage; MD, mild damage; ND, no damage. Asterisks indicate statistically significant differences respect to WT (***P<0.005). Differences in the proportion of damaged roots were assessed with a two-sided Fisher's test. Values for all pairwise comparisons are provided in Table S4. Data are generated from three independent replicates (n>25). (J) Quantification of QC divisions after 24 h of bleomycin treatment and 24 additional hours of recovery. ND, QC non-divided; PD, QC partially divided; D, QC totally divided. Asterisks indicate statistically significant differences with respect to WT (**P<0.01, ***P<0.005). Differences in division frequencies were assessed with a two-sided Fisher's test. Values for all pairwise comparisons are provided in Table S5. Data are generated from three independent replicates (n>24). Scale bar: 50 µm.

from surrounding cells. Thus, we found that BRI1 signaling in the QC is necessary, but not sufficient to promote QC self-renewal, and highlight BRI1 as the main driving factor for this process. Despite the fact that BRL activity is also partially downregulated in pWOX5:BRI1-amiR lines, in agreement with our data, previous results showed that *brl1brl3* double mutants have a normal BR-induced QC division (Fábregas et al., 2013). On the other hand, *bri1-116* mutants, which have intact *BRL1* and *BRL3* genes, retain a quiescent QC, even upon application of high doses of BL (Gonzalez-Garcia et al., 2011) (Fig. 2M; Table S1). Our results relegate BRL receptors to a supporting action for BRI1, which in turn acts as the main promoter of QC divisions in normal conditions. Moreover, QC division frequency also has an impact on the growth of primary roots, as the roots of pWOX5:BRI1-amiR lines are slightly shorter than those of the WT (Fig. 4H; Fig. S2C). Congruently, the *bri1-116* mutant lines that overexpressed BRI1 or BES1 in the QC (i.e. pWOX5:BRI1-YFP;*bri1-116* and pWOX5:bes1-D-YFP;bri1-116) not only partially recovered BR signaling in the QC, but also partially recovered seedling root length compared with that in the *bri1-116* mutant (Fig. S2D). This latter fact prompted us to hypothesize that some spontaneous QC divisions under basal conditions are required to sustain optimal root growth – presumably for replenishment of the stem cell niche.

BR signaling acts in a paracrine manner to trigger QC division

It is known that the QC divides in response to environmental stresses such as the presence of DNA-damaging agents (Vilarrasa-Blasi et al., 2014) or changes in the homeostasis of reactive oxygen

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species (ROS) (Yu et al., 2016). In the root, DNA-damaging agents preferentially harm vascular and columella stem cells. Cells that are unable to repair this damage activate programmed cell death (PCD) and undergo apoptosis (Fulcher and Sablowski, 2009), thereby subsequently promoting QC divisions to replenish the stem cell niche and maintain meristematic activities (Heyman et al., 2016; Vilarrasa-Blasi et al., 2014). We took advantage of this property to analyze the receptor requirements of the signaling that causes QC division. Interestingly, we found that the BRI1 receptor is necessary to trigger QC divisions after vascular cell death (Fig. 6), although we cannot discard a major contribution of BRLs under this stress scenario. Furthermore, we discounted the idea that QC quiescence observed in the pWOX5:BRI1-amiR line after damage is due to a slower cell cycle (Fig. 5; Fig. S5C), as is the case for the *bri1-116* mutant. Although it has been demonstrated that downregulation of BRAVO is implicated in this type of QC division (Vilarrasa-Blasi et al., 2014), the exact nature of signal progression from the damaged cell to the QC is still unclear. Even if we cannot discern between BRI1 and the BRLs perceiving this signal, results obtained by treating the pWOX5:BRI1-amiR lines with bleomycin have revealed that these signals are perceived by BR receptors acting in the stem cell niche, so the signal should be of a steroid nature and act in a paracrine manner.

It is known that by stimulating paracrine signaling, human stem cells can promote wound healing and cancer progression (Dittmer and Leyh, 2014), but in plants, the mechanisms behind autocrine and paracrine signaling are only just being uncovered (Qi et al., 2017). It has been proposed that BRs can regulate stem cell division in the roots via long-range signals originating at the epidermis (Hacham et al., 2011). However, although changes in QC markers (e.g. AGL42) were observed in response to epidermal signaling, no effect on QC divisions was reported (Hacham et al., 2011). This therefore limits direct readout of BR-mediated signaling in the QC to short-range signals. Indeed, in contrast to other hormones that act over long distances, it is accepted that BRs act at a more local level (Fridman et al., 2014) and our findings indicate that the signals that promote QC divisions come from the nearby stem cell microenvironment rather than from the outer cell layers. Nevertheless, where exactly the BR signals are driven from remains a controversy.

In summary, our findings show that (1) QC cell division activity is promoted by BES1 transcription factor in the QC; (2) BRI1 is required in both the QC and nearby cells to trigger division; and (3) paracrine steroid signaling may be regulated by the hormone's availability in the stem cell niche (Fig. 7). A plausible way to control the hormone levels in the stem cell microenvironment of the root could be to upregulate the genes controlling its biosynthesis. However, the spatial regulation of the enzymes responsible for BR biosynthesis is still poorly understood. As such, further efforts in this area are crucial for elucidating the nature and origin of BR signals, where they are synthesized and where they are driven.

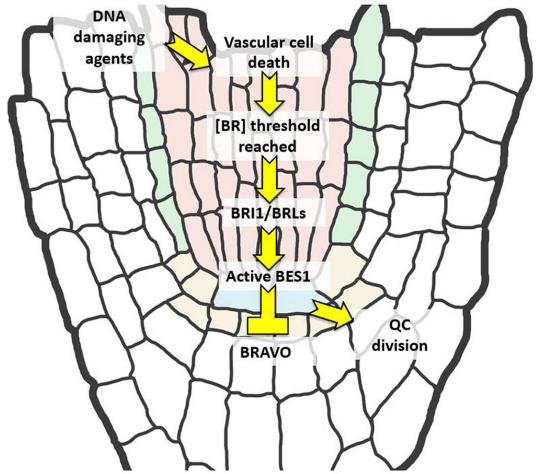

Fig. 7. Working model: BR concentration as a limiting factor for QC divisions. In order to promote QC divisions when needed, a threshold concentration of BRs has to be reached in the root apical meristem. Upon reaching this threshold, the signal is transduced via BRI1 with enough strength to promote BES1 dephosphorylation. Dephosphorylated BES1, in turn, inhibits BRAVO and triggers QC division.

MATERIAL AND METHODS

Plant material and growth conditions

All lines used in this study, along with their references are listed in Table S6. We used *Arabidopsis thaliana* (L.) Heyhn, ecotype Columbia-0 (Col-0) as the control background line.

Seeds were surface sterilized using 35% bleach, and subsequently washed five times with distilled sterile water. Seeds were vernalized at 4°C in the dark for 48 h before sowing. Plants were grown in vertical plates containing half-strength Murashige and Skoog (MS) medium with vitamins but no sucrose supplements (0.5×MS−), in long day conditions (LD, 16 h light:8 h dark) at 22°C and 60% relative humidity.

amiRNA design and cloning

We designed the artificial miRNA using Web MicroRNA Designer (WMD2) as previously described (Ossowski et al., 2008; Schwab et al., 2006). Briefly, the nucleotides encoding the mature miRNA sequence, GCCCCTATCTAAGTG-TCAGTT, were engineered in the miR319a precursor as described (Schwab et al., 2006). This was then subcloned under the control of the WOX5 QC promoter in the binary plasmid pH7m24GW,3, and transformed into Arabidopsis using the floral dip method (Zhang et al., 2006). In this work, we used two independent homozygous T4 lines named pWOX5:BRI1-amiR#2 and pWOX5:BRI1-amiR#3, both of which express the specific amiRNA against BRI1 under the *WOX5* promoter (4.2 kb upstream of the *WOX5* start codon). For the pWOX5:BRI1-YFP construct, the coding sequence of the *BRI1* gene was cloned under the control of the *WOX5* promoter and fused to *YFP*, all inside the binary plasmid pB7m34GW. All constructs were cloned using Gateway technology (Invitrogen) according to the manufacturer's instructions.

Confocal microscopy

For QC division analysis, 6-day-old seedlings were fixed, clarified and counterstained using modified Pseudo Schiff–propidium iodide (mPS-PI) staining (Truernit and Haseloff, 2008). Then, each seedling was mounted onto a microscope slide with a drop of Hoyer's solution (30 g gum arabic, 200 g chloral hydrate, 20 g glycerol and 50 ml water). Images were obtained using a FV 1000 confocal microscope (Olympus, Tokyo, Japan). The QC division phenotypes were scored as in Vilarrasa-Blasi et al. (2014). Differences in QC division frequencies were statistically evaluated with a two-sided Fisher's exact test (Tables S2–S4).

For bleomycin assays, the percentage of damaged roots was scored after 24 h of treatment, which is a qualitative classification depending on the amount of death cells in the vasculature, identified by the incorporation of PI inside the cells: no damage means that cells did not uptake PI; mid damage indicates that some cells in the stem cell niche area were stained; hard damage indicates that all cells in the stem cell niche and some cells in the vascular system stained with PI. The percentage of QC divisions was scored after 24 h of bleomycin treatment and 24 h of recovery.

Hormone and drug treatments

For brassinolide (BL) treatment, BL (C28H48O6; Wako, Osaka, Japan) previously dissolved in ethanol was added to medium at a final concentration

of either 4 nM or 0.04 nM. For bleomycin treatment, seedlings were transferred to vertical plates supplemented with 0.6 µg/ml bleomycin (Calbiochem) 4 days after sowing. For recovery, plants were transferred back to control medium after 1 day of growth in bleomycin-containing medium and quantified under a confocal microscope after 24 h.

EdU staining

For evaluating EdU staining, we used the Click-iT EdU Alexa Fluor 555 Imaging Kit (Thermo Fisher). Five days after sowing, seedlings were transferred to vertical plates supplemented with 10 µg/ml EdU. After 24 h, seedlings were fixed in a solution containing 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 in 1× PBS for 1 h in a vacuum. After fixation, the seedlings were washed twice with 3% (w/v) BSA in 1× PBS, and subsequently incubated in the Click-iT reaction cocktail (as described in the protocol of Invitrogen EdU Click-iT Reaction Imaging Kit) for 1 h in the dark. For counterstaining, seedlings were washed twice with 3% BSA in 1× PBS and incubated for 30 min with 1 µg/ml DAPI in 1× PBS in the dark. Finally, the seedlings were washed a final time in 3% BSA in 1× PBS.

Root measurements and fluorescence quantification

For root length measurements, images of seedlings were taken with a Nikon D7000 camera and roots were measured with ImageJ software (http://imagej.nih.gov/ij/). For meristem cell counts, 6-day-old seedlings were stained with 10 µg/ml PI and the images were obtained using a FV 1000 confocal microscope (Olympus, Tokyo, Japan), using a 20× objective. Then cells were counted by tracking the cortex, starting from QC cells. The end of the meristem was considered when a cell had >75% increase in cell length (longitudinally) than the previous one. Cell measurements were performed with ImageJ. For root stele width, measures were taken at 50 µm upstream of the QC in the root longitudinal axis. The separation between pericycle cell files (stele) was measured perpendicular to the root longitudinal axis. Measures were made with ImageJ. For fluorescence quantifications, the mean pixels/area of fluorescence in the green channel (to quantify GFP) or the red channel (to quantify EdU incorporation) were quantified with ImageJ, either on complete images for the EdU-stained samples or by measuring only the area of expression of the BRLs.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: F.L.-E., A.P.-R., A.I.C.-D.; Methodology: F.L.-E., A.P.-R., J.V.-B., R.S., A.I.C.-D.; Validation: F.L.-E., A.P.-R., J.V.-B., A.I.C.-D.; Formal analysis: F.L.-E., J.V.-B., A.I.C.; Investigation: F.L. -E., A.P.-R., J.V.-B., A.I.C.-D.; Resources: F.L.-E., A.P.-R., R.S., A.I.C.-D; Data curation: F.L.-E., A.P.-R., R.S., A.I.C.-D.; Writing - original draft: F.L.-E., A.P.-R., A.I.C.-D.; Writing - review & editing: F.L.-E., A.P.-R., J.V.-B., R.S., A.I.C.-D.; Visualization: A.I.C.-D.; Supervision: A.I.C.-D.; Project administration: A.I.C.-D.; Funding acquisition: A.I.C.-D.

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Supplementary information

Supplementary information available online at
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REVIEW

Brassinosteroid signaling in plant development and adaptation to stress

Ainoa Planas-Riverola[1,*], Aditi Gupta[1,*], Isabel Betegón-Putze[1], Nadja Bosch[1], Marta Ibañes[2,3] and Ana I. Caño-Delgado[1,‡]

ABSTRACT

Brassinosteroids (BRs) are steroid hormones that are essential for plant growth and development. These hormones control the division, elongation and differentiation of various cell types throughout the entire plant life cycle. Our current understanding of the BR signaling pathway has mostly been obtained from studies using *Arabidopsis thaliana* as a model. In this context, the membrane steroid receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1) binds directly to the BR ligand, triggering a signal cascade in the cytoplasm that leads to the transcription of BR-responsive genes that drive cellular growth. However, recent studies of the primary root have revealed distinct BR signaling pathways in different cell types and have highlighted cell-specific roles for BR signaling in controlling adaptation to stress. In this Review, we summarize our current knowledge of the spatiotemporal control of BR action in plant growth and development, focusing on BR functions in primary root development and growth, in stem cell self-renewal and death, and in plant adaption to environmental stress.

KEY WORDS: Brassinosteroid, Growth, Root, Stem cell, Stress

Introduction

Brassinosteroids (BRs) are phytohormones that were originally discovered in *Brassica napus* pollen based on their ability to promote growth (Mitchell et al., 1970). Since their discovery, the main components of the canonical BR signaling pathway have been identified through multiple genetic and biochemical screens (Vert et al., 2005; Zhu et al., 2013). BR perception occurs at membrane-localized receptors and downstream cytosolic regulators transduce BR-mediated signals to the nucleus where they activate the transcription of BR-responsive genes that drive cellular growth (Belkhadir and Jaillais, 2015; Zhao and Li, 2012). Accordingly, mutations in genes encoding the main components of the BR synthesis and signaling pathways result in severe dwarfism, impaired organ growth and development, and limited plant fertility and yield (Li and Chory, 1997; Singh and Savaldi-Goldstein, 2015). Despite such knowledge of BR pathway components, many questions remain unclear, including how BRs function in a cell-specific manner, how the BR pathway interacts with other hormonal pathways under normal and environmentally challenging scenarios, and in which tissues BR synthesis occurs (Caño-Delgado and Blázquez, 2013; Vukasinovic and Russinova, 2018).

Over the past few decades, BR hormones have been shown to be essential for cell elongation and, as such, initial studies on hypocotyl elongation have been very rewarding in terms of understanding the transcriptional responses that trigger elongation (Clouse and Sasse, 1998). However, since the discovery that BRs also play a role in cell division (González-García et al., 2011; Hacham et al., 2011), studies have switched focus in an attempt to understand how BRs modulate growth and development in plants, using the primary root of *Arabidopsis thaliana* as a model. In this context, techniques such as fluorescence-activated cell sorting (Brady et al., 2007), and tools that allow the local expression of signaling components (Marquès-Bueno et al., 2016) and the visualization of cell-specific protein-protein interactions (Long et al., 2017), have been instrumental in elucidating novel BR signaling components and cell-specific signals (Fàbregas et al., 2013; Vilarrasa-Blasi et al., 2014; Vragović et al., 2015). More recent work on BRs has also begun to decode the mechanisms by which BR-mediated signaling regulates adaptation to biotic (De Bruyne et al., 2014) and abiotic (Lozano-Durán and Zipfel, 2015; Nolan et al., 2017a) stresses. Here, we review these recent advances that aim to decipher the spatiotemporal control of BR action. First, we provide an overview of the BR signal transduction pathway and then discuss how BRs regulate root growth and development in a cell-specific fashion. We also highlight how BRs function within some of the most special cells of the plant, the root stem cells. Finally, we review our current understanding of the roles of BRs and their crosstalk with other hormones in mediating adaptation to abiotic stresses, such as drought, temperature changes and salinity.

Brassinosteroid ligand perception and signal transduction

BR hormones are perceived extracellularly by members of the BRI1 (BRASSINOSTEROID INSENSITIVE 1) leucine-rich repeat receptor-like kinase (LRR-RLK) family (Li and Chory, 1997; Wang et al., 2001). The BR hormone binds directly to a 93-amino-acid region located within the extracellular domain of membrane-bound BRI1 (Hothorn et al., 2011; Kinoshita et al., 2005; Sun et al., 2013). Direct binding triggers the formation of a BRI1-BAK1 [BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1, also known as SERK3 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3)] heterodimer, which in turn initiates an intracellular phosphorylation relay cascade (Li and Nam, 2002; Russinova et al., 2004). The cascade (Fig. 1A) culminates in promotion of the activity and stability of the plant-specific transcription factors BZR1 (BRASSINAZOLE RESISTANT 1) (Wang et al., 2002) and BES1 (BRI1-EMS-SUPPRESSOR 1) (Yin et al., 2002), which directly control the transcription of thousands of BR-responsive genes and hence regulate a plethora of developmental events in the plant (He et al., 2002; Sun et al.,

[1]Department of Molecular Genetics, Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Barcelona E-08193, Spain. [2]Departament de Física de la Matèria Condensada, Universitat de Barcelona, Barcelona 08028, Spain. [3]Universitat de Barcelona Institute of Complex Systems (UBICS), Universitat de Barcelona, Barcelona 08028, Spain.
*These authors contributed equally to this work

‡Author for correspondence (ana.cano@cragenomica.es)

A.I.C., 0000-0002-8071-6724

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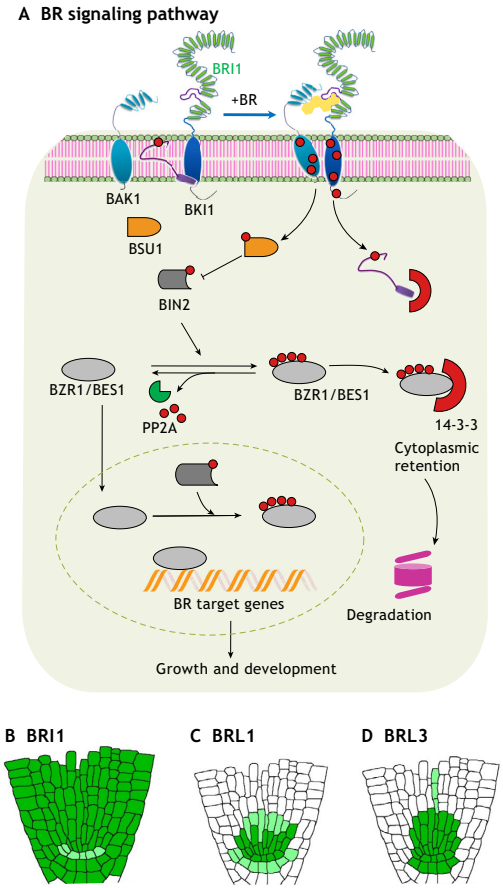

Fig. 1. An overview of the BR signaling pathway. (A) Schematic of the BRI1 signaling pathway. In the absence of BR, BIN2 phosphorylates BZR1 and BES1 proteins, inactivating them by promoting their binding to 14-3-3 proteins, leading to their cytoplasmic retention and degradation. When BRI1 perceives BR molecules, it heterodimerizes with BAK1, initiating an intracellular phosphorylation relay cascade that ends with the dephosphorylation and consequent activation of BZR1 and BES1. (B-D) Schematics of the root tissue-specific expression of BR receptors. BRI1 is expressed throughout the root (B), whereas BRL1 (C) and BRL3 (D) exhibit a more discrete expression pattern, being active mainly in the root stem cell niche area. Dark green represents high expression of the protein, whereas light green represents lower expression.

2010). When BRs are absent, the GSK3-like kinase BIN2 (BRASSINOSTEROID-INSENSITIVE 2) phosphorylates BZR1/BES1 proteins and inactivates them, promoting their binding to 14-3-3 proteins and leading to their cytoplasmic retention and degradation (Gampala et al., 2007; Li and Nam, 2002; Peng et al., 2008). This thereby inhibits their ability to bind DNA and causes pathway inactivation.

Based on the presence of the extracellular BR-binding domain, there are three membrane-localized BRI1-like homologs named BRL1, BRL2 and BRL3 (BRI1-LIKE 1, 2 and 3). Whereas BRL1 and BRL3 are functional BR receptors that, like BRI1, can bind to steroid molecules with high affinity, BRL2 appears not to be a functional BR receptor (Caño-Delgado et al., 2004). Furthermore, whereas BRI1 is expressed nearly ubiquitously in the root (Friedrichsen and Chory, 2001) (Fig. 1B), the BRLs are found only in some specific tissues (Fig. 1C,D). For example, BRL1 and BRL3 are localized in vascular stem cells, where they govern cell-specific BR-response pathways (Caño-Delgado et al., 2004; Fàbregas et al., 2013; Salazar-Henao et al., 2016). Under native conditions, both BRL1 and BRL3 can heterodimerize with the BAK1 co-receptor, but not with BRI1, and form a complex (Fàbregas et al., 2013). These studies suggest that BRI1 and the BRLs are able to form different receptor complexes in different cell types, thereby performing different signaling roles, but the specific downstream components of the BRL1 and BRL3 pathways remain unknown.

The primary root as a model for deciphering cell-specific brassinosteroid signaling

Owing to its simple and radial organization of cell types, the primary root of *Arabidopsis* provides an excellent model for dissecting signaling mechanisms with cell-specific resolution (Dolan et al., 1993; Scheres et al., 1994). Indeed, a number of studies of the primary root have shown that BRs control specific cellular processes in distinct root cell types (Fig. 2).

BRs play an important role in overall root development; both an excess and a lack of BRs are detrimental to primary root growth and development. On the one hand, mutants lacking BR compounds or BR receptors exhibit short roots, indicating that BRI1 signaling is required for root growth (Chaiwanon and Wang, 2015; González-García et al., 2011; Hacham et al., 2011; Mussig et al., 2003). On the other hand, short roots are also observed in *bes1-D* (gain-of-function) mutants, or in plants treated with high concentrations of BRs (González-García et al., 2011; Mussig et al., 2002). The short roots of mutants with impaired BR biosynthesis can be rescued by treatment with low concentrations of BR (Chaiwanon and Wang, 2015). Moreover, supporting the notion that BRs can promote root growth, it has been shown that wild-type roots treated with low concentrations of BRs increase their length (González-García et al., 2011; Mussig et al., 2003), although this enlargement is small and not always detectable (Chaiwanon and Wang, 2015). Altogether, these results suggest that, rather than controlling root growth in a linear fashion, the correct balance of BR levels appears to be crucial for normal root growth and development (González-García et al., 2011).

Root growth also depends on cell proliferation at the meristem and on cell elongation prior to differentiation. BRs impinge on both of these processes. BRs modulate meristematic proliferation (González-García et al., 2011; Hacham et al., 2011) and have been proposed as key regulators in the optimal control of cell cycle progression (González-García et al., 2011). BRs have been also proposed to be crucial for optimal cell expansion (Chaiwanon and Wang, 2015; Clouse and Sasse, 1998). Recent mathematical and computational modeling has further demonstrated that root growth features depend on the mechanism by which cell elongation terminates, e.g. whether cells stop elongating according to their spatial position along the root, according to a time interval, and/or according to their cell size (Pavelescu et al., 2018). Quantification of cell length in single roots, together with mathematical and computational modeling, suggests that the dominant mechanism for cell elongation termination is a size-based mechanism whereby root cells stop expanding when they reach a determined length, and that BRI1 facilitates this mechanism (Pavelescu et al., 2018). In addition, this suggests that BR signaling at least partially controls these three separate functions: cell division, cell elongation rate and termination of cell elongation (Pavelescu et al., 2018). Indeed, plants treated with high concentrations of BR increase expansion at

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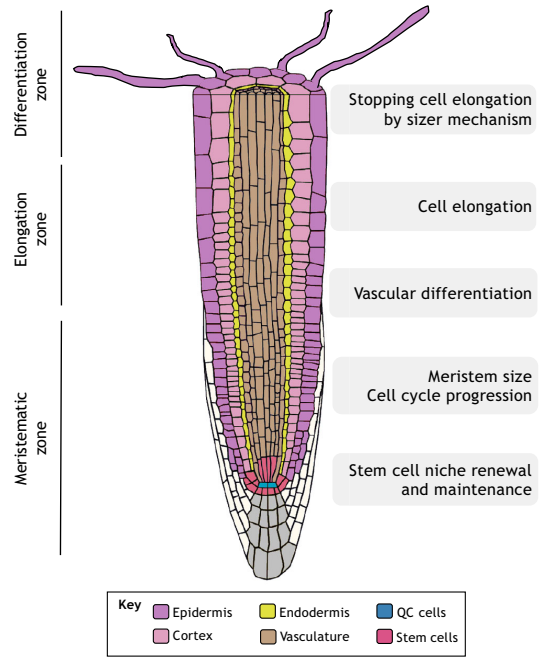

Fig. 2. BR functions in the primary root. BRs are involved in a variety of cell-specific processes that occur within the different zones of the root. These include processes such as cell cycle division, cell elongation and cell differentiation.

the meristem and reduce the number of meristematic cells, but do not exhibit an increase in meristem cell length (Chaiwanon and Wang, 2015).

The control of root growth by BR signaling is also spatially segregated throughout the root. BR signaling is not found homogeneously throughout the root, with BZR1 being more strongly activated at the transition from the meristem to the elongation zones and in the elongation zone itself (Chaiwanon and Wang, 2015). Moreover, BR signaling induces target genes in the epidermis (the outer layer of the root) but mostly represses genes in the stele (the inner layer) (Vragović et al., 2015), highlighting that BR signaling can elicit tissue-specific responses. Based on these results, it has been proposed that BR signaling can function in a non-cell-autonomous manner, signaling from the epidermis to inner cells (Hacham et al., 2011; Vragović et al., 2015). Interestingly, the differential expression of BRI1 between hair and non-hair epidermal cells controls the length of mature cells as well as their sensitivity to BR hormonal treatment (Fridman et al., 2014). Furthermore, it was recently shown that expressing BRI1 under the control of cell-specific promoters of the protophloem (a component of the stele) such as pMAKR5 (MEMBRANE-ASSOCIATED KINASE REGULATOR 5) and pCVP2 (COTYLEDON VASCULAR PATTERN 2) rescues the phenotypic defects of *bri1 brl1 brl3* triple receptor mutants, suggesting that a phloem-derived signal can non-autonomously drive root growth (Kang et al., 2017). These results point to the complexity of BR signaling and highlight some level of directionality – from inner to outside cell layers and vice versa – of BR signaling in the root. This signaling directionality likely depends on the cell-specific expression and site of action of BR receptors, which could promote specific signals and thus contribute differentially to overall root development. Given that BRL receptors function in the phloem (Caño-Delgado et al., 2004) and the recent proposed role for BRL3 in root mobilization of osmoprotectant metabolites to confer drought resistance (Fàbregas et al., 2018), we propose that that BR receptors expressed in the inner layers of the root may selectively promote growth under stress.

BR signaling is also involved in the development of vascular tissues within the plant. Early studies in *Zinnia elegans* cells indicate that BR synthesis increases prior to, and is necessary for, tracheary element differentiation (Yamamoto et al., 2001), and in *Arabidopsis* suspension cultures BRs induce VND7-mediated xylem cell wall differentiation (Yamaguchi et al., 2010). In *Arabidopsis*, BR-deficient plants harboring mutations in genes such as *CPD* (*CONSTITUTIVE PHOTOMORPHOGENIC DWARF*) and *DWF7* (*DWARF 7*) have abnormal xylem development (Choe et al., 1999; Szekeres et al., 1996). BR receptor mutants also exhibit abnormal vascular differentiation, a process in which BRI1 and the BRLs have redundant functions (Caño-Delgado et al., 2004). In the primary root, BR suppresses radial vascular cell divisions (Fàbregas et al., 2013; Kang et al., 2017). In line with this, the *brl1 brl3 bak1-3* triple mutant is hypersensitive to BR in the stele, showing greater stele narrowing than that of wild-type, *bak1* or *brl1 brl3* mutant plants upon BR treatment (Fàbregas et al., 2013). In addition, the wider stele of the *bri1 brl1 brl3* triple mutant increases when BRI1 is expressed in the stele and decreases when BRI1 is expressed in the epidermis (Kang et al., 2017). Thus, the control of formative asymmetric divisions in the stele can be controlled both cell-autonomously and non-cell-autonomously in an opposite manner, implying that the nature of the stele divisions might depend on the localization of the instructing signal. Conversely, the control of formative asymmetric cell divisions in the epidermis appears to be cell-autonomous, as expression of BRI1 in the epidermis restores the wider phenotype of the *bri1 brl1 brl3* triple receptor mutant (Kang et al., 2017). Of note, BRs together with auxins are also involved in establishing the periodic pattern of vascular bundles in the *Arabidopsis* shoot (Ibanes et al., 2009); the quantification of this pattern, together with mathematical modeling, supports the notion that cell numbers, which are controlled by BRs, are relevant for vascular patterning. However, despite these various lines of evidence linking BRs and vascular development, little is known about the contribution of different BR receptors and downstream transcriptional players in the formation of functional vascular tissues and overall organ growth.

The role of brassinosteroid signaling in stem cell self-renewal and differentiation

The root stem cell niche comprises a small group of stem cells located at the base of the meristem in the root apex. These cells are essential for sustaining root growth, as they continuously provide the precursors of more-specialized cells, and to replace tissues that have been damaged (Dolan et al., 1993; Sabatini et al., 2003; van den Berg et al., 1997). The core of the niche contains a group of cells with very low mitotic activity that are collectively known as the quiescent center (QC). The QC maintains the undifferentiated state of the surrounding stem cells (Sarkar et al., 2007; van den Berg et al., 1997) whilst maintaining its own stemness, but it can also act as a reservoir of cells that can replenish damaged ones (Heyman et al., 2013; Vilarrasa-Blasi et al., 2014). As we discuss below,

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BRs play a key role in maintaining the identity and quiescence of QC cells (González-García et al., 2011), and thereby affect the maintenance of the root stem cell niche.

BR signaling acts within the root stem cell niche by modulating BRAVO (BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER) (Vilarrasa-Blasi et al., 2014). This transcription factor, also named MYB56, belongs to the R2R3-MYB family and is expressed specifically in vascular initials and QC cells (Vilarrasa-Blasi et al., 2014). Phenotypic analyses have shown that BRAVO represses QC cell divisions (Fig. 3A), as *bravo* mutants show a significant increase in QC division frequency. However, when BR signaling is activated, for example following DNA damage (Fig. 3B), the BR downstream effector BES1 becomes activated and downregulates the levels of *BRAVO* transcript. It also heterodimerizes with BRAVO protein itself, strongly inhibiting its action and promoting the division of QC cells (Vilarrasa-Blasi et al., 2014). This constitutes a regulatory circuit that controls QC division via interactions at both the transcriptional and protein levels. Another transcription factor that acts as a co-repressor of BRAVO is TPL (TOPLESS), which can bind to the *BRAVO* promoter as well as interact with BES1 via its ERF-associated amphiphilic repression (EAR) motif (Espinosa-Ruiz et al., 2017). Future studies aiming to dissect the cell-specific gene regulatory networks controlled by BRAVO in the stem cell niche will be instrumental for uncovering how and when QC cells divide.

BRs can also induce the expression of ERF115 (ETHYLENE RESPONSE FACTOR 115), a transcription factor that belongs to the ethylene response factor family and plays a key role in root growth and development. Specifically, ERF115 acts as a limiting factor for QC divisions as it regulates the expression of PSK5 (PHYTOSULFOKINES 5), a peptide hormone that enhances the frequency of QC divisions (Heyman et al., 2016, 2013). Collectively, BR signaling represses BRAVO activity and, at the same time, activates ERF115 to promote QC divisions when needed (Fig. 3B). However, it is still unknown where the signals that activate the BR pathway originate from, i.e. if they come from external tissues or if this process is carried out in a cell-autonomous way. Nonetheless, a

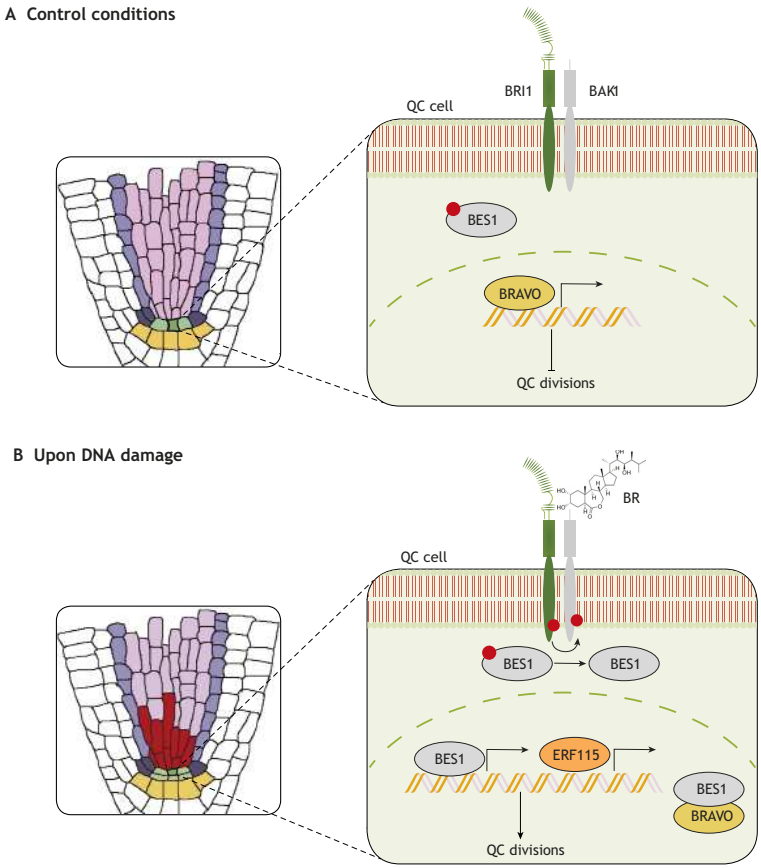

Fig. 3. Functional role of BRs in stem cell regeneration. (A) In normal ('control') conditions, BR signaling in the QC is not active. This maintains BES1 in a phosphorylated and inactive state, permitting BRAVO to act and repress QC divisions. (B) In the presence of DNA damaging agents, vascular cells die and produce a local pool of BR that is sensed by BRI1/BRLs in a paracrine way in the QC. This leads to the dephosphorylation of BES1, the inactivation of BRAVO (both transcriptionally and via BRAVO heterodimerization with BES1) and the induction of ERF115 expression, which together promote the division of QC cells to replenish the dying cells.

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recent study has shed some light on this matter, revealing that QC cell division is an autonomous process that needs BRI1 action within the stem cell niche (Lozano-Elena et al., 2018). This study also suggested that a paracrine signal leads to the activation of BES1 in QC cells in order to promote their division when needed. Thus, when the root suffers damage and stem cells undergo programmed cell death, the plant detects this scenario and starts promoting QC cell divisions to replenish damaged cells and to assure its survival. Although the mechanism underlying this response remains to be elucidated, it appears to involve a steroid paracrine signal from dead cells to the QC and that is perceived by BRI1 and transduced by BES1 (Lozano-Elena et al., 2018). We hypothesize that one such mobile signal could be the BR molecule itself, and that the increase in BR concentration in the stem cell niche could be due to a possible increase in BR biosynthetic genes, such as those encoding CPD and DWF4 (DWARF 4) (Lozano-Elena et al., 2018). However, further studies of BR synthesis and mobility are required to shed light on this matter (Vukasinovic and Russinova, 2018).

BRs also promote the differentiation of columella stem cells (CSCs), cells which are located distally to the QC. This occurs in a dose-dependent manner (González-García et al., 2011; Lee et al., 2015) via the transcription factor WOX5 (WUSCHEL-RELATED HOMEOBOX 5). WOX5 is a homolog of WUSCHEL, a transcription factor that maintains the identity of stem cells in the shoot (Mayer et al., 1998). In the root, WOX5 is required to maintain the identity of stem cells (Sarkar et al., 2007), and its transcript expression is restricted to the QC through external signals (Ding and Friml, 2010; Zhang et al., 2015) where it represses CYCD activity to establish quiescence (Forzani et al., 2014). *wox5* mutants show increased QC divisions and differentiated CSCs in the root apex (Sarkar et al., 2007). Importantly, the expression of WOX5 is regulated by BR; WOX5 expression decreases in *bri1-116* mutants (lacking the BRI1 receptor) and in plants treated with brassinazole (an inhibitor of BR biosynthesis). In contrast, WOX5 expression increases in plants treated with brassinolide (a bioactive form of BR) and in *bes1-D* or BRI1 overexpressor mutants (González-García et al., 2011).

In summary, BR levels are essential for regulating both cellular quiescence and the differentiation of stem cells in the root apex. Further studies, including cell-specific 'omics' approaches, will be key to decipher, for example, BRAVO partners and targets in the stem cell niche. It will also be interesting to decipher which receptors are involved in this context, and if the promotion of QC divisions is mediated primarily by BRI1 or if BRL1/3 play a major role as a consequence of their expression pattern throughout the root.

Brassinosteroid signaling in adaptation to environmental stress

The ability of a plant to tolerate stress, such as changes in water availability, temperature or soil salinity, depends on its ability to switch between growth activation and repression in unfavorable conditions (Bechtold and Field, 2018; Feng et al., 2016). A key pathway that controls responses to environmental stresses is the abscisic acid (ABA) signaling pathway (Yoshida et al., 2014; Zhu et al., 2017). However, compelling evidence indicates that BRs also play a prominent role in controlling the balance between normal growth and resistance against environmental assaults, acting either via crosstalk with the ABA pathway or independently (Fig. 4). Several mechanisms have been proposed to explain how BR signaling mediates adaptation to stress. These include: (1) fine-tuning stress-responsive transcript machineries (Ye et al., 2017); (2) activating antioxidant machineries (Kim et al., 2012; Lima and Lobato, 2017; Tunc-Ozdemir and Jones, 2017; Xia et al., 2009; Zou et al., 2018); and (3) promoting the production of osmoprotectants (Fàbregas et al., 2018). As we discuss below, these various mechanisms contribute to BR-mediated adaptation to drought, cold, heat and salinity.

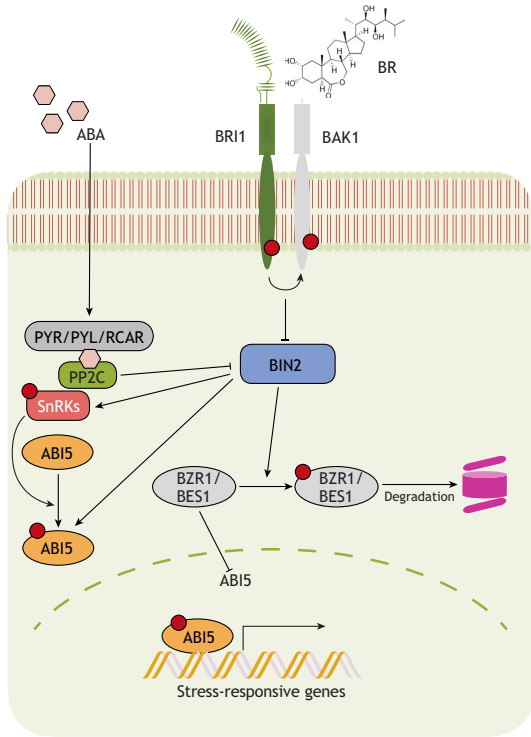

Fig. 4. BR-ABA crosstalk during the regulation of stress responses. Schematic of the crosstalk between the BR and ABA pathways. ABA is perceived by PYR/PYL/RCAR receptors and promotes the phosphorylation and activation of SnRKs, thereby relieving them from PP2C-mediated repression. SnRKs, in turn, phosphorylate downstream transcription factors such as ABI5 that regulate the transcription of various stress-responsive genes. BIN2, which is a negative regulator of BR signaling, can also directly phosphorylate and activate SnRKs and ABI5, while PP2C is able to inactivate BIN2. ABI5 is also a direct target of BZR1, which represses its transcription to negatively regulate stress-responsive gene expression.

BRs and ABA perform mostly antagonistic physiological functions, converging at the level of BIN2 and BZR1 (Cai et al., 2014; Hu and Yu, 2014). Whereas BIN2 acts a repressor of BR signaling (as discussed above), it enhances ABA-mediated stress responses by phosphorylating SnRK2 (SNF1-RELATED PROTEIN KINASE 2), leading to ABA-responsive gene expression (Cai et al., 2014). In addition, exogenous BR treatment inhibits the ABA-mediated induction of *RD26* (*RESPONSIVE TO DESICCATION 26*), a gene encoding a transcriptional activator of stress-inducible gene expression (Chung et al., 2014). This reciprocal antagonism between BR signaling and ABA-responsive transcription factors is key for coordinating plant growth and drought tolerance in *Arabidopsis* (Fig. 4). Indeed, it has been shown that RD26 is also a direct target of BES1 and is repressed by BR under drought conditions; reciprocally, RD26 modulates the transcription of BES1-regulated genes to inhibit BR function (Ye et al., 2017). The transcription factors WRKY46,

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54 and 70 also interact with BES1 directly to promote BR-regulated plant growth while repressing drought-inducible global transcripts to inhibit drought tolerance (Chen and Yin, 2017). BIN2 phosphorylates and destabilizes WRKY54 to negatively regulate its effect on the BES1-mediated BR response (Chen et al., 2017). Recently, it was revealed that BR signaling via BIN2 interacts with autophagy pathways to coordinate plant growth and survival under drought stress and starvation (Nolan et al., 2017b) (Fig. 5A). In this context, BIN2 phosphorylates and activates the ubiquitin receptor protein DSK2, which further interacts with BES1 and targets it for degradation via autophagy (Nolan et al., 2017b). Together, these findings highlight the complexity of BR-mediated responses to drought. Future investigations are clearly needed to unravel the roles of individual BR signaling components and to understand how they switch the balance between normal versus drought-adapted growth and development.

BR signaling also modulates plant adaptation to different temperature stresses (Fig. 4B,C). The BR-regulated basic helix-loop-helix (bHLH) transcription factor CESTA activates the expression of C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTOR (CBF) transcriptional regulators, which control the transcription of core cold responsive (COR) genes (Eremina et al., 2016). Another BR-regulated transcription factor, BR-ENHANCED EXPRESSION 1 (BEE1), promotes cold acclimation by indirectly influencing the transcription of MYB-bHLH-WD40 complex components (Petridis et al., 2016) (Fig. 5B). In line with these findings, BIN2 overexpression has been shown to cause hypersensitivity to freezing stress under both non-acclimated and acclimated conditions, whereas *bin2-3 bil1 bil2* triple mutants, as well as the gain-of-function *bzr1-1D* and *bes1-D* mutants, have enhanced tolerance for freezing stress (Li et al., 2017). BZR1 dephosphorylation is also induced upon cold treatment and can regulate COR genes, either directly or indirectly by binding to CBF1 and CBF2, and thereby affect the transcription of their downstream targets (Li et al., 2017) (Fig. 5B). BR signaling is also involved in regulating plant growth under high temperature stress (Fig. 5C). Upon elevated temperature, BZR1 accumulates in the nucleus and induces the expression of growth-promoting genes, either directly or via binding to the promoter of *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) to regulate thermomorphogenesis (Ibañez et al., 2018; Oh et al., 2012). Elevated temperature has been shown to increase the accumulation of active PIF4, thereby shifting the balance of nuclear protein complexes towards BES1-PIF heterodimers instead of BES1 homodimers (Martinez et al., 2018). The subsequent reduced availability of active BES1 homodimers causes de-repression of BR biosynthesis and feedback inhibition of BR signaling output. In contrast, abundant levels of BES1-PIF4 complexes activate the genes involved in thermomorphogenesis (Martinez et al., 2018). Elevated ambient temperatures can also reduce BRI1 levels and affect primary root elongation growth (Martins et al., 2017). Recently, the kinase-defective BRI1 protein from *bri1-301* mutants was found to show less stability and biochemical activity under elevated temperature (29°C). A mutated version of this protein undergoes temperature-enhanced protein misfolding and degradation via an as-yet-unknown mechanism (Zhang et al., 2018). Together, these studies highlight a clear involvement of both BR receptors and downstream signaling components in regulating growth responses under fluctuating temperatures.

BR signaling is also able to mediate salt tolerance. It does so via the regulation of ethylene biosynthesis and signaling (Fig. 5D). Under salinity stress conditions, BR pre-treatment induces ethylene production, and hence signaling, by enhancing the activity of 1-aminocyclopropane-1-carboxylate synthase (ACS), an ethylene synthesis enzyme (Tao et al., 2015; Zhu et al., 2016). Conversely, blocking ethylene production and/or signaling components inhibits BR-induced antioxidant enzyme activities and salt tolerance (Tao et al., 2015; Zhu et al., 2016). The role for BR signaling in regulating salt stress tolerance may be mediated by BRI1; inhibiting the endoplasmic reticulum-associated protein degradation system is able to partially rescue the salt hypersensitivity of *bri1-9* mutants, providing evidence for the involvement of a membrane-bound BRI1 signaling complex in the salinity response (Cui et al., 2012). In contrast, *bin2-1* mutants are hypersensitive to salinity stress, and this correlates with inhibited induction of stress-responsive genes (Zeng et al., 2010). High salinity also causes growth quiescence in roots by suppressing nuclear accumulation of BZR1 and subsequent BR signaling functions (Geng et al., 2013). It is evident from the above-mentioned reports that exogenous application of BR helps plants to cope better under high salinity conditions by modulating both BR and ethylene signal outputs.

In addition to the crosstalk and mechanisms discussed above, the interplay between BR signaling and redox signaling appears to be crucial for plant development under stress (Fig. 5A). It is known that BR induces the antioxidant system during abiotic stress tolerance (Jiang et al., 2012; Zhou et al., 2014). BR has also been reported to utilize hydrogen peroxide (H_2O_2)- and nitric oxide (NO)-mediated mechanisms to provide stress tolerance (Cui et al., 2012; Xia et al., 2009). For example, during oxidative stress, BR increases ABA production through NO-mediated machinery (Zhang et al., 2011). BR-mediated transient H_2O_2 production via NADPH oxidase also triggers ABA biosynthesis, which, along with enhanced H_2O_2 production, acts as a positive-feedback mechanism for prolonged heat and oxidative stress tolerance (Zhou et al., 2014). The over-accumulation of superoxide anions (O_2^-) in the BR biosynthesis-defective mutant *det2-9* highlights yet another node of crosstalk between the BR and reactive oxygen species (ROS) pathways that is implicated in controlling root growth and development (Lv et al., 2018). Interestingly, this BR-mediated control of O_2^- accumulation was found to occur through the peroxidase pathway rather than the NADPH oxidase pathway (Lv et al., 2018). H_2O_2-mediated oxidative modifications enhance the transcriptional activity of BZR1 and promote its interaction with ARF6 and PIF4. In contrast, the thioredoxin TRXh5 interacts with BZR1 and catalyzes its reduction (Tian et al., 2018) (Fig. 5A). Exogenous BR application also increases H_2O_2 production in the root stem cell niche, contributing to BR-induced QC division and cell elongation (Tian et al., 2018).

Nutrient availability in the soil microenvironment is another limiting factor for optimal root growth. BR signaling components were recently shown to regulate root growth behavior under low iron or phosphate levels (Singh et al., 2018). Specifically, it was found that BR signaling becomes activated upon iron deficiency and promotes root growth, and similarly that perturbed BR signaling affects iron distribution in *Arabidopsis* roots. In contrast, low phosphate levels cause enhanced iron accumulation, inhibiting BR signaling activation and subsequent root growth acceleration. The BRI1 negative regulator BKI1 was found to be the center point of this signal interplay, with BZR1/BES1, along with their direct target LPR1, which is a ferroxidase, acting at more downstream steps in this response (Singh et al., 2018). Moving forward, obtaining a more comprehensive understanding of the complex interplay between BR signaling, cellular redox status and the surrounding microenvironment will undoubtedly prove beneficial for

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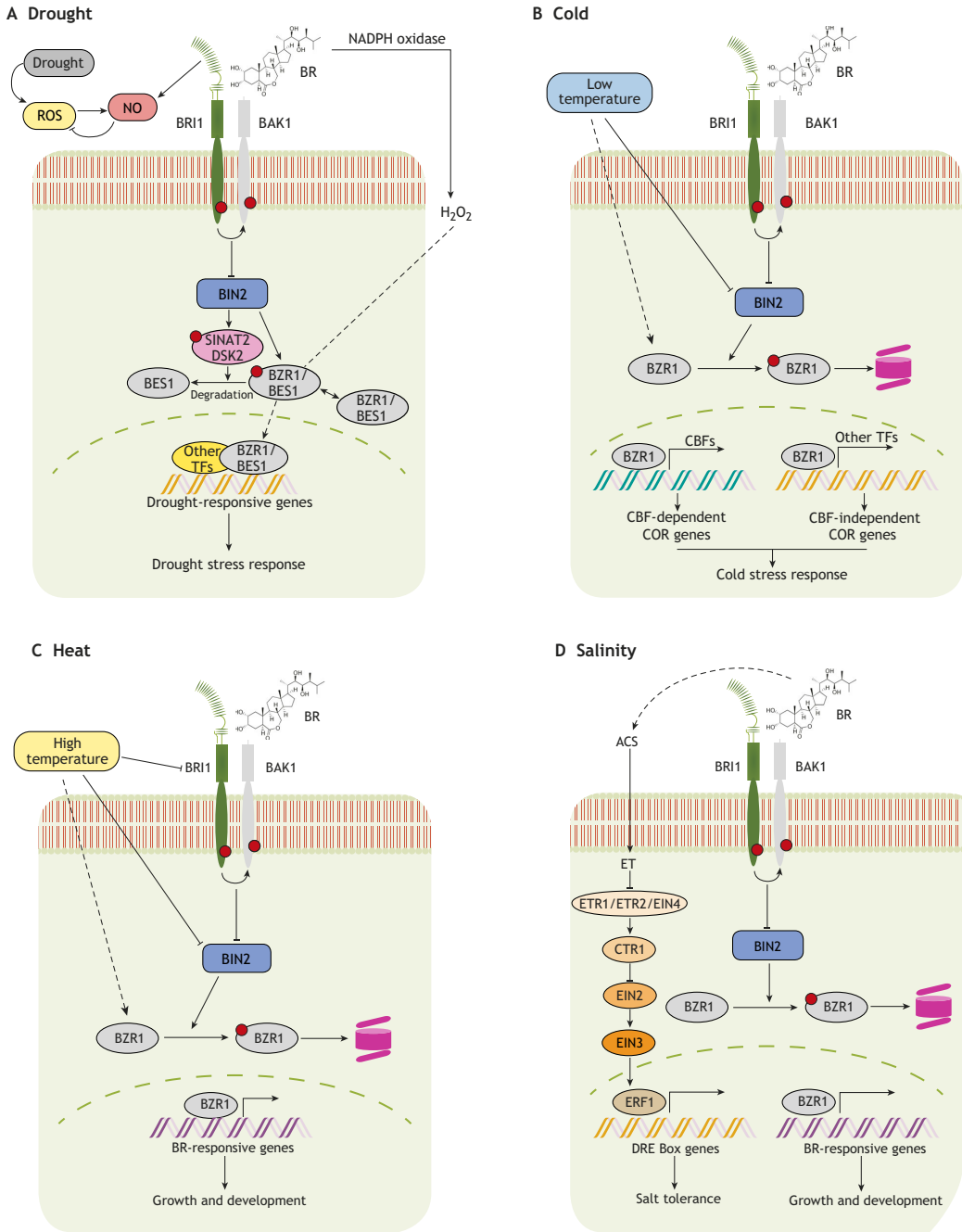

Fig. 5. BR signaling controls the switch between growth and abiotic stress responses. (A-D) Schematics of cellular BR actions and crosstalk under conditions of drought (A), cold (B), heat (C) and high salinity (D). Notably, BRs act to control the balance between plant growth and stress responses. The BR and stress signaling pathways show multi-level crosstalk via their receptors, via the downstream kinase BIN2 and/or via transcription factors such as BZR1/BES1, depending on external as well as cellular environments. ET, ethylene.

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understanding the mechanisms of plant survival and growth adaptation in suboptimal growth conditions.

Many of the BR-regulated stress adaptation responses discussed above have been described at the whole-plant survival level. However, recent technological advances are now allowing us to deconstruct the complexity of stress traits in a more spatiotemporal fashion. This approach has been instrumental in identifying the spatiotemporal roles of other phytohormones during stress responses in plants (Dinneny and Benfey, 2008; Geng et al., 2013; Iyer-Pascuzzi et al., 2011). Recently, a role for the vascular cell-specific activation of BR signaling in regulating drought adaptation in different developmental stages of root and shoot organs was uncovered using a multi-omics approach (Fàbregas et al., 2018). This study revealed that the quadruple BR receptor mutant (*bri1 brl1 brl3 bak1*) exhibits enhanced drought tolerance at the expense of overall growth. However, the overexpression of vascular-localized BRL3 receptors significantly improves drought tolerance without penalizing growth. In this case, BRL3 receptor accumulation in vascular tissues triggers the transcription of canonical water stress-response genes and osmoprotectant metabolism genes under both normal as well as water-deprived conditions. Metabolomic analyses confirmed that BRL3-overexpressing roots are enriched in osmoprotectant sugars and amino acids, and analysis of the transcriptome showed that it is enriched in genes involved in abiotic stress responses (Fàbregas et al., 2018). Altogether, these changes indicate that BRL3-overexpressing plants are better prepared for any upcoming stress, which in this case is drought (Fàbregas et al., 2018). This finding is corroborated by previous results reporting that BRs regulate metabolic flux, flavonol accumulation and anthocyanin synthesis during cold acclimation (Petridis et al., 2016), and other studies showing that BR application and BZR1 overexpression promote carotenoid, soluble sugar and ascorbic acid accumulation (Liu et al., 2014). Another example of spatiotemporal compartmentalization of BR signaling has recently been reported (Lozano-Elena et al., 2018). This study highlighted that paracrine BR signals from damaged cells can activate QC division and stem cell replenishment to compensate for root growth arrest upon genotoxic stress. Given the tissue-specific localization and regulation of different BR signaling components, combined with the complexity and diversity of stress-responsive mechanisms, it is likely that decisions of growth versus adaptation are made by signal activation/suppression on spatiotemporal scales. Understanding how these spatiotemporal variations in the activity of BRs control growth and plant adaptation to various environmental stresses is essential for understanding the mechanism by which plants balance growth with adaptation to ensure survival.

Conclusions and perspectives

BRs are key for maintaining proper plant growth, both under normal conditions and in response to environmental stress, and ample evidence now supports the idea that modifying the BR response pathway can be a powerful strategy for designing better-adapted crops. However, our understanding of the main functions of BR signaling during stress is only generic, and the investigation of precise spatiotemporal- and context-specific regulatory mechanisms has only just begun (Kang et al., 2017; Lozano-Durán and Zipfel, 2015; Lozano-Elena et al., 2018; Vragović et al., 2015). Further studies are clearly required to obtain a more mechanistic understanding of the global and local actions of the BR pathway. With such knowledge, we could improve both the growth rates of plants and their adaptation to the environment by only changing the BR signal in specific tissues, making, for example, plants that are resistant to drought without altering their growth. Such an approach will be important to meet the food demands of an exponentially growing world population, especially when increasing plant yield in environmentally challenging conditions becomes essential (Food & Agriculture Organization, 2017).

Excitingly, studies have indicated that the local activities of the different BR receptors – BRI1 and the BRLs – and their effects on root development vary (Kang et al., 2017; Vragović et al., 2015); this could be one of the mechanisms through which BRs execute their pleiotropic effects on growth and stress adaptation. We propose a scenario (Fig. 6) in which, under normal conditions, BRI1-mediated signals drive the growth and development of roots and, subsequently, of the whole plant. This idea is supported by the finding that the lack of this receptor produces dwarf and sterile plants with shorter roots (Li and Chory, 1997). In contrast, the BRL1/BRL3 receptors seem to have little impact on these physiological processes, as mutants of both receptors (*brl1 brl3* mutants) do not show any visible phenotype (Caño-Delgado et al., 2004). However, these BR receptors, which exhibit tissue/cell-specific expression patterns, could be more involved during stress responses and adaptation. For example, vascular BRL3 expression is able to confer drought resistance, driving the accumulation of osmoprotectant metabolites in the root by promoting the activity of genes involved in their production. Moreover, it is known that *brl1 brl3* and *brl1 brl3 bak1* mutants have phenotypes associated with

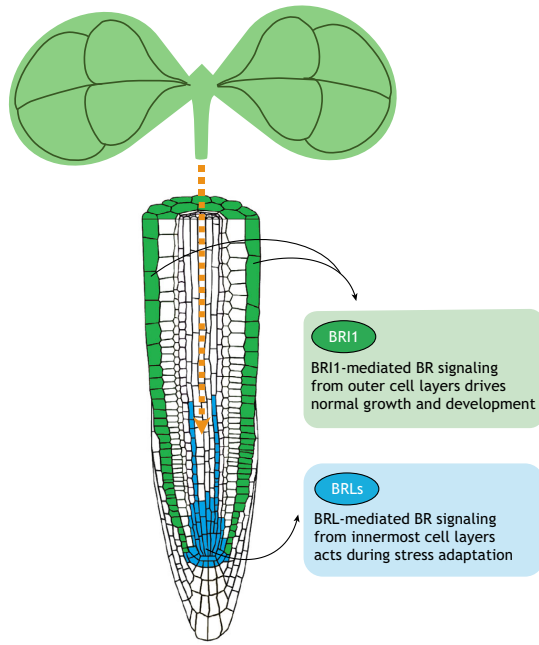

Fig. 6. Model depicting the tissue-specific actions of BR receptors during growth and stress responses. Schematic of a scenario in which BRI1-based BR signaling from outer tissues (green) primarily regulates normal growth and development, whereas the signaling mediated by BRLs situated in the innermost cell layers (e.g. in the QC, the stem cell niche and vascular tissues; blue) controls stress adaptation responses. BRLs might also be involved in facilitating the mobilization of metabolic signals (orange arrow) from the shoot to root to provide stress tolerance.

hydrotropism that are independent of the BRI1 pathway (Fàbregas et al., 2018), suggesting that the response to different stresses might be driven by BR receptors in specific cell types, such as stem cells and vascular tissues (Fig. 6). The identification of these BR receptor-driven differential signals will not only illustrate how different tissues coordinate their organ growth, but may prove to be useful for engineering new plants that have improved adaptation to the environment without modified growth.

Finally, it will also be important to capture the canonical as well as non-canonical signaling dynamics that function downstream of different BR receptor complexes. Examining these over different time and spatial scales may enable the identification of novel candidates that are relevant for adaptation upon stress-induced damage. A more precise and quantitative visualization of BRI1- and/or BRL-mediated cellular responses, such as ROS and NO production, stress-responsive transcription factor activation and downstream transcript regulation in different root tissues, will also help establish how BR executes stress protection and subsequent growth recovery. Overall, studies of the mechanisms underlying BR-regulated growth, in both optimal and stress conditions, will bring us closer to understanding the trade-off between growth and adaptation, and will help us strategize new approaches for creating smart root systems with efficient water and nutrient uptake abilities that can sustain crop biomass and yield.

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Competing interests
The authors declare no competing or financial interests.

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Spotlight

New Role for LRR-Receptor Kinase in Sensing of Reactive Oxygen Species

Ainoa Planas-Riverola,[1] Enara Markaide,[1] and Ana I. Caño-Delgado[1,*]

Understanding how reactive oxygen species (ROS) are sensed could help engineer plants with better stress responses that are relying on the production of ROS. Here, we summarize the latest research in ROS signaling with focus on the discovery by Wu *et al.* of a leucine-rich repeat receptor kinase (LRR-RK) as a hydrogen peroxide (H_2O_2) sensor.

Introduction

For decades, ROS were considered as harmful byproducts generated by metabolism in aerobic organisms. However, recent evidence demonstrates that low levels of ROS have key regulatory roles in cell fate signaling, including plant growth and developmental processes, as well as in plant biotic and abiotic stress responses. Environmental stress conditions induce the overproduction of ROS in plant cells and, under these circumstances, ROS molecules can trigger signal transduction events leading to elicitation of a bulk of different specific cellular responses, which are sensitive to redox situation. Every type of ROS is unique, due to differences in chemical properties and in individual half-life times. Singlet oxygen ($^1O_2$) can oxidize lipids, proteins and guanidine residues of DNA, whereas superoxide ($O_2^{\bullet-}$) reacts with Fe-S proteins, with both types of molecule being relatively stable. Hydroxyl ($OH^{\bullet}$) radicals are extremely reactive and generally unstable. By contrast, H_2O_2 is more stable, which is why it is considered the predominant ROS involved in cellular signaling [1].

Although ROS biosynthesis and signaling have been intensively studied, new mechanisms that control or interact with these pathways are still being unraveled. The most recent breakthrough in the field was the discovery of the first sensor of H_2O_2 in plants. Here, we summarize the latest research in ROS biosynthesis, signaling and sensing, with focus on the relevance of LRR-RK in these pathways. Understanding how ROS molecules are formed and sensed can be helpful for engineering plants with improved adaptation to adverse situations in which ROS signals have a key role.

ROS Biosynthesis Pathways

In plants, ROS can be generated in nearly every subcellular compartment, and numerous enzymatic reactions have evolved to actively generate ROS. Plant NADPH oxidases (NOX) (also termed respiratory burst oxidase homologs; RBOHs) are a major component of the production of extracellular ROS, specifically of extracellular (e)H_2O_2 [2]; therefore, activity is strictly controlled to avoid damaging consequences of unrestricted H_2O_2 production [3]. Apoplastic peroxidases are also important components in the production of extracellular ROS, especially during plant immunity; however, the molecular mechanisms that regulate their activity remain unknown.

Arabidopsis (*Arabidopsis thaliana*) has ten *RBOH* genes, from *AtRBOHA* to *AtRBOHJ*. A recent study using an heterologous expression system of human HEK293T cells served to uncover the role of each of the RBOHs family members in plants [4]. Results revealed some functional redundance between all of the arabidopsis RBOHs. The EF-hand motif in the N terminus, where Ca^{2+} can bind and promote their activity, is conserved (Figure 1). *AtRBOHI* carries a point mutation in this motif, leading to decreased ROS production compared with the others, revealing the importance of this position. The study also revealed the functional diversification of the RBOH family in arabidopsis with different activity levels for H_2O_2 production. This, together with the lack of functional complementation, suggests that the RBOHs family members evolved to accommodate specific roles in different cell types [4].

AtRBOHD is the best-characterized RBOH and has been reported to be involved in both biotic and abiotic stress responses. Receptor-like cytoplasmic kinases, such as BOTRYTIS-INDUCED KINASE 1 (BIK1), are responsible, among other several protein kinases, for phosphorylating the N-terminus region of RBOHD [4] (Figure 1). BIK1 is activated as an immunity response triggered by FLAGELLIN-SENSING 2 (FLS2), a LRR-RK that recognizes the bacterial peptide flagellin (flg22). When FSL2 is activated by flg22, it forms a complex with BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1), which leads to a succession of *trans*-phosphorylation events between several intracellular kinases, including BIK1. Furthermore, the FLS2–BAK1 union also activates other downstream signaling, including the mitogen-activated protein kinase (MAPK) cascades, which also control H_2O_2 and are regulated by it (Figure 1). Interestingly, another recent study also uncovered that, upon bacterial pathogen infection, CYSTEINE-RICH receptor-like protein kinase (CRK2), which exists in a preformed complex with RBOHD, phosphorylates its C-terminal region and regulates its H_2O_2-production activity *in vivo* (Figure 1). While previous studies only reported phosphorylation of the N-terminal region as a unique way of RBOHD regulation, these new results uncover a novel

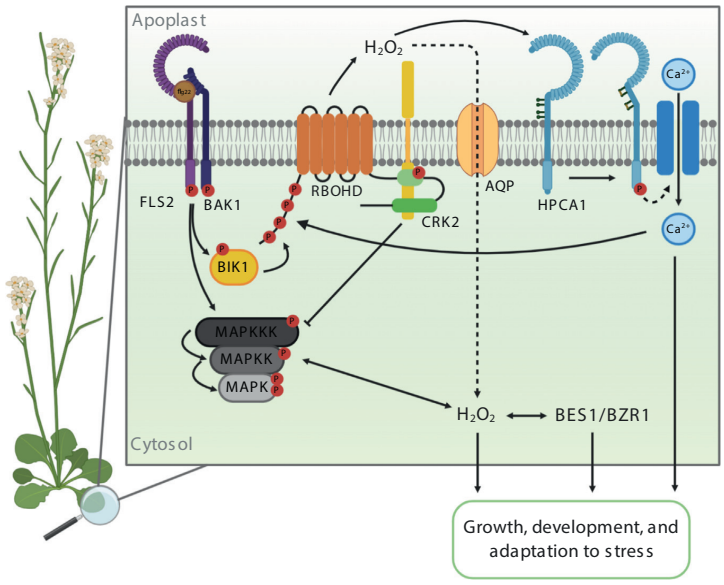

Figure 1. Current Model for Reactive Oxygen Species (ROS) Production, and Sensing and Signaling Pathways. Extracellular ROS, mainly hydrogen peroxide (H_2O_2), is produced by respiratory burst oxidase homologs (RBOHs). Specifically, RBOHD can be activated through phosphorylation of its N-terminus or C-terminus domains by interaction with different molecules and receptors. BOTRYTIS-INDUCED KINASE 1 (BIK1) and Ca^{2+} have been shown to interact with the N terminus and CYSTEINE-RICH receptor-like protein kinase (CRK2) with the C terminus. BIK1 activation is dependent on the activity of the complex FSL2–BAK1, which can sense pathogen peptides, such as flagellin 22 (flg22). Moreover, FSL2–BAK1 activation also leads to the activation of the mitogen-activated protein kinase (MAPK) cascade directly controlling ROS activity. Extracellular (e)H_2O_2 can be sensed by HYDROGEN-PEROXIDE-INDUCED *Ca^{2+}* INCREASES 1 (HPCA1), a leucine-rich repeat receptor kinase (LRR-RK), activated by the direct oxidation of two Cys residues. The creation of disulfide bounds leads to conformational changes that cause autophosphorylation of HCPA1, eventually triggering Ca^{2+}-channel gating. eH_2O_2 can also enter the cells via aquaporins (AQP) and can then interact with different transcription factors in the cytosol, such as BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1). These interactions lead to developmental changes and adaptation to stresses, which, through different mechanisms, can also be triggered by Ca^{2+} influx. Figure created using BioRender (https://biorender.com/). Abbreviations: BAK1, BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1; FLS2, FLAGELLIN-SENSING 2.

mechanism of regulation and highlight a key role of CRK2 in the control of the apoplastic H_2O_2 burst in response to biotic stress [5].

ROS Signaling Pathways

H_2O_2 can be transported into the cell by aquaporins (Figure 1) and, once inside, acts as a signaling molecule up and downstream from many other secondary messengers, such as Ca^{2+}, MAPKs, and transcription factors to initiate specific responses to developmental and environmental stimuli. H_2O_2 interplays with several phytohormone signals that can trigger conformational modifications of targeted proteins to regulate development and stress responses. As an example, a recent study unveiled that brassinosteroid (BR) signaling is affected positively by H_2O_2, as transcription factors acting as effectors at the end of this phytohormone pathway, BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1), have to be oxidized to be active [6] (Figure 1). However, crosstalk between ROS and hormonal pathways is not limited to oxidizing pathway components, because hormones can also directly affect ROS production. For example, BRs can trigger the accumulation of H_2O_2 [6] (Figure 1). Conversely, an interlink exists between ethylene and BR controlling O^{-2} production, revealing the control of ROS through the peroxidase pathway rather than via the NADPH oxidase pathway [7].

ROS-Sensing Pathways

ROS sensing by plant cells remained a mystery until recently. The HYDROGEN-PEROXIDE-INDUCED *Ca^{2+}* INCREASES 1 (HPCA1) was identified as the first known cell surface-specific sensor for eH_2O_2 in plants [8]. HPCA1 encodes an LRR-RK that contains two special pairs of cysteine (Cys) residues in its extracellular H_2O_2 domain. The thiol groups of Cys residues are a target for oxidation to sulfenic acids by H_2O_2 and, in this way, they are considered as sites of eH_2O_2 sensing. This covalent modification leads to activation of the HPCA1 kinase domain by autophosphorylation, which subsequently triggers Ca^{2+}-channel gating (through unknown mechanisms) and Ca^{2+} influx, followed by activation of intrinsic and systemic signaling pathways (Figure 1).

Remarkably, eH_2O_2 sensing by LRR-RK does not resemble any eH_2O_2 receptors or sensors previously reported across the phyla. Such a specific cell membrane sensor may serve to integrate the external and internal stresses or signals on the eH_2O_2 status to robustly trigger signaling pathways. Before the discovery of HPCA1, the LRR-RK GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1 (GHR1) was proposed as a ROS sensor, because it acts downstream of ROS production but upstream of the Ca^{2+}signaling cascade [9]. However, current thinking is that GHR1 acts downstream of HPCA1 and, instead, cysteine-rich receptor-like

kinases have been proposed to also function in eH_2O_2 sensing and signaling [10].

Despite this breakthrough discovery, the molecular mechanisms involved in the prevention of the oxidation of the Cys residues remain uncharacterized. However, because HPAC1 is an exposed extracellular protein, it is likely to become oxidized and then some mechanism should prevent constitutively activated sensors. Future structural analyses of ROS receptors are necessary to further understand the sensing machinery and any implications for downstream functions.

Concluding Remarks and Future Directions

Gaining a deep understanding of all the pathways in which ROS are involved is essential for understanding plant growth and development as well as how plants adapt to the changing environment, because ROS are crucial players in all these processes. Recent studies have elucidated that spatiotemporal regulation of signaling pathways can be beneficial to drive plant adaptive changes without compromising growth [11]. Considering that RBOHs probably diverged to gain specific functions in different cell types, and the likely roles of LRR-RKs in many aspects of ROS signaling and sensing, points to different signaling networks driving cell type-specific responses. Indeed, several studies point to a coordination and network formation of LRR-RKs and that their activities vary depending on their partners [12]. Further studies on the relationship between ROS biosynthesis, signaling, and sensing, and LRR-RKs should focus on how these pathways are regulated. This knowledge, in addition to understanding the connection of ROS and Ca^{2+} signals, which is still not known, would be valuable for engineering plants with improved agronomic traits.

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[1]Center for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Barcelona 08193, Spain

*Correspondence:
ana.cano@cragenomica.es (A.I. Caño-Delgado).

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Forum

Pedospheric Microbial Nitric Oxide Production Challenges Root Symbioses

Bin Hu,[1,*] Emmanouil Flemetakis,[1,2] and Heinz Rennenberg[1]

Recent studies indicate that a multitude of microbial processes are involved in nitric oxide production and consumptions in the pedosphere. Due to its dual function as a toxic metabolite and signaling compound, we speculate that this pedospheric nitric oxide of microbial origin can significantly interact with mycorrhizal symbioses and symbiotic nitrogen fixation of legumes.

Pedospheric Nitric Oxide

High nitric oxide (NO) concentrations are a general phenomenon in many soils, because pedospheric NO production mostly originates from denitrification (Figure 1), a ubiquitous microbial process [1]. In a recent case study [2], high NO concentrations in the soil of a Norway spruce forest were reported and semicontinuous measurements showed that these high NO concentrations are maintained throughout the year in different soil depths, in line with the previous sporadic NO measurements in different forest soils [2]. Recent studies also showed that NO production takes place in different biosynthetic/detoxification routes in fungi, similarly to other organisms, such as bacteria, plants, and mammals [3]. High soil NO concentrations may be particularly challenging for roots, because of the dual role of NO in plants, as a toxic metabolite and a regulatory compound that interacts with developmental and